Effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial on weanling pig growth performance and nutrient digestibility

Jaron R. Lewton†, Adrienne D. Woodward‡, Ronny L. Moser†, Kyan M. Thelen*, Adam J. Moeser*, Nathalie L. Trottier†, Robert J. Tempelman†, and Dale W. Rozeboom†2

†Department of Animal Science, Michigan State University, East Lansing, Michigan 48824, USA;
‡United Animal Health, Sheridan, Indiana 46069, USA;
*Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824, USA

1The authors acknowledge United Animal Health (Sheridan, IN) for providing the direct-fed microbial product and for giving to the Michigan State University Swine Nutrition & Production Management Master’s Degree Fund which supported J. R. Lewton.

2Corresponding author: rozeboom@msu.edu
ABSTRACT

A study was conducted to evaluate the effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM) on growth performance and apparent nutrient digestibility of nursery pigs. Eighty pigs, of equal number of barrows and gilts (initial BW: 7.0 ± 0.60 kg), were weaned at 21 ± 1 d and randomly allotted to one of sixteen pens, with five pigs per pen. Two dietary treatments were implemented, a basal control (CON) and a control plus DFM (DFM). Both diets were corn, soybean meal, and distillers dried grains based. Diets were fed for 42 d and growth performance measures were recorded weekly. On d 21 and 42 of the experiment, one pig per pen, with equal number of males and females, was randomly selected and euthanized. Digestibility of nitrogen (N), amino acids (AA) and energy were evaluated within the duodenum, jejunum, ileum, ascending and distal colon. Relative to CON, DFM tended to increase ADG during wk 2 ($P = 0.08$), and significantly increased ADFI during wk 2 ($P = 0.04$) and wk 3 ($P = 0.02$). In addition, DFM decreased G:F during wk 6, relative to CON ($P = 0.04$). Within the jejunum, pigs fed DFM had greater digestibility of tryptophan ($P = 0.04$) and cysteine ($P = 0.04$), and tended to have greater digestibility of lysine ($P = 0.07$), methionine ($P = 0.06$), and threonine ($P = 0.08$), relative to CON. The content pH in ascending colon did not differ between DFM and CON. Compared to CON, apparent total tract digestibility (ATTD) of energy of DFM did not differ while ATTD of nitrogen of DFM was lower ($P = 0.05$). The addition of a multi-strain *Bacillus subtilis*-based DFM appears to impact growth performance, AA and N digestibility depending upon location in the gastrointestinal tract, with primary AA differences occurring within the mid-jejunum.

**Key words:** direct-fed microbial, amino acid, digestibility, weanling pig
LIST OF ABBREVIATIONS

AA = Amino acid

ADF = Acid detergent fiber

ADFI = Average daily feed intake

ADG = Average daily gain

CON = Control

CP = Crude protein

DFM = Direct-fed microbial

DM = Dry matter

GE = Gross energy

G:F = Gain to feed ratio, feed efficiency

GIT = Gastrointestinal tract

N = Nitrogen

NDF = Neutral detergent fiber

Ti = Titanium

VFA = Volatile fatty acid
INTRODUCTION

With the concerns regarding antibiotic resistant bacteria reducing antibiotic effectiveness in humans, the use of antibiotics in swine is increasingly regulated (Aarestrup et al., 2010; Schultz and Rademacher, 2017). Alternative ways to achieve similar health and performance include the use of direct-fed microbials (DFM) (Chen et al., 2005). Feeding DFM composed of *Bacillus subtilis* improves pig growth performance (Kim et al., 2019) and nutrient digestibility (Lee et al., 2014; Blavi et al., 2018). Strains within the *Bacillus subtilis* species have considerable genomic diversity which imparts a range of strain-specific capabilities, likely contributing to the ability of this species to inhabit a myriad of terrestrial and aquatic environments including the mammalian gastrointestinal tract (Earl et al. 2008). A two-strain *Bacillus subtilis* combination comprised of strains isolated from intestinal epithelial scrapings of high-performing pigs showed promising performance benefits for nursery pigs, increasing gain by 5-10% and lowering feed/gain ratio up to 5% (Augspurger et al., 2016), however the mechanisms for improved growth were not elucidated. Given the gastrointestinal origin, it was hypothesized that improved digestibility of nutrients may be one mechanism by which the multi-strain combination may provide benefit. Moreover, while there are several previous studies involving either a single-strain *Bacillus subtilis* or multiple strains within the *Bacillus* genus, there are no published studies involving multiple strains of this specific species. Therefore, the specific objective of this study was to evaluate the effect of a multi-strain *Bacillus subtilis*-based DFM on growth performance and specific nutrient digestibility of the 21-d old weanling pig. The hypothesis was that pigs fed a diet supplemented with *Bacillus subtilis* have improved growth performance and greater digestibility of nitrogen (N), amino acids (AA), and gross energy (GE) within the different segments of the gastrointestinal tract (GIT).
MATERIALS AND METHODS

Animals, housing, and experimental design

The Institutional Animal Care and Use Committee at Michigan State University reviewed and approved the protocol (PROTO201900154) for this experiment. The animal study was structured as a completely randomized design and conducted between the months of August and September 2019 at the Michigan State University Swine Teaching and Research Center. Eighty crossbred pigs (PIC 359 × Yorkshire) equally balanced by sex. Pigs were weaned at 21 ± 1 d (7.0 ± 0.6 kg, initial BW) and randomly allotted into 16 cohorts with five pigs per cohort. Cohorts were randomly allotted to one of 16 pens (1.22 × 1.83 m) located in one of four mechanically ventilated identical nursery rooms. Cohort allotment was based on litter (dam), weight, and sex, and maintaining a similar average weight in each pen. Treatments were randomly assigned to each pen. Each pen held five pigs with four of the pens within each treatment containing three gilts and two barrows and the remaining four pens containing three barrows and two gilts. Pens were equipped with round-rod steel flooring, vertical-rod, fiberglass fencing and gates, single-sided two-hole feeders, and one nipple drinker. Pen to pen cross-contamination between the two treatments was considered minimal as pens and alleyways were cleaned on a regular basis. Rooms were operated on an all-in/all-out system and were disinfected using bleach (15.6 mL/L) two to five days before pigs were placed in the pens. All dams were vaccinated pre-breeding for parvovirus, leptospirosis, and erysipelas. Processing of newborn pigs on days one and two included ear notching, tail docking, and 1.0 mL iron dextran (200 mg/mL). All pigs received an additional 1.0 mL iron dextran (200 mg/mL) between d 7 and 10, and males were castrated. Pigs were vaccinated at weaning for the prevention of circovirus and erysipelas. All water nipples had a flow rate of 25 ± 1 mL/sec.
Diets and feeding

Two dietary treatments were used: a control diet with no DFM supplementation (CON) and diet with supplementation of a multi-strain *Bacillus subtilis*-based DFM (DFM) (United Animal Health, Sheridan, IN) comprised of a dried spore preparation having a guaranteed count of $1.48 \times 10^8$ CFU/g and included at a rate of 0.5 g/kg of feed to provide a final count of at least $7.35 \times 10^4$ CFU/g of complete feed. Treatments were imposed over three dietary phases (d 0-14, 14-28, and 28-42) with d 0 representing the day of weaning (21 ± 1 d of age). All diets were based on requirements published by the NRC (2012) and formulated according to example diets made available online by Kansas State University (https://www.asi.k-state.edu/research-and-extension/swine/premix-and-diet-recommendations.html, accessed: August 25, 2019) (Table 1). Dietary Cu and Zn were maintained at requirement (NRC, 2012). An indigestible marker, Titanium (Ti) was included in phases two and three in the form of titanium dioxide, at an inclusion rate of 0.1% of the complete diet. Diets were mixed at the Michigan State University swine farm using a 113-kg paddle ribbon mixer. The mixer was emptied and wiped clean between each batch to minimize cross-contamination. Analyzed feed values were obtained for each dietary phase, from composite samples of individual feeders from the same treatment (Table 3).

