Effects of in-feed additives on performance, gut microbe ecology, and antimicrobial susceptibility of enterobacteria on nursery pigs

by

Hayden Ervin Williams

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Approved by:

Co-Major Professor
Dr. Joel DeRouchey

Approved by:

Co-Major Professor
Dr. Jason Woodworth

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Abstract

Two experiments using a total of 720 nursery pigs were used to determine the effects of Elarom SES, in-feed antibiotics, zinc, or copper on nursery pig growth performance and fecal consistency. Two experiments using a total of 1,534 nursery pigs were used to determine the effects of formaldehyde inclusion, lysine level, and synthetic amino acid inclusion on nursery pig performance, amino acid utilization, and gut microbial community. One experiment using a total of 300 nursery pigs were used to determine the effects of chlortetracycline (CTC) or a probiotic inclusion on nursery pig growth performance and antimicrobial susceptibility. Experiment 1 determined the effect of Elarom SES, in-feed antibiotics, or zinc on nursery pig performance and fecal consistency. The addition of Elarom SES or ZnO alone reduced ADG, but G:F was poorest when all three additives were fed in combination. Addition of in-feed antibiotics increased ADG and G:F throughout the study. Experiment 2 determined the effects of Elarom SES or copper inclusion on nursery pig performance and fecal consistency. The addition of Elarom SES or increasing copper did not provide consistent benefits in performance. In both experiments, there were no individual or overall treatment effects or treatment × day interactions observed for fecal consistency. Experiment 3 compared the effects of formaldehyde source and lysine level on nursery pig growth performance. Regardless of source or lys level, the inclusion of formaldehyde in nursery pig diets marginally reduced ADG and resulted in poorer G:F. Experiment 4 compared the effects of formaldehyde and synthetic amino acid inclusion level on nursery pig growth performance, amino acid utilization, and gut microbial community. The inclusion of Sal CURB in diets reduced ADG and ending BW while inclusion decreased ADFI. ADFI response was dependent on synthetic amino acid level in the diet. Sal CURB inclusion in diets reduced total and available lysine, but reduced bacterial microflora in treatment feed. Experiment 5

determined the effects of CTC or a probiotic on nursery pig performance and antimicrobial susceptibility. The addition of CTC to diets improved ADG, ADFI, and ending BW. The addition of Poultry Star improved ADFI and d 14 BW, but benefits did not carry throughout the study.

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Finally, to Mom, Dad, Hayley, Hadley, Peyton, Holden, Hudson, Hesson, and Kirstin, I would not be who I am without your love, support, and patience. I am forever grateful for this.

Dedication

This thesis is dedicated to my parents Noel and Jacque Williams.

Chapter 1 - Evaluation of Elarom SES with or without dietary in-feed antibiotics and pharmacological levels of zinc or copper on nursery pig performance

ABSTRACT

Weanling pigs (n=720; 200 × 400 DNA, Columbus, NE; initially 5.2 ± 0.04 kg BW in Exp.1; 6.0 ± 0.13 kg BW in Exp. 2) were used in 2 separate 42-d growth trials to evaluate the effects of feeding Elarom SES in combination with pharmacological levels of Zn, Cu, or in-feed antibiotics on nursery pig performance and fecal consistency. Elarom SES (Trouw Nutrition USA, Highland, IL) is a commercially available blend of fatty acids and slow release organic acids designed to enhance pig performance and gut health. Pigs were weaned (~21 d of age) and allotted to pens based on initial BW in a completely randomized block design. Experimental diets were fed in 3 phases (d 0 to 7; d 7 to 21 and d 21 to 42). In Exp.1, dietary treatments were arranged as a 2 × 2 × 2 factorial with main effects of Elarom SES (none vs. 0.20%), additional Zn from ZnO (none vs. 3,000 mg/kg in Phase 1, 2,000 mg/kg in Phase 2, and none in Phase 3), and in-feed antibiotic regimen (none vs. 400 mg/kg Chlortetracycline and 35 mg/kg Tiamulin in Phase 1 and 50 mg/kg Carbadox in Phases 2 and 3). Overall, an Elarom SES × Zn × antibiotic interaction was observed for ADG ($P = 0.043$) and G:F ($P = 0.009$). In general, the antibiotic regimen increased ($P < 0.013$) ADG and G:F, but the response to added Zn was dependent on the addition of the antimicrobials. When Elarom SES or ZnO were added alone, ADG was poorest, while G:F was poorest when all three ingredients were added in combination. There were no individual or overall treatment effects or treatment × day interactions observed for fecal consistency. In Exp. 2, experimental diets were arranged as a 2 × 3 factorial with main effects of