Data recording and sample collection

Weekly performance data was collected by weighing each pig individually each wk to estimate pen average daily gain (ADG). Pen feed disappearance was measured by vacuuming out remaining feed and subtracting that from total weekly feed additions to represent average daily feed intake (ADFI). Feed efficiency (G:F) was calculated by dividing pen ADG by the corresponding pen ADFI.
At the end of wk 3 and 6, one pig per pen, was humanely euthanized for analysis of nutrient digestibility and pH of ascending colon content. An equal number of males and females were euthanized from both treatments to leave 4 pigs per pen at the conclusion of wk 3, and to maintain 2 of each sex per pen for the remainder of the study. One pig was euthanized at a time alternating between CON and DFM. Pigs were sedated using a combination of Telazol (2.5 mg/kg), Ketamine (1.25 mg/kg), and Xylazine (1.25 mg/kg) in a single intramuscular injection with a 22 G needle. Pigs were then euthanized using sodium pentobarbital (1 mL/4.5 kg) in a single intra-cardiac injection with an 18-G needle.

Immediately after confirmation of death, pigs were opened lengthwise and the cecum was located. Immediately anterior to the cecum, the ileum was sealed off with string and cut along the mesentery for approximately 50 cm. Digesta anterior to this section, and within the ileum was manually pushed into the 50 cm section before sealing off the proximal end and removing that section of the ileum. The ascending colon was then removed in a similar manner, sealing the proximal end, immediately distal to the cecum, and measuring a 50 cm section, then sealing the distal end and removing the whole segment of ascending colon, stretching it lengthwise by cutting along the mesentery. The distal colon segment was collected by sealing off both proximal and distal ends and removing the entire distal colon, from the beginning of the descending colon to the rectum. This section was about 20 cm in length. The jejunum was located by cutting along the mesentery for approximately 8 m proximal to the cecum to ensure that proper location of the mid-jejunum. Beginning at this location a 30 m section of jejunum was removed after sealing both proximal and distal ends. Before sealing the proximal end, additional jejunal digesta was manually moved into this 30 cm section similar to that performed in the ileum. The duodenum was located by first locating the distal end of the stomach and sealing off the duodenum just distal to the gastroduodenal
junction. Approximately 20 cm distal this point, the duodenum was sealed a second time before removing the entire section.

Upon removal of each individual segment, digesta was collected from each of the five sections (duodenum, jejunum, ileum, ascending and distal colon) for GE, N, complete AA profile, and Ti analyses. All digesta samples were collected into labeled, plastic 50-mL tubes. Ileal, jejunal, and ascending colon digesta were collected into two separate tubes. Digesta was removed by cutting off one of the tied ends and gently stripping the digesta lengthwise from the tissue into each tube. Digesta from the duodenum and distal colon were limited and little to no digesta could be obtained from the duodenum. Only small amounts were collected from the distal colon from a limited number of animals. For those, the small amount was placed into a single tube for GE, N, and Ti analysis. After collection, each tubes cap was wrapped in paraffin paper and placed on ice, and then frozen at -20°C until analysis.

After removal of digesta from the ascending colon, the pH was immediately recorded using a pH reader equipped with a probe (Mettler Toledo, Columbus, OH).

Chemical analysis of feed and digesta

Feed samples were prepared as described below, and then shipped to the University of Missouri Experimental Station Chemical Laboratory (Columbia, MO) for nutrient analysis (Table 3). The following analyses were performed: Dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose, cellulose, lignin, and total dietary fiber, crude fiber, N, ether extract, individual AA, Ca and P, and ash. Neutral detergent fiber was determined by use of neutral detergent and heat stable amylase according to the methods of Van Soest et al. (1991). Hemicellulose was calculated as NDF-ADF. Titanium in the feed was calculated according to Myers et al. (2004). Individual AA were determined in accordance with the standard methods of AOAC (2007).
Samples of digesta were prepared for analysis of GE, N, Ti, and individual AA. All samples were freeze dried (HarvestRight 115V, 3/4HP Salt Lake City, UT). Immediately after being removed from the freezer, whole tube weights were recorded and tubes were thawed placing digesta in appropriately labeled weigh boats or whirl pack baggies to increase surface area. Samples were then refrozen before placing in the freeze drier. After complete drying of individual samples, samples were finely ground using a Willey mill micro grinder (Swedesboro, NJ) with a one mm-mesh sized screen.

Titanium in the digesta was analyzed using an adjusted protocol based on that of Myers et al. (2004). Samples were weighed (150 mg) into a 100 mL Digesdahl flasks. Four mL of concentrated H$_2$SO$_4$ was added to each flask, swirled to cover all digesta and kept over-night to digest. Flasks were then placed on a Digesdahl burner (Model 23130-20, Loveland, CO) and vacuum system to boil the acid for six minutes, followed by the addition of 10 mL of 50% H$_2$O$_2$. After completely burning off H$_2$O$_2$, flasks were cooled and then diluted to the 100 mL mark with distilled water. Upon water dilution to the 100 mL mark, 160 µL of standards and individual samples were transferred to micro plates in duplicate. Standard concentrations used for Ti analysis were 0, 0.5, 1.0, 1.5, and 2.0 mg/dL. Plates were read at an absorbance of 460 nm on a well plate reader (Molecular Devices SpectraMax Plus 384, San Jose, CA). All sample duplicates having a CV < 5 were averaged for a final Ti concentration, while those exceeding CV of 5 were analyzed a third time.

Due to limited sample availability from the distal colon, N digestibility of all GIT segments was also analyzed on site according to Hach et al. (1987) to confirm with the analyzed values from the jejunum, ileum, and ascending colon obtained by the University of Missouri. Upon confirmation digesta values for N collected on site, were used in the final analysis when calculating apparent digestibility.
Gross energy was analyzed using an Adiabatic Bomb Calorimeter (115VParr model 12141 Parr Instrument Co., Moline, IL) according to manufacturer's instructions.

Individual AA were determined for digesta samples by the University of Missouri Experimental Station Chemical Laboratory (Columbia, MO) as described above. As available sample was limited, proximate analysis of digesta was limited to N.

**Calculations and statistical analysis**

Apparent digestibility was calculated for GE, N, and AA as follow:

\[
\text{% digestibility} = \left[1 - \left(\frac{\text{nutrient digesta}}{\text{nutrient feed}}\right) \times \left(\frac{\text{marker feed}}{\text{marker digesta}}\right)\right] \times 100,
\]

where marker represents analyzed Ti values in both the feed and digesta, and nutrient represents the analyzed value of individual nutrients in both the feed and digesta.

Data was analyzed using PROC GLIMMIX procedure in SAS 9.4 (SAS Institute, Inc., Cary, NC) specifying pen as the experimental unit. Data was analyzed as repeated measures over time (week) for performance. Digestibility data was analyzed as double repeated measures over both time (week) and space (GIT segment). The model included the fixed effects of dietary treatment, week, and GIT segment and all possible two-way interactions. Pens nested within treatments were specified to be random effects. Compound symmetry with heterogeneous variances was used to account for greater variation in the ascending colon, compared to other GIT segments and was found to be a better fitting model compared to a homogeneous variance specification. Because of this heterogeneous variance specification, all analyses incorporated the Kenward-Rogers adjusted degrees of freedom. Treatment means were separated using the Tukey-Kramer multiple comparison test. Differences were considered significant at \( P < 0.05 \) and tendencies at \( 0.05 < P < 0.10 \).
RESULTS AND DISCUSSION

Morbidity, Mortality, and Growth Performance

This study was designed to evaluate the effects of a multi-strain *Bacillus subtilis*-based DFM on growth performance and nutrient digestibility. There was no mortality and only two pigs were treated, both belonging to the DFM treatment, one due to lameness and the other due to weight loss, for a total morbidity of 2.5%. These pigs were monitored and considered to have fully recovered and were gaining weight within a few days of treatment and therefore remained on test for the duration of the study. Overall, there were no differences in growth performance with means ± SD of 0.51 ± 0.05 kg/d, 0.79 ± 0.05 kg/d and 0.66 ± 0.05 for average daily gain, average daily feed intake, and feed efficiency (G:F), respectively. However, there were significant differences within individual weeks. Compared to CON, ADG tended to be greater during wk 2 (*P* = 0.08), and ADFI was greater during wk 2 (*P* = 0.04) and 3 (*P* = 0.02) for DFM. Compared to CON, feed efficiency was lower during wk 6 for DFM (*P* = 0.04) (Table 4). The study did not confirm performance results previously obtained by Augspurger et al. (2016), who observed a 5-10% greater gain and 1.4-5% greater feed conversion with addition of the same multi-strain *Bacillus subtilis*-based DFM. In the current study, with pen being defined as the experimental unit, eight pens were assigned to each treatment, as this was considered enough statistical power to detect differences in nutrient digestibility of 5% or 10% (Lee et al., 2014), however, this was not expected to provide enough power to mimic the differences in growth performance obtained by Augspurger et al. (2016). Furthermore, pen density, with five pigs per pen, favored maximum performance. Animals performed exceptionally well compared to other similar studies (Guo et al., 2006; Walsh et al., 2007; Lee et al., 2014; Tang et al., 2019).
**Amino Acid Digestibility**

Differences between treatments were found within the jejunum only (Table 5; Table 6). Because of insufficient amounts of digesta from the duodenum and distal colon, differences in AA digestibility between treatments for those segments could not be assessed. Amino acid digestibility in the large intestinal segments were determined as a proxy to microbial N metabolism. While there is little evidence for AA absorption across the large intestine of the pig, significant lysine transport across the apical membrane of the proximal colon was reported in the growing pig (Woodward et al., 2012). Thus, AA digestibility and therefore disappearance from the large intestine may also indicate *in situ* AA utilization by the colonocytes rather than complete absorption. Although the role of hindgut in the global AA and N metabolism of the pig has been given little attention, its relevance in the context of the microbiota and the microbiome cannot be ignored. Therefore, AA and N digestibility values in segments of the large intestine were also evaluated. In the jejunum, compared to CON, digestibility was greater for tryptophan (11%, *P = 0.04*) and cysteine (17%, *P = 0.04*) and tended to be greater for lysine (*P = 0.07*), methionine (*P = 0.06*), and threonine (*P = 0.08*) (Table 5). These results indicate potential activity of *Bacillus subtilis* by the middle of the small intestine, specifically the jejunum.

Treatment differences in AA digestibility may have begun as early as the end of wk 3 of the study. Compared to wk 6, there was a consistent greater numerical difference between treatments during wk 3 for the digestibility of nearly all indispensable AA, including arginine, histidine, isoleucine, lysine, methionine, threonine, tryptophan, and valine (Table 7). In some cases, the numerical difference in % digestibility between treatments was more than double at the end of wk 3 compared to wk 6. This was true for arginine (11.5% vs. 4.5%), histidine (12% vs 4.5%), and tryptophan (16.5% vs. 5.5%). These results indicate an early
impact of the DFM on jejunal AA digestibility, at least within three weeks of supplementation.

Isaacson and Kim (2012) showed differences in the microbiota within different GIT segments, with the jejunum being composed of primarily bacteria belonging to the *Firmicutes* phyla (> 90%), and the ileum composed of a mix of *Firmicutes* and *Proteobacteria*. Their findings agree with the effects of *Bacillus subtilis* on digestibility within the jejunum in the current study. As a member of the *Firmicutes* phyla, *Bacillus subtilis* may have helped restore the preferred microbiome of the jejunum, being a naturally *Firmicutes* dominate environment. There are limited studies evaluating the effects of *Bacillus subtilis* or other similar DFM on AA digestibility. Kaewtapee et al. (2017) recently evaluated AA digestibility of growing pigs with and without a mixed *Bacillus* spp. DFM containing one strain of both *Bacillus subtilis* and *Bacillus licheniformis*. When fed with a wheat, barley, and soybean meal-based diet, DFM addition did not impact the digestibility of any AA by the terminal end of the ileum. Similarly, in the present study, no differences were observed in ileal digestibility of AA.

*Bacillus subtilis* may alter digestibility of AA and other nutrients through enzyme secretion. Some of the enzymes it has been known to secrete include α-amylase, arbinase, cellulase, dextranase, lavansucrase, maltase, alkaline protease, neutral protease, and β-glucanase (Priest, 1977). Blavi et al. (2018) reported that increases in apparent total tract digestibility of energy may be due to the ability of *Bacillus subtilis* to secrete α-amylase, which catalyzes the hydrolysis of glycosidic bonds in starch. They suggested that increases in energy utilization may be the result of increased digestion of fiber due to other enzymes secreted by *Bacillus subtilis* including pectinase and xylanase. However, differences in GE digestibility were not observed in this study. Other research attributes changes in growth performance and digestibility to the proteases produced by *Bacillus subtilis* (Tang et al.,
or other unidentified enzymes aiding in the breakdown of crude protein (CP), non-starch polysaccharides, or other substrates present in soybean meal and other common swine feed ingredients (Giang et al., 2012; Upadhaya et al., 2015). The mechanisms of action for each single strain or mixed cocktail appear to differ. The DFM used in the current study appears to improve digestibility of specific AA, rather than directly impacting energy digestibility. This may be an indication that the *Bacillus subtilis* strains involved in this study secrete unique proteases, specifically improving absorption of cysteine and tryptophan, and potentially improving absorption of lysine, methionine, and threonine (Table 5). Recent research has been conducted to evaluate dietary supplementation of proteases in nursery pig diets. Two separate studies conducted in 2016 (Pan et al., 2016; Yu et al., 2016) found improved AID of several AA both indispensable (arginine, histidine, isoleucine, leucine, lysine, methionine, and threonine), and dispensable (alanine, cysteine, and tyrosine) with protease supplementation. However, neither protease improved digestibility of tryptophan, the AA most greatly affected by the DFM used in the current study.

The DFM used herein appeared to have a more pronounced impact on indispensable AA digestibility, compared to dispensable AA (Table 5). Improvements in indispensable AA digestibility may be correlated with improved growth performance as demonstrated in previous research (Nortey et al., 2007; Min et al., 2009). Thus, greater utilization of many of the most limiting AA in corn, soybean meal, and DDGS based diets may have played an important role in the improvements in gain and feed efficiency observed in the studies conducted by Augspurger et al. (2016).

Regarding the AA digestibility in the large intestine, dietary supplementation of DFM had no effect. We acknowledge that the extensive metabolism of AA by the microbiota, including utilization and synthesis, may have hampered finding distinct differences between AA digestibility, however, as discussed below, N digestibility was affected.
Digestibility of Gross Energy and Nitrogen

Limited studies have determined nutrient digestibility in multiple segments of the GIT. In the present study, digestibility of GE and N numerically increased from proximal to distal segments with CON digestibility values of 52.0, 62.1, 70.2, and 77.6% for GE in the jejunum, ileum, ascending colon, and distal colon, respectively (Table 8). The same trend was found for N digestibility values for CON with 43.2, 55.8, 57.4, and 71.3% for the jejunum, ileum, ascending colon, and distal colon, respectively (Table 8). While apparent nutrient digestibility values in small intestinal segments represent apparent nutrient absorption and post-gut availability to the animal, those of the large intestine are a consequence of microbial utilization and metabolism, and indicative of urea-N recycling across the large intestinal wall. The values obtained from CON appear to be small, yet similar to those obtained from other weanling pig studies. Giang et al. (2012) fed weaned pigs a lactic acid bacteria supplement in addition to *Bacillus subtilis* and observed greater digestibility values for N than the current study, with an AID of N approaching 80% and ATTD close to 85-90%. In a different study, ATTD was found to be close to 70% for N and 80% for GE, 4 wk into the nursery phase in pigs fed *Bacillus subtilis* fermentation biomass (Lee et al., 2014). The ATTD of both N and GE fell between 70 and 80%, with a slight decrease in both measures from d 14 to 42 post-weaning in pigs fed a multi-strain DFM composed of 1 strain of *Bacillus subtilis* and 2 strains of *Bacillus amyloliquefaciens* (Cai et al., 2015). Tang et al. (2019) suggested that the *Bacillus subtilis* may have a greater impact on digestibility in pigs fed low protein diets. In their study, low protein combined with *Bacillus subtilis* led to a 5% increase in ATTD of N (75% vs 80%). Each of these studies observe a slight increase in either AID or ATTD with addition of a *Bacillus subtilis*-based DFM which is in contrast with the results of the current study. These variations could be the result of differences in strains of bacteria, pig age and growth performance, dietary composition, inclusion rate, and/or interaction with other feed additives.
(Chesson, 1994; Chen et al., 2005). The diets fed in the current study were composed of corn, soybean meal, and distillers dried grains (DDGS). Inclusion of DDGS has been shown to significantly decrease AID of GE and ATTD of GE, N, and several essential AA (Agyekum et al., 2016) and may explain the relatively low digestibility values observed here.