Elarom SES (none vs. 0.20% in all phases) and tribasic copper chloride (none, 108, or 183 mg/kg of Cu in phase 3 only; TBCC, Micronutrients, Indianapolis, IN). Overall, there was no treatment differences observed for ADG, ADFI, or fecal consistency, but a tendency for an Elarom SES × TBCC interaction was observed for G:F (quadratic, $P = 0.058$). The interaction was a result of G:F being improved at the intermediary level of TBCC without Elarom SES inclusion, but G:F was improved at the highest TBCC inclusion when Elarom SES was present. In conclusion, no consistent benefit was observed from supplementing diets with Elarom SES with or without antibiotics, zinc or copper. The improvements in performance observed from feeding in-feed antibiotics or high levels of zinc oxide were similar to previous studies.

Key words: antibiotic, copper, Elarom SES, growth performance, nursery, zinc

INTRODUCTION

The addition of in-feed antibiotics and pharmacological levels of Cu and Zn to nursery pig diets is routinely practiced in the U.S. swine industry. Producers add in-feed antibiotics at sub-therapeutic levels to capture the growth promoting benefits, even in the absence of a health challenge. The efficacy of in-feed antibiotics has long been established in nursery pig diets (Elliot et al., 1964; Stahly et al., 1980; Roof and Mahan, 1982). Furthermore, addition of pharmacological levels of Cu and Zn to nursery diets has been shown to enhance growth performance (Smith et al., 1997; Hill et al., 2001; Armstrong et al., 2004). However, as movements towards antibiotic-free diets as well as environmental concerns with pharmacological concentrations of Cu and Zn increase, new technologies to help offset losses in growth performance need to be developed and their efficacy confirmed.

Elarom SES (Trouw Nutrition USA, Highland, IL) is a technology that combines the use of slow-release medium and short chain fatty acids, phenolic compounds, and slow release organic acids to serve as a potential alternative strategy to the addition of in-feed antimicrobials and pharmacological concentrations of Cu and Zn. Preliminary research has shown the inclusion of Elarom SES in nursery pig diets increased ADG and improved G:F compared to control diets without in-feed antibiotics (Trouw Nutrition, 2016); however, the effect of Elarom SES included in diets with or without in-feed antibiotics or pharmacological levels of Cu or Zn has not been characterized.

Therefore, the objective of these trials were to compare the growth performance and fecal consistency of nursery pigs fed diets containing Elarom SES, in-feed antibiotics, Zn, and/or Cu.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this experiment.

General

Similar protocols were used in both experiments. Pigs (200 × 400, DNA, Columbus, NE) were weaned at approximately 21 d of age and allotted to pens based on initial BW. Both experiments were conducted at the Kansas State University Segregated Early Weaning Facility, Manhattan, KS. Each pen (1.22 × 1.22 m) had metal tri-bar flooring, one 4-hole self-feeder, and a cup waterer to provide ad libitum access to feed and water. Dietary treatments were fed for 42 d in 3 different phases (Phase 1: d 0 to 7; Phase 2: d 7 to 21; Phase 3: d 21 to 42). Pigs and feeders were weighed on d 0, 7, 14, 21, 28, 35, and 42 to calculate ADG, ADFI, and G:F.

Fecal scoring of pens occurred on d 0, 4, 7, 14, 21, 28, 35, and 42 by visual appraisal of the pen floors. Fecal scores were conducted before weighing on weigh days and were replicated

by 3 individuals each day. Pens were scored on a scale from 1 to 5 with 1 indicating hard pellet type feces; 2 indicating firm, formed feces; 3 indicating soft, moist feces that retained shape; 4 indicating soft, unformed feces; and 5 indicating watery, liquid feces.

Diet Preparation

All diets were prepared at the O.H. Kruse Feed Technology and Innovation Center located in Manhattan, KS. Dietary treatments were corn-soybean meal-based and fed in meal form. Phase 1 and 2 diets contained specialty protein ingredients and all diets were formulated according to the Nutrient Requirements of Swine (NRC, 2012) to be at or be above the pigs' daily nutrient requirement estimates as not to limit growth performance. Each diet contained 110 ppm of added Zn from ZnO and 17 ppm added Cu from $CuSO_4$ from the trace mineral premix. The treatment ingredients were substituted for an equivalent amount of corn in the respective diets to form the experimental diets. During feed manufacturing, when bagging the experimental diets, feed samples were collected from the 5^{th}, 10^{th}, 15^{th}, 20^{th}, 25^{th}, 30^{th}, 35^{th}, and 40^{th} bags, and these samples were pooled and used for nutrient analysis.

Chemical analysis

One sample of each dietary treatment from the pooled feed samples was sent to a commercial laboratory (Ward Laboratories, Kearney, NE) for analysis of DM (AOAC 935.29; 2012), CP (AOAC 990.03; 2012), Ca (AOAC 965.14/985.01, 2012), P (AOAC 965.17/985.01, 2012), Zn, and Cu. Samples for Zn and Cu were prepared using the method outlined by the AOAC (2012) and analyzed using an iCAP 6000 series ICP Emission Sprectrometer (Thermo Electron Corporation, Marietta, OH).

Experiment 1

A total of 360 weanling pigs (initially 5.2 ± 0.04 kg BW) were used to evaluate the effects of Elarom SES, ZnO, or antibiotic regimen on nursery pig performance and fecal consistency in a 42-d growth trial. Pigs were weaned and allotted to pens based on initial BW to 1 of 8 dietary treatments arranged in a 2 × 2 × 2 factorial. There were 9 pens per treatment and 5 pigs per pen. The 8 dietary treatments (Table 1-1) consisted of added Zn from ZnO (none vs. 3,000 ppm Zn from d 0 to 7, 2,000 ppm Zn from d 7 to 21, and no additional Zn above that provided in the trace mineral premix from d 21 to 42), in-feed antibiotics (none vs. 400 mg/kg CTC (Zoetis Services, LLC., Florham Park, NJ) and 35 mg/kg Denagard (Elanco Animal Health, Greenfield, IN) from d 0 to 7 and 50 mg/kg Mecadox-2.5 (Phibro Animal Health, Teaneck, NJ) from d 7 to 42), or Elarom SES (none vs. 0.20% from d 0 to 42; Trouw Nutrition USA, LLC., Highland, IL).

Experiment 2

A total of 360 nursery pigs (initially 6.0 ± 0.13 kg BW) were used to evaluate the effects of Elarom SES in nursery diets with the inclusion of varying levels of TBCC (Intellibond C, Micronutrients, Indianapolis, IN) on nursery pig performance and fecal consistency. Pigs were weaned and allotted to pens based on initial BW to 1 of 6 dietary treatments arranged in a 2 × 3 factorial. There were 12 pens per treatment and 5 pigs per pen. The 6 dietary treatments (Table 1-2) consisted of added Elarom SES (none vs. 0.20% from d 0 to 42) or added Cu from TBCC (none vs. 108 vs. 183 ppm Cu from d 21 to 42). From d 0 to 21, all diets only contained the Cu provided by the trace mineral premix.