Relative to CON, overall N digestibility decreased ($P = 0.05$) in the distal colon with the addition of DFM (71.3 vs. 58.9 ± 4.2 %) (Table 8). Others reported greater fecal or ATTD of N when supplementing a *Bacillus subtilis*-based DFM (Lee et al., 2014; Cai et al., 2015; Tang et al., 2019). The decrease in N digestibility may have been related to changes in the richness or diversity of the microbiome of the distal colon, leading to increased N recycling and endogenous secretions. Bacteria utilize and synthesize AA and other N containing metabolites (Davila et al., 2013). Up to 25% of the urea produced in the liver enters the intestinal lumen, mostly within the small intestine but also in the colon in growing pigs (Bergen and Wu, 2009). Microbes are essential for the hydrolysis of urea into ammonia and carbon dioxide, and subsequent conversion of ammonia to form glutamate and glutamine (Bergen and Wu, 2009). Glutamate digestibility was not measured within the distal colon due to limited sample volume. Glutamate digestibility values may have provided support in interpreting the difference in N digestibility in the distal colon.

**Colonic Contents pH**

Agyekum et al. (2016) measured colonic pH as a secondary indication of changes in the microbiome or hindgut fermentation and production of volatile fatty acids (VFA). As the primary site of bacterial fermentation and microbial communities in nonruminants, we expected to see the greatest impact of *Bacillus subtilis* on N digestibility, VFA production, and pH within the colon (Tajima and Aminov, 2015). However, no differences were observed in the pH or nutrient digestibility of ascending colon content in this study. Over the course of
the 42-d study, both treatments were fed increasing amounts of DDGS, 5, 10, and 20% for phases one, two, and three, respectively. Other studies have demonstrated the effects of DDGS on the microbiota of nursery pigs, finding that inclusion of 30% DDGS led to a significant decrease in the *Firmicutes:Bacteroidetes* ratio, mostly due to a decrease in many *Lactobacillus* species (Burrough et al., 2015). As they are known for producing lactic acid and reducing digest pH, a decrease in *Lactobacillus* within the ascending colon could result in an increase in digesta pH. However, this is not supported as pH was numerically decreased 5.8 vs 5.6 in the control diet when increasing DDGS inclusion from 10 to 20% for pigs euthanized on wk 3 and 6, respectively. Between wk 3 and 6, colonic pH of CON and DFM appear to move in opposite directions, possibly indicating changes occurring within the microbiome with prolonged addition of *Bacillus subtilis*. While *Lactobacillus* experiences optimal growth at a lower pH, *Bacillus subtilis* has been shown to prefer an environment with a higher pH. Some studies suggest an optimal pH of 5.5, while others suggest a pH much closer to 6.5, depending on the specific strains and activity of interest (Chantawannakul et al., 2002; Koni et al., 2017).

In conclusion, supplementation of a multi-strain *Bacillus subtilis*-based DFM appeared to have some beneficial effects on both growth performance and nutrient digestibility of nursery pigs. Digestibility was improved specifically within the jejunum, increasing the digestibility of cysteine and tryptophan, while tending to improve that of lysine, methionine, and threonine. These improvements in AA digestibility could help explain the improvements in ADG and G:F observed in previous studies conducted by Augspurger et al. (2016), which utilized the same DFM as the present study. *Bacillus subtilis* also appears to lead to changes in hindgut digestibility and fermentation, observed through decreased digestibility of N within the distal colon relative to CON. The current study evaluated nutrient digestibility across multiple segments of the GIT. No previous DFM studies have
evaluated digestibility in the jejunum and ascending colon, thus the data provided herein are novel information. While enzyme secretions related to protein digestion were not evaluated, we speculate that the Bacillus subtilis may be secreting enzymes that improve digestibility of specific AA, that may in some cases impact overall nitrogen digestibility. Additional studies are needed to identify specific mechanisms by which multi-strain Bacillus subtilis-based DFM may be improving AA digestibility in the jejunum and other GIT segments.

DISCLOSURES

Michigan State University has no financial interests in the product evaluated or in the company providing that product. Because of the perception of a conflict of interest and in the interest of full transparency, we are disclosing 1) that employees of the company providing said product were co-investigators or researchers, and are co-authors of this manuscript, 2) the company provided the product gratis, and 3) the company had previously donated to the Michigan State University Swine Nutrition & Production Management Master’s Degree program which supported J. R. Lewton.
LITERATURE CITED

Aarestrup, F.M., V.F. Jensen, H-D. Emborg, E. Jacobsen, and H.C. Wegener. 2010. Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark. Am. J. Vet. Res. 71:726-733. doi:10.2460/ajvr.71.7.726.

Agyekum, A.K., A. Regassa, E. Kiarie, and C.M. Nyachoti. 2016. Nutrient digestibility, digesta volatile fatty acids, and intestinal bacterial profile in growing pigs fed a distillers dried grains with solubles containing diet supplemented with a multi-enzyme cocktail. Anim. Feed Sci. Technol. 212:70-80. doi:10.1016/j.anifeedsci.2015.12.006.

AOAC. 2007. Official methods of analysis. 18th ed.Rev. 2. W. Hortwitz and G. W. Latimer Jr, editors. Gaithersburg, MD: AOAC International.

Augspurger, N.R., J.D. Spencer, S. Son, J.A. Ley, and M.R. King. 2016. Improved growth performance of nursery pigs fed diets supplemented with a Bacillus subtilis-based direct-fed microbial feed additive. J. Anim. Sci. 94(Suppl. 2):76(Abstr.) doi:10.2527/msasas2016-162.

Ballevre, O., A. Cadenhead, A.G. Calder, W.D. Rees, G.E. Lobley, M.F. Fuller, and P.J. Garlick. 1990. Quantitative partition of threonine oxidation in pigs: effect of dietary threonine. Am. J. Physiol. 259:E483-E491. doi:10.1152/ajpendo.1990.259.4.E483.

Bergen, W.G., and G. Wu. 2009. Intestinal nitrogen recycling and utilization in health and disease. J. Nutr. 139:821-825. doi:10.3945/jn.109.104497.

Blavi, L., J.N. Jorgensen, and H.H. Stein. 2018. Effects of Bacillus amyloliquefaciens and Bacillus subtilis on ileal digestibility of AA and total tract digestibility of CP and gross energy in diets fed to growing pigs. J. Anim. Sci. 97: 727-734. doi:10.1093/jas/sky432.
Burrough, E.R., B.L. Arruda, J.F. Patience, and P.J. Plummer. 2015. Alterations in the colonic microbiota of pigs associated with feeding distillers dried grains with solubles. PLoS One. 10. doi:10.1371/journal.pone.0141337.

Cai, L., S. Indrakumar, E. Kiarie, I.H. Kim. 2015. Effects of a multi-strain Bacillus species-based direct-fed microbial on growth performance, nutrient digestibility, blood profile, and gut health in nursery pigs fed corn-soybean meal-based diets. J. Anim. Sci. 93:4336-4342. doi:10.2527/jas2015-9056.

Chantawannakul, P., A. Oncharoena, K. Klanbuta, E. Chukeatiroteb, and S. Lummyong. 2002. Characterization of proteases of Bacillus subtilis strain 38 isolated from traditionally fermented soybean in northern Thailand. Sci. Asia. 28:241-245.