Statistical analysis

For both experiments, growth data were analyzed as a randomized complete block design with pen as the experimental unit using the GLIMMIX procedure of SAS (SAS Institute, Cary, NC). Treatment was considered the fixed effect and a random effect of block was used in analysis. Differences between treatments were determined by using least squares means. Results were considered to be significant with *P*-values ≤ 0.05 and were considered marginally significant with *P*-values ≤ 0.10.

For Exp. 1 the main effects of Zn, Elarom SES, and in-feed antibiotics, as well as their interactions, were evaluated using preplanned CONTRAST statements. For Exp. 2 the main effects of Elarom SES and linear and quadratic effects of TBCC, as well as their interactions, were evaluated using preplanned CONTRAST statements. For both trials, fecal consistency scores were analyzed using the MIXED procedure of SAS with pen as the experimental unit and a random effect of barn used in analysis.

RESULTS

Chemical Analysis

In Exp. 1, results of DM, CP, Ca, P, and Zn analysis closely matched formulated values (Table 1-3). Analyzed diets confirmed diets manufactured with no added ZnO contained approximately 110 ppm ZnO from the trace mineral premix, and phase 1 and phase 2 diets manufactured with added ZnO contained increased levels of Zn as expected.

Similarly, for Exp. 2, results of the analysis closely matched those of formulated levels (Table 1-4). Analyzed diets confirmed diets manufactured with no added Cu contained approximately 17 ppm Cu from the trace mineral premix, and phase 3 diets manufactured with added Cu from TBCC increased in a step-wise fashion as expected.

Growth Performance

Experiment 1

From d 0 to 7, an Elarom SES × ZnO interaction ($P = 0.016$) was observed for G:F (Table 1-5). The interaction occurred because pigs fed Elarom SES in combination with ZnO had improved G:F compared to pigs fed diets containing only Elarom SES or ZnO with the control diet intermediate. Pigs fed diets containing in-feed antimicrobials had improved ADG ($P = 0.05$) compared to those without. There was no response detected for ZnO or Elarom SES for ADG or ADFI.

From d 7 to 21, the inclusion of added ZnO increased ($P < 0.001$) ADG, ADFI, and d 21 BW. Additionally, diets containing in-feed antibiotics had increased ($P < 0.001$) ADG, ADFI, and G:F.

On d 21, the high level of ZnO was removed from diets for pigs fed the ZnO treatments. From d 21 to 42, an Elarom SES × ZnO × antibiotic interaction ($P = 0.006$) was observed for G:F. This interaction occurred because G:F was poorest for the treatment where all three feed additives were fed in combination compared to all other treatments. An Elarom SES × antibiotic interaction for ADFI ($P = 0.013$) was observed. This was the result of ADFI being similar to control values when Elarom SES was fed alone ; however, when antimicrobial was added to the diet, ADFI was increased, with the diet with both antimicrobial and Elarom SES being intermediate.

Overall (from d 0 to 42), an Elarom SES × ZnO × antibiotic interaction was observed for ADG ($P = 0.043$) and G:F ($P = 0.009$). The ADG interaction was the result of the poorest ADG observed when Elarom SES or ZnO were added alone compared to when antibiotic was added alone. Furthermore, this interaction occurred because pigs fed the combination of all three

additives had poorer ADG than pigs fed the combinations of Elarom SES added with ZnO or ZnO added with antibiotic. The G:F interaction was a result of a combination of observations. First, the poorest G:F was observed when all three additives were fed in combination compared to the control or diets with Elarom SES or antibiotic only. Pigs fed all three additives also had poorer G:F than pigs fed diets containing Elarom SES and ZnO in combination or ZnO and antibiotic in combination. Overall, ADFI was increased ($P < 0.001$) when in-feed antimicrobials were added.