Chen, Y.J., K.S. Son, B.J. Min, J.H. Cho, O.S. Kwon, and I.H. Kim. 2005. Effects of dietary probiotic on growth performance, nutrients digestibility, blood characteristics and fecal noxious gas content in growing pigs. Asian-Australas. J. Anim. Sci. 18:1464-1468. doi:10.5713/ajas.2005.1464.

Chesson, A. 1994. Probiotics and other intestinal mediators. In: D.J.A. Cole, J. Wiseman and M.A. Varley, editors, Principles of pig science. Nottingham Univ. Press, Loughborough, UK. p. 197-214.

Davila, A.N., F. Blachier, M. Gotteland, M. Andriamihaja, P.H. Benetti, Y. Sanz, and D. Tome. 2013. Re-print of intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharm. Res. 69:114-126. doi:10.1016/j.phrs.2012.11.005.

Earl, A.M., R. Losick, and R. Kolter. 2008. Ecology and genomics of Bacillus subtilis. Trends Microbiol. 16:269. doi:10.1016/j.tim.2008.03.004.
Giang, H.H., T.Q. Viet, B. Ogle, and J.E. Lindberg. 2012. Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with a complex of lactic acid bacteria alone or in combination with Bacillus subtilis and Saccharomyces boulardii. Livest. Sci. 143:132-141. doi:10.1016/j.livsci.2011.09.003.

Groff, J.L., and S.S. Gropper. 2000. Advanced nutrition and human metabolism. 3rd ed. Wadsworth/Thomson Learning, Belmont, CA.

Guo, X., D. Li, W. Lu, X. Piao, X. Chen. 2006. Screening of Bacillus strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of Bacillus subtilis MA139 in pigs. Antonie Van Leeuwenhoek. 90:139–146. doi:10.1007/s10482-006-9067-9.

Hach, C.C., B.K. Bowden, A.B. Kopelove, and S.V. Brayton. 1987. More powerful peroxide Kjeldahl digestion method. J. AOAC Int. 70:783–787. doi:10.1093/jaoac/70.5.783.

Henry, Y., B. Sève, Y. Colléaux, P. Ganier, C. Saligaut, P. Jégo. 1992. Interactive effects of dietary levels of tryptophan and protein on voluntary feed intake and growth performance in pigs, in relation to plasma free amino acids and hypothalamic serotonin. J. Anim. Sci. 70:1873-1887. doi:10.2527/1992.7061873x.

Isaacson, R. and H.B. Kim. 2012. The intestinal microbiome of the pig. Anim. Health Res. Rev. 13:100-109. doi:10.1017/S1466252312000084.

Johansson, C. 1974. Studies of gastrointestinal interactions. VII. characteristics of the absorption pattern of sugar, fat and protein from composite meals in man. a quantitative study. Scand. J. Gastroenterol. 10:33-42.
Kaewtapee, C., K. Burbach, G. Tomforde, T. Hartinger, A. Camarinha-Silva, S. Heinritz, J. Seifert, M. Wiltafsky, R. Mosenthin, and P. Rosenfelder-Kuon. 2017. Effect of *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. J. Anim. Sci. Biotechol. 8,37. doi:10.1186/s40104-017-0168-2.

Kim, K., Y. He., X. Xiong, A. Ehrlich, X. Li, H. Raybould, E.R. Atwill, E.A. Maga, J. Jorgensen, and Y. Liu. 2019. Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. J. Anim. Sci. Biotechol. 10. doi:10.1186/s40104-019-0364-3.

Kim, Y., S. Ingale, J. Kim, K. Kim, and B. Chae. 2011. Effects of dietary lysine and energy levels on growth performance and apparent total tract digestibility of nutrients in weanling pigs. Asian-Australas. J. Anim. Sci. 24:1256-1267. doi:10.5713/ajas.2011.11134.

Koni, T.N.I., Rusman, C. Hanim, and Zuprizal. 2017. Effect of pH and temperature on *Bacillus subtilis* FNCC 0059 oxalate decarboxylase activity. Pak. J. Biol. Sci. 20:436-441. doi:10.3923/pjbs.2017.436.441.

Kwon, Y.H., H. Wang, E. Denou, J-E. Ghia, L. Rossi, M.E. Fontes, S.P. Bernier, M.S. Shajib, S. Banskota, S.M. Collins, M.G. Surette, and W.I. Khan. 2019. Modulation of gut microbiota composition by serotonin signaling influences intestinal immune response and susceptibility to colitis. Cell. Mol. Gastroenterol. Hepatol. 7:709-728. doi:10.1016/j.jcmgh.2019.01.004.
Lee, S.H., S.L. Ingale, J.S. Kim, K.H. Kim, A. Lokhande, E.K. Kim, I.K. Kwon. 2014. Effects of dietary supplementation with *Bacillus subtilis* LS 1-2 fermentation biomass on growth performance, nutrient digestibility, cecal microbiota and intestinal morphology of weanling pig. Anim. Feed Sci. Technol. 188:102-110. doi:10.1016/j.anifeedsci.2013.12.001.

Lewton, J.R., A.D. Woodward, R.L. Moser, K.M. Thelen, A.J. Moeser, N.L. Trottier, R.J. Tempelman, and D.W. Rozeboom. 2020. Effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial on immunity markers, intestinal morphology, and microbial communities in diets fed to weanling pigs. J. Anim. Sci. 98(Suppl. 4):105-106(Abstr.). doi:10.1093/jas/skaa278.193.

Mao, X., X. Zeng, S. Qiao, G. Wu, and D. Li. 2011. Specific role of threonine in intestinal mucosal integrity and barrier function. Front. Biosci. 3:1192-1200. doi:10.2741/e322.

Meister, A. and M.E. Anderson. 1983. Glutathione. Annu. Rev. Biochem. 52:711-760.

Menegat, M.B., R.D. Goodband, J.M. DeRouchey, M.D. Tokach, J.C. Woodworth, and S.S. Dritz. 2019. Example of swine nursery diets. Kansas State University Swine Extension. https://www.asi.k-state.edu/research-and-extension/swine/premix-and-diet-recommendations.html, accessed: August 25, 2019.

Min, B.J., J.H. Cho, Y.J. Chen, H.J. Kim, J.S. Yoo, Q. Wang, I.H. Kim, W.T. Cho, and S.S. Lee. 2009. Effects of replacing soy protein concentrate with fermented soy protein in starter diet on growth performance and ileal amino acid digestibility in weaned pigs. Asian-Australas. J. Anim. Sci. 22: 99-106. doi:10.5713/ajas.2009.70306.
Myers, W.D., P.A. Ludden, V. Nayigihugu, and B.W. Hess. 2004. Technical Note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide. J. Anim. Sci. 82:179-183. doi:10.2527/2004.821179x.

Nieto, R., R. Barea, L. Lara, P. Palma-Granados, and J.F. Aguilera. 2015. Lysine requirement relative to total dietary protein for optimum performance and carcass protein deposition of Iberian piglets. Anim. Feed Sci. Technol. 206:48-56. doi:10.1016/j.anifeedsci.2015.05.002.

Nortey, T.N., J.F. Patience, P.H. Simmins, N.L. Trottier, and R.T. Zijlstra. 2007. Effects of individual or combined xylanase and phytase supplementation on energy, amino acid, and phosphorus digestibility and growth performance of grower pigs fed wheat-based diets containing wheat millrun. J. Anim. Sci. 85:1432-1443. doi:10.2527/jas.2006-613.

NRC. 2012. Nutrient requirements of swine. 11th rev. ed. Natl. Acad. Press, Washington, DC. p. 210-213.

Pan, L., P.F. Zhao, Z.Y. Yang, S.F. Long, H.L. Wang, Q.Y. Tian, Y.T. Xu, X. Xu, Z.H. Zhang, and X.S. Piao. 2016. Effects of coated compound proteases on apparent total tract digestibility of nutrients and apparent ileal digestibility of amino acids for pigs. Asian-Australas. J. Anim. Sci. 29:1761-1767. doi:10.5713/ajas.16.0041.