While there was no treatment or treatment × day effects observed on fecal consistency, a day effect was observed ($P = 0.001$; Table 1-7) resulting from pigs exhibiting softer stool on d 0, 4, and 7 with stools improving to a firmer stool in the subsequent collection days.

Experiment 2

From d 0 to 21, d 21 to 42, and overall, there were no differences in growth performance observed between pigs fed any of the dietary treatments. A tendency for an Elarom × TBCC interaction ($P = 0.058$) was observed (Table 1-6). The interaction was a result of G:F being improved at the intermediary level of TBCC without Elarom SES inclusion, but G:F was improved at the highest TBCC inclusion when Elarom SES was present.

There were no treatment or treatment × day effects observed on fecal consistency, but a day effect was observed ($P = 0.001$; Table 1-8) resulting from pigs at d 0 exhibiting firmer stool that transitioned to a softer stool in the subsequent collection days.

DISCUSSION

An abundance of research has been conducted in regard to nursery pig feeding strategies and dietary supplementation of feed additives to improve gut health and nutrient uptake. This research is in response to growth and health challenges of the weanling pig. These challenges can

be attributed to social, environmental, and physiological changes that pigs experience during this post-weaning period (Heo et al., 2013). The weaning process causes a social stressor to nursery pigs because it removes constant social interaction with the sow and disrupts established social hierarchy within the littermates by mixing pigs from other litters. Nursery pigs also must adapt to a new environment and establish new routines for eating and drinking (Lalles et al., 2007). Furthermore, nursery pigs undergo significant changes to their gut morphology during the subsequent time post-weaning. These morphological changes attributed to weaning stress include villous atrophy, reduction in brush-border enzyme activity, and a reduction in the absorption of nutrients in the small intestine (Miller et al.,1986; Pacha et al., 2000). Because of these stresses, a post-weaning lag is observed in nursery pigs that is marked by a decrease in feed intake, reduced performance, and noticeable post-weaning diarrhea (Pluske et al., 1997).

Elliot (1964) produced the earliest research on the effects of in-feed antibiotics supplementation to nursery diets. This early study demonstrated that addition of in-feed antibiotics to nursery diets resulted in improved ($P < 0.05$) ADG, ADFI, and G:F compared to pigs fed diets not containing in-feed antibiotics. Subsequent research has shown consistent results for growth promoting aspects of in-feed antibiotics that result in improved ADG, ADFI, and G:F which in turn produces a 0.6 to 0.9 kg heavier pig at the end of the nursery phase (Stahly et al., 1980; Roof and Mahan, 1982). These results are consistent with the in-feed antibiotics effect found in the current study with improved ADG, ADFI, G:F, and ending BW similar to that of previous research.

The supplementation of Zn, most commonly in the form of ZnO, to nursery diets is practiced in an effort to reduce the occurrence of diarrhea associated with weaning and to capture growth promotion effects similar to that of in-feed antimicrobials. Holm (1988) observed that the

addition of up to 4,000 mg/kg Zn in nursery diets improved growth and reduced mortality rates of piglets compared to piglets fed control diets. Hahn and Baker (1993) observed similar improvements in ADG and ADFI in pigs fed either 3,000 or 5,000 mg/kg of ZnO compared to pigs fed basal diets.

Management of dietary Zn addition to nursery diets in regards to duration must be considered. A study conducted by Carlson et al. (1999) found that phase feeding ZnO to early- and traditionally weaned pigs for a minimum of 2 weeks after weaning is needed to improve growth rates. The researchers found pigs fed high ZnO for 2 weeks or 4 weeks after weaning had the greatest ADG compared to pigs fed a control diet for 4 weeks. Hill et al. (2001) further discovered that in a 28-d growth trial, early- or traditionally weaned pigs had improvements in growth parameters that plateaued at a ZnO supplementation level of 2,000 ppm. A 35-d study conducted by Buff et al. (2005) found that in phase 1 (d 0 to 14) pigs fed diets supplemented with 2,000 ppm ZnO had greater ADG and G:F compared to pigs fed control diets, but only saw a tendency for improved ADG in phase 2 (d 14 to 35). The results from the current study are similar to previous research with pigs fed diets containing pharmacological levels of ZnO having improved ADG and ADFI compared to pigs fed diets not containing supplemental ZnO in the first 3 weeks. Although in our study overall performance was similar for pigs fed ZnO, this could be due to the removal of ZnO in phase 3 of pigs fed diets formerly containing ZnO. No conclusive evidence exists as to why pigs on this specific treatment elicited this observation and this has not been observed in prior research.