Priest, F.G. 1977. Extracellular enzyme synthesis in the genus Bacillus. Bacteriol. Rev. 41:711-753.

Redmond, H.P., P.P. Stapleton, P. Neary, and D. Bouchier-Hayes. 1998. Immunonutrition: the role of taurine. Nutrition. 14:599–604. doi:10.1016/S0899-9007(98)00097-5.
Rincker, M.J., S.D. Carter, D.E. Real, J.L. Nelssen, M.D. Tokach, R.D. Goodband, S.S.
Dritz, B.W. Senne, R.W. Fent, L.A. Pettey, and K.Q. Owen. 2003. Effects of increasing dietary l-carnitine on growth performance of weanling pigs. J. Anim. Sci. 81:2259-2269. doi:10.2527/2003.8192259x.

Schultz, L.L., and C.J. Rademacher. 2017. Food and drug administration guidance 209 and 213 and veterinary feed directive regulations regarding antibiotic use in livestock: a survey of preparation and anticipated impacts in the swine industry. J. Swine Health Prod. 25:247-255.

Silk, D.B.A., G.K. Grimble, and R.G. Rees. 1985. Protein digestion and amino acid and peptide absorption. Proc. Nutr. Soc. 44:63-72. doi:10.1079/PNS19850011.

Stein, H.H., B. Sève, M.F. Fuller, P.J. Moughan, and C.F.M. de Lange. 2007. Invited review: amino acid bioavailability and digestibility in pig feed ingredients: terminology and application. J. Anim. Sci. 85:172-180. doi:10.2527/jas.2005-742.

Tajima, K., and R. Aminov. 2015. Structure and function of a nonruminant gut: a porcine model. A.K. Puniya, R. Singh, and D.N. Kamra, editors, Rumen Microbiology: From Evolution to Revolution. Springer, New Delhi, India, p. 47-79.

Tang, W., Y. Qian, B. Yu, T. Zhang, J. Gao, J. He, Z. Huang, P. Zheng, X. Mao, J. Luo, J. Yu, and D. Chen. 2019. Effects of Bacillus subtilis DSM32315 supplementation and dietary crude protein level on performance, gut barrier function and microbiota profile in weaned piglets. J. Anim. Sci. 97:2125-2138 doi: 10.1093/jas/skz090.

Upadhaya, S.D., S.C. Kim, R.A. Valientes, and I.H. Kim. 2015. The Effect of Bacillus-based Feed Additive on Growth Performance, Nutrient Digestibility, Fecal Gas Emission,
and Pen Cleanup Characteristics of Growing-finishing Pigs. Asian-Australas. J. Anim. Sci. 28, 7: 999-1005. doi:10.5713/ajas.15.0066.

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597. doi:10.3168/jds.S0022-0302(91)78551-2.

Waclawiková, B. and S.E. Aidy. 2018. Role of microbiota and tryptophan metabolites in the remote effect of intestinal inflammation on brain and depression. Pharmaceuticals. 11:63. doi:10.3390/ph11030063.

Walsh, M.C., K.L. Saddoris, D.M. Sholly, R.B. Hinson, A.L. Sutton, T.J. Applegate, B.T. Richert, and J.S. Radcliffe. 2007. The effects of direct-fed microbials delivered through the feed and/or in a bolus at weaning on growth performance and gut health. Livest. Sci. 108:254-257. doi:10.1016/j.livsci.2007.01.051.

Woodward, A.D., M.Z. Fan, R.J. Geor, L.J. McCutcheon, N.P. Taylor, and N.L. Trottier. Characterization of L-lysine transport across equine and porcine jejunal and colonic brush border membrane. J. Anim. Sci. 90:853-862. doi:10.2527/jas.2011-4210.

Yin, J., L. Yuying, H. Han, Z. Liu, X. Zeng, T. Li, and Y. Yin. 2018. Long-term effects of lysine concentration on growth performance, intestinal microbiome, and metabolic profiles in a pig model. Food Funct. 9:4153-4163. doi:10.1039/C8FO00973B.

Yu, G., D. Chen, B. Yu, J. He, P. Zheng, X. Mao, Z. Huang, J. Luo, Z. Zhang, and J. Yu. 2016. Coated protease increases ileal digestibility of protein and amino acids in weaned piglets. Anim. Feed Sci. Technol. 214:142-147. doi:10.1016/j.anifeedsci.2016.02.006.
Table 1. Composition of diets, control and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases, as-fed basis\textsuperscript{1,2}

| Ingredient % | Phase 1, d 0-14 | Phase 2, d 14-28 | Phase 3, d 28-42 |
|--------------|----------------|-----------------|-----------------|
| Corn         | 40.18          | 48.25           | 50.89           |
| Soybean meal, 47.5% CP | 17.30          | 21.15           | 22.65           |
| Corn DDGS, 7.5% oil | 5.00           | 10.00           | 20.00           |
| Dried whey, 72% lactose | 25.00          | 10.00           | -               |
| Fish meal    | 3.00           | 4.50            | -               |
| Spray-dried bovine plasma | 4.00           | -               | -               |
| Corn oil     | 3.00           | 3.00            | 3.00            |
| Calcium carbonate, 38.5% Ca | 0.65           | 0.60            | 0.85            |
| Monocalcium phosphate, 21.5% P | 0.55           | 0.55            | 0.45            |
| Salt         | 0.30           | 0.55            | 0.60            |
| L-Lysine HCl | 0.35           | 0.50            | 0.65            |
| DL-Methionine| 0.15           | 0.15            | 0.14            |
| L-Threonine  | 0.13           | 0.19            | 0.21            |
| L-Tryptophan | 0.03           | 0.06            | 0.06            |
| L-Valine     | 0.07           | 0.10            | 0.10            |
| VTM premix\textsuperscript{3} | 0.25           | 0.25            | 0.25            |
| Phytase      | 0.05           | 0.05            | 0.05            |
| Titanium dioxide\textsuperscript{4} | -              | 0.10            | 0.10            |
| Total, 100%  | 100            | 100             | 100             |

\textsuperscript{1}CON and DFM diets differed only by the inclusion of DFM at 0.05 g/kg or 1.48 × 10\textsuperscript{8} CFU/g of complete feed

\textsuperscript{2}Direct-fed microbial (United Animal Health, Sheridan, IN) had a guaranteed count of 1.48 × 10\textsuperscript{8} CFU/g and was included at a rate of 0.5 g/kg of feed to provide a final count of at least 7.35 × 10\textsuperscript{4} CFU/g of complete feed

\textsuperscript{3}VTM premix provided the following vitamin and microminerals concentrations per kilogram of premix: Zinc 83.4 g, iron 66.7 g, manganese 33.4 g, copper 10 g, iodine 0.3 g, selenium 0.2 g, vitamin A 7,363 KIU, vitamin D 1,177 KIU, vitamin E 44,112 IU, menadione 1.5 g, vitamin B12 0.02 g, riboflavin 4.7 g, pantothenic acid 14.7 g, niacin 29.4 g, thiamine 0.7 g, pyridoxine 2.9 g, folic acid 1.1 g, and biotin 0.1 g.

\textsuperscript{4}Titanium dioxide was included as an indigestible marker in phases two and three at 0.1% of the diet
Table 2. Calculated analysis of diets, control and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases, as-fed basis\(^1\,\,^2\)

| Item                | Phase 1, d 0-14 | Phase 2, d 14-28 | Phase 3, d 28-42 |
|---------------------|-----------------|------------------|------------------|
| ME, kcal/kg         | 3,477           | 3,439            | 3,417            |
| CP, %               | 21.40           | 21.70            | 21.60            |
| Lys SID, %          | 1.40            | 1.35             | 1.30             |
| His SID, %          | 0.48            | 0.47             | 0.48             |
| Ile SID, %          | 0.77            | 0.76             | 0.70             |
| Leu SID, %          | 1.65            | 1.62             | 1.69             |
| Met + Cys SID, %    | 0.78            | 0.76             | 0.73             |
| Thr, SID, %         | 0.88            | 0.85             | 0.82             |
| Trp SID, %          | 0.27            | 0.26             | 0.25             |
| Val SID, %          | 0.97            | 0.93             | 0.90             |
| Ca, %               | 0.78            | 0.74             | 0.59             |
| STTD P, %           | 0.63            | 0.56             | 0.43             |
| Ca:P, ratio         | 1.12            | 1.11             | 1.09             |
| Phytase, FTU/kg     | 257.5           | 257.5            | 257.5            |
| Na, %               | 0.51            | 0.38             | 0.31             |
| Cl, %               | 0.69            | 0.62             | 0.51             |

\(^1\)CON and DFM diets differed only by the inclusion of DFM at 0.05 g/kg or 1.48 × 10\(^8\) CFU/g of complete feed
\(^2\)Direct-fed microbial (United Animal Health, Sheridan, IN) had a guaranteed count of 1.48 × 10\(^8\) CFU/g and was included at a rate of 0.5 g/kg of feed to provide a final count of at least 7.35 × 10\(^4\) CFU/g of complete feed.
Table 3. Analyzed composition of control diet and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\), across all three dietary phases

| Item                  | Phase 1, d 0-14 | Phase 2, d 14-28 | Phase 3, d 28-42 |
|-----------------------|-----------------|------------------|------------------|
| DM, %                 | 91.45           | 91.40            | 90.34            |
| GE, kcal/kg           | 4,608           | 4,611            | 4,597            |
| CP, %                 | 21.70           | 21.40            | 22.00            |
| Crude Fat, %          | 4.50            | 4.30             | 5.20             |
| Crude Fiber, %        | 1.60            | 1.70             | 2.10             |
| NDF, %                | 6.20            | 6.20             | 8.30             |
| ADF, %                | 2.90            | 2.50             | 4.00             |
| Titanium, ppm         | -               | -                | 684              |
| **Indispensable AA, %** |                 |                  |                  |
| Arg                   | 1.10            | 1.10             | 1.10             |
| His                   | 0.51            | 0.51             | 0.50             |
| Ile                   | 0.91            | 0.92             | 0.91             |
| Leu                   | 1.85            | 1.89             | 1.83             |
| Lys                   | 1.58            | 1.53             | 1.56             |
| Met                   | 0.50            | 0.42             | 0.45             |
| Met + Cys             | 0.91            | 0.81             | 0.76             |
| Phe                   | 0.98            | 0.97             | 0.98             |
| Thr                   | 1.00            | 1.03             | 0.97             |
| Trp                   | 0.30            | 0.30             | 0.28             |
| Val                   | 1.13            | 1.15             | 1.08             |
| **Dispensable AA, %**  |                 |                  |                  |
| Ala                   | 1.06            | 1.07             | 1.11             |
| Asp                   | 1.94            | 1.93             | 1.86             |
| Cys                   | 0.41            | 0.39             | 0.31             |
| Glu                   | 3.28            | 3.27             | 3.32             |
| Gly                   | 0.80            | 0.80             | 0.88             |
| Pro                   | 1.17            | 1.19             | 1.23             |
| Ser                   | 0.84            | 0.84             | 0.80             |
| Tyr                   | 0.66            | 0.68             | 0.65             |

\(^1\)Direct-fed microbial (United Animal Health, Sheridan, IN) had a guaranteed count of \(1.48 \times 10^8\) CFU/g and was included at a rate of 0.5 g/kg of feed to provide a final count of at least \(7.35 \times 10^4\) CFU/g of complete feed.
Table 4. Weekly growth performance of control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\)

| Item | Control | DFM   | SEM  | P-value |
|------|---------|-------|------|---------|
| ADG, kg |         |       |      |         |
| Wk 1 | 0.170   | 0.160 | 0.01 | 0.31    |
| Wk 2 | 0.295\(^y\) | 0.341\(^x\) | 0.02 | 0.08    |
| Wk 3 | 0.428   | 0.446 | 0.02 | 0.54    |
| Wk 4 | 0.581   | 0.573 | 0.03 | 0.82    |
| Wk 5 | 0.722   | 0.751 | 0.02 | 0.35    |
| Wk 6 | 0.832   | 0.809 | 0.03 | 0.59    |
| Overall | 0.507   | 0.513 | 0.01 | 0.67    |
| ADFI, kg |         |       |      |         |
| Wk 1 | 0.274   | 0.260 | 0.01 | 0.25    |
| Wk 2 | 0.399\(^b\) | 0.436\(^a\) | 0.01 | 0.04    |
| Wk 3 | 0.630\(^b\) | 0.693\(^a\) | 0.02 | 0.02    |
| Wk 4 | 0.854   | 0.877 | 0.02 | 0.49    |
| Wk 5 | 1.146   | 1.165 | 0.03 | 0.63    |
| Wk 6 | 1.359   | 1.401 | 0.04 | 0.41    |
| Overall | 0.777   | 0.805 | 0.02 | 0.23    |
| G:F   |         |       |      |         |
| Wk 1 | 0.619   | 0.618 | 0.02 | 0.98    |
| Wk 2 | 0.735   | 0.779 | 0.03 | 0.25    |
| Wk 3 | 0.680   | 0.642 | 0.02 | 0.28    |
| Wk 4 | 0.679   | 0.653 | 0.02 | 0.28    |
| Wk 5 | 0.630   | 0.646 | 0.01 | 0.44    |
| Wk 6 | 0.613\(^a\) | 0.577\(^b\) | 0.01 | 0.04    |
| Overall | 0.659   | 0.653 | 0.01 | 0.58    |

\(^1\)Direct-fed microbial (United Animal Health, Sheridan, IN), performance data taken from n=8 pens per treatment, 5 pigs per pen from d 0-21, 4 pigs per pen from d 21-42

\(^a,b\)Values in a common row lacking a common superscript differ (P ≤ 0.05)

\(^x,y\)Values in a common row lacking a common superscript tend to differ (P ≤ 0.10)
Table 5. Apparent jejunal digestibility of indispensable and dispensable amino acids, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\)

| Item          | Control | DFM | SEM  | P-value |
|---------------|---------|-----|------|---------|
| **Indispensable AA, %** |         |     |      |         |
| Arg           | 57.00   | 64.96| 3.64 | 0.13    |
| His           | 47.20   | 55.51| 3.64 | 0.11    |
| Ile           | 51.26   | 57.67| 3.20 | 0.17    |
| Leu           | 54.27   | 58.58| 3.20 | 0.35    |
| Lys           | 61.62\(\text{y}\) | 70.61\(\text{x}\) | 3.40 | 0.07    |
| Met           | 67.09\(\text{y}\) | 73.44\(\text{x}\) | 2.22 | 0.06    |
| Met+Cys       | 46.20\(\text{y}\) | 55.31\(\text{x}\) | 3.70 | 0.07    |
| Phe           | 54.63   | 59.81| 2.88 | 0.21    |
| Thr           | 49.36\(\text{y}\) | 58.47\(\text{x}\) | 3.61 | 0.08    |
| Trp           | 56.92\(\text{b}\) | 68.02\(\text{a}\) | 3.34 | 0.04    |
| Val           | 49.77   | 57.63| 3.56 | 0.13    |
| **Dispensable AA, %** |         |     |      |         |
| Ala           | 47.37   | 53.11| 3.44 | 0.24    |
| Asp           | 43.57   | 52.06| 3.83 | 0.13    |
| Cys           | 12.55\(\text{b}\) | 29.41\(\text{a}\) | 5.32 | 0.04    |
| Glu           | 49.70   | 54.84| 3.11 | 0.25    |
| Gly           | -8.69   | -1.47| 8.84 | 0.57    |
| Pro           | 44.17   | 49.66| 3.87 | 0.32    |
| Ser           | 43.88   | 51.94| 3.76 | 0.14    |
| Tyr           | 51.59   | 58.13| 3.37 | 0.18    |
| All indispensable AA, % | 57.89   | 62.31| 3.17 | 0.31    |
| All dispensable AA, % | 45.00   | 48.14| 3.93 | 0.55    |
| All AA, %     | 47.87   | 54.44| 3.43 | 0.18    |