Supplementation of high levels of Cu is a feeding strategy considered in the nursery because of its antimicrobial effects that are potentially similar to that of antibiotics and the ability of Cu to counter the effects of the post-weaning lag. Stahly et al. (1980) conducted a series of

trials to determine the effect of Cu supplementation to nursery diets. The researchers found that addition of 250 ppm Cu to nursery diets improved ($P < 0.05$) ADG and G:F. Further studies by Cromwell et al. (1978) found that Cu fed as $CuSO_4$ at levels of 125 or 250 ppm stimulated growth rate and efficiency of nursery pigs in a manner more effective than higher levels of Cu or Cu oxide as the Cu source. The Cu source used in experiment 2 of this series of studies was tribasic copper chloride (TBCC). Tribasic copper chloride has been shown to have the potential to be more efficient at promoting nursery pig growth when supplemented to diets at lower levels than $CuSO_4$. Cromwell et al. (1998) observed a tendency of improvement in G:F when feeding 100 or 200 ppm of TBCC. A series of experiments conducted by Shelton et al. (2011) observed the addition of 150 ppm TBCC from d 0 to 42 post-weaning improved ($P < 0.007$) ADG and ADFI. The results from experiment 2 of the current study are inconsistent with previous research. A possible reason for this lack of Cu response could be due to not including copper prior to phase 3 of the study. This would suggest that the duration of Cu feeding within the nursery stage of production plays a role in observing a Cu response. No linear or quadratic effects of TBCC supplementation to nursery diets were observed. The lack of response was similar to the observations of a study conducted by Stansbury et al. (1990) that found no effect of dietary chelated Cu source or $CuSO_4$ on nursery pig performance.

Elarom SES is a proprietary blend of short and medium chain fatty acids, phenolic compounds, and slow release organic acids that is designed as a potential antibiotic alternative to improve nursery pig growth performance. The potential improvement in growth performance is thought to be due to stabilization of gut microbiota and strengthened intestinal barrier against enteric pathogens commonly associated with weaning. Preliminary research has shown improvements in ADG and G:F of nursery pigs fed diets supplemented with Elarom SES

compared to pigs fed control diets (Trouw Nutrition, 2016). Limited research has been conducted in the areas of how Elarom SES and in-feed antibiotics and ZnO and Cu supplementation to nursery diets interact. The literature on MCFAs, phenolic compounds, and organic acids shows inconsistencies in growth responses when these additives are supplemented to nursery diets. These inconsistencies support the finding in the current study in that the addition of Elarom SES to diets containing in-feed antimicrobials does not elicit an additive effect. This is evident from the Elarom SES × antimicrobial interaction that occurred from d 21 to 42. The inclusion of antibiotics alone improved ADG, ADFI, and G:F compared to pigs fed diets containing Elarom SES and antibiotics in combination.