\(^1\)Direct-fed microbial (United Animal Health, Sheridan, IN), digestibility coefficients within jejunum, n=6-8 representative pigs per treatment at both d 21 and 42, n=3-4 for Trp d 21 due to lack of sufficient sample collection

\(^\text{a,b}\)Values in a common row lacking a common superscript differ (\(P \leq 0.05\))

\(^\text{x,y}\)Values in a common row lacking a common superscript tend to differ (\(P \leq 0.10\))
Table 6. Apparent digestibility of indispensable amino acids, across segments of the gastrointestinal tract, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\)

| Item | %     | Control | DFM   | SEM  | P-value |
|------|-------|---------|-------|------|---------|
| Arg  |       |         |       |      |         |
| Jejunum | 57.00 | 64.96   | 3.64  | 0.13 |
| Ileum | 74.80 | 76.27   | 3.34  | 0.76 |
| Ascending colon | 74.63 | 70.74   | 4.50  | 0.54 |
| His  |       |         |       |      |         |
| Jejunum | 47.20 | 55.51   | 3.64  | 0.11 |
| Ileum | 66.56 | 66.02   | 3.69  | 0.92 |
| Ascending colon | 69.21 | 65.60   | 4.93  | 0.60 |
| Ile |       |         |       |      |         |
| Jejunum | 51.26 | 57.67   | 3.20  | 0.16 |
| Ileum | 67.87 | 68.27   | 3.66  | 0.94 |
| Ascending colon | 58.83 | 57.31   | 6.35  | 0.86 |
| Leu  |       |         |       |      |         |
| Jejunum | 54.27 | 58.58   | 3.20  | 0.35 |
| Ileum | 68.74 | 68.02   | 3.54  | 0.88 |
| Ascending colon | 65.95 | 62.23   | 5.27  | 0.61 |
| Lys  |       |         |       |      |         |
| Jejunum | 61.62\(^y\) | 70.61\(^x\) | 3.40 | 0.07 |
| Ileum | 73.28 | 75.99   | 3.35  | 0.57 |
| Ascending colon | 68.89 | 66.80   | 5.02  | 0.76 |
| Met  |       |         |       |      |         |
| Jejunum | 67.09\(^y\) | 73.44\(^x\) | 2.22 | 0.06 |
| Ileum | 76.23 | 78.43   | 2.64  | 0.55 |
| Ascending colon | 63.96 | 62.25   | 5.48  | 0.82 |
| Phe  |       |         |       |      |         |
| Jejunum | 54.63 | 59.81   | 2.88  | 0.21 |
| Ileum | 68.16 | 68.38   | 3.52  | 0.96 |
| Ascending colon | 65.16 | 61.04   | 5.36  | 0.58 |
| Thr  |       |         |       |      |         |
| Jejunum | 49.36\(^y\) | 58.47\(^x\) | 3.61 | 0.08 |
| Ileum | 62.50 | 62.31   | 4.10  | 0.97 |
| Ascending colon | 62.81 | 57.21   | 5.91  | 0.49 |
| Trp  |       |         |       |      |         |
| Jejunum | 56.92\(^b\) | 68.02\(^a\) | 3.34 | 0.04 |
| Ileum | 72.16 | 73.53   | 3.29  | 0.78 |
| Ascending colon | Val | Jejunum | Ileum | Ascending colon |
|-----------------|-----|---------|-------|----------------|
| 76.94           | 76.12| 3.58    | 0.87  |                |
| 49.77           | 57.63| 3.56    | 0.13  |                |
| 65.64           | 65.99| 3.93    | 0.95  |                |
| 57.78           | 56.46| 6.54    | 0.88  |                |

Direct-fed microbial (United Animal Health, Sheridan, IN), all values are overall LSmeans of d 21 and 42 combined data, total amino acids were not reported in distal colon due to insufficient sample size, n=12 to 16 representative pigs per treatment, n=8 to 11 for Trp due to insufficient sample collection.

*Values in a common row lacking a common superscript differ (P ≤ 0.05)*

*1Values in a common row lacking a common superscript tend to differ (P ≤ 0.10)*
Table 7. Apparent digestibility of indispensable amino acids, by week within the jejunum, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\)

| Item | Control | DFM | SEM | P-value |
|------|---------|-----|-----|---------|
| Arg  | Wk 3    | 46.98 | 58.51 | 4.77 | 0.53 |
|      | Wk 6    | 67.01 | 71.41 | 4.24 | 0.98 |
| His  | Wk 3    | 34.62 | 46.37 | 5.20 | 0.60 |
|      | Wk 6    | 59.78 | 64.65 | 4.53 | 0.97 |
| Ile  | Wk 3    | 41.52 | 50.04 | 4.66 | 0.79 |
|      | Wk 6    | 61.00 | 65.31 | 4.07 | 0.97 |
| Leu  | Wk 3    | 44.22 | 49.18 | 4.69 | 0.97 |
|      | Wk 6    | 64.31 | 67.98 | 4.07 | 0.99 |
| Lys  | Wk 3    | 52.30 | 62.46 | 4.61 | 0.64 |
|      | Wk 6    | 70.94 | 78.75 | 4.03 | 0.74 |
| Met  | Wk 3    | 60.81 | 67.69 | 3.24 | 0.70 |
|      | Wk 6    | 73.36 | 79.19 | 2.82 | 0.69 |
| Phe  | Wk 3    | 43.79 | 50.67 | 4.19 | 0.85 |
|      | Wk 6    | 65.47 | 68.95 | 3.65 | 0.98 |
| Thr  | Wk 3    | 38.63 | 49.82 | 5.20 | 0.65 |
|      | Wk 6    | 60.09 | 67.11 | 4.53 | 0.88 |
| Trp  | Wk 3    | 47.07 | 63.53 | 5.28 | 0.35 |
|      | Wk 6    | 66.78 | 72.50 | 3.84 | 0.93 |
| Val  | Wk 3    | 40.64 | 50.28 | 5.17 | 0.77 |
|      | Wk 6    | 58.90 | 65.00 | 4.51 | 0.93 |

\(^{1}\)Direct-fed microbial (United Animal Health, Sheridan, IN), digestibility coefficients within jejunum, n=6-8 representative pigs per treatment at both d 21 and 42, n=3-4 for Trp d 21 due to lack of sufficient sample collection

\(^{a,b}\)Values in a common row lacking a common superscript differ (P ≤ 0.05)

\(^{x,y}\)Values in a common row lacking a common superscript tend to differ (P ≤ 0.10)
Table 8. Apparent digestibility of gross energy, nitrogen, and total amino acids across segments of the gastrointestinal tract, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)\(^1\)

| Item, % | Control | DFM  | SEM  | P-value |
|---------|---------|------|------|---------|
| Gross Energy |         |      |      |         |
| Jejunum  | 52.00   | 53.59| 3.26 | 0.73    |
| Ileum    | 62.13   | 60.00| 3.77 | 0.69    |
| Ascending colon | 70.18 | 65.20| 4.53 | 0.42    |
| Distal colon | 77.60 | 69.15| 3.52 | 0.13    |
| Nitrogen |         |      |      |         |
| Jejunum  | 43.17   | 45.18| 4.01 | 0.73    |
| Ileum    | 55.77   | 59.21| 4.53 | 0.59    |
| Ascending colon | 57.39 | 53.50| 6.25 | 0.65    |
| Distal colon | 71.31   | 58.91| 4.22 | 0.05    |
| Total amino acids |         |      |      |         |
| Jejunum  | 47.87   | 54.44| 3.43 | 0.18    |
| Ileum    | 65.01   | 64.96| 3.85 | 0.99    |
| Ascending colon | 65.93 | 61.22| 5.33 | 0.52    |

\(^1\)Direct-fed microbial (United Animal Health, Sheridan, IN). all values are overall LSmeans of d 21 and 42 combined data, total amino acids were not reported in distal colon due to insufficient sample collection, n=6 to 8 representative pigs per treatment for both d 21 and 42.

\(^{a,b}\)Values in a common row lacking a common superscript differ \((P \leq 0.05)\)

\(^{x,y}\)Values in a common row lacking a common superscript tend to differ \((P \leq 0.10)\)