Medium chain fatty acids (MCFAs) are comprised of 6-12 carbons long fatty acids. These fatty acid chains exhibit known antimicrobial effects against gut pathogens (Dierick et al., 2002) and can be found in various fats such as coconut and palm oil. Medium chain fatty acids have the ability to reduce inflammation of the gut associated with weaning by decreasing activation of inflammatory factors and exhibit antimicrobial effects by inactivating pathogenic bacteria and viruses through membrane destabilization (Zentek, 2011). Although this technology is viewed as a potential alternative to supplementing antimicrobials to nursery diets, results are varying. Cera et al. (1990) observed that addition of MCFAs to nursery diets had poorer growth rates comparative to pigs fed a control diet. Furthermore, Devi and Kim (2014) observed that nursery pigs fed diets supplemented with MCFAs performed similar to nursery pigs fed control diets. In comparison to these experiments with little to no response, Hong et al. (2012) found that addition of MCFAs to diets of nursery pigs improved ($P < 0.05$) ADG compared to pigs fed control diets.

Phenolic compounds, otherwise known as phytogenic compounds, are plant-derived products that are used as feed additives in swine diets. These plant-based products are theorized to contain antioxidant properties and have the potential to exert antimicrobial effects against pathogens (Windisch et al., 2008). The efficacy of supplementation to swine diets with these plant-based products is inconsistent. Research has shown that phytogenic compounds have no effect and in some cases negative effects on nursery pig growth performance in comparison to more common additives such as antibiotics (Manzanilla et al., 2006; Namkung et al., 2004; Lien et al., 2007). Although, some research indicates an improvement in performance is observed when phytogenics are supplemented to nursery pig diets compared to diets fed without these plant-based products (Upadhaya et al., 2016). The current studies indicate that inconsistencies still exist for phytogenics because of the lack of response with the addition of Elarom SES to nursery pig diets.

Organic acids are short chains of carbohydrates that have the potential to exhibit antimicrobial effects on pathogens through decreasing the pH of the gut. The decrease in pH allows organic acids to convert to a more dissociated form which allows for diffusion into pathogen cells. This allows the organic acids to disrupt nutrient pathways essential for pathogen survival (Partanen and Mroz, 1999). These structures can be found in the form of formic, acetic, propionic, and butyric acid. Because of this antimicrobial nature, organic acids are viewed as a potential replacement for antimicrobials in swine diets. Giesting and Easter (1985) conducted a series of experiments that investigated the supplementation of various organic acids to nursery diets and their impacts on performance. The researchers found a tendency for improved G:F ($P < 0.07$) with the addition of 2% propionic, fumaric, or citric acid to nursery diets compared to pigs fed control diets, while propionate decreased feed intake ($P < 0.05$). The response to

supplementation of organic acids to nursery diets is inconsistent with some researchers finding no response to organic acid supplementation. (Biagi et al., 2005). This lack of response to organic acid supplementation is consistent with the results obtained in the current study. A potential reason for this may be that the inclusion rate of individual components within Elarom SES may not have been high enough to elicit a response.

In summary, these studies have provided evidence that a consistent response is observed when in-feed antibiotics and pharmacological levels of ZnO are supplemented to nursery diets. The absence of an Elarom SES response in the current studies further show the inconsistencies in response that occur when blends of short and medium chain fatty acids, slow release organic acids, and phenolic compounds are supplemented to nursery diets. Further research needs to be conducted to determine why these inconsistencies in performance are observed in the nursery stage of production.

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Table 1-1. Diet composition, Exp. 1 (as-fed basis)[1]

Ingredient, %	Phase 1	Phase 2	Phase 3
Corn	36.25	51.80	62.81
Soybean meal, 48% CP	20.65	27.25	32.57
Dairylac 80[2]	15.00	---	---
Corn DDGS, 6-9% oil[3]	5.00	---	---
HP 300[4]	5.00	5.00	---
Spray dried whey	8.00	5.00	---
Monocalcium Phosphate, 21% P	1.13	1.00	1.18
Limestone	1.03	1.00	1.08
Sodium chloride	0.30	0.30	0.35
Choice white grease	1.00	1.00	1.00
L-Lys HCl	0.30	0.38	0.35
DL-Met	0.17	0.20	0.14
L-Thr	0.10	0.15	0.13
L-Val	---	0.05	---
Vitamin premix	0.25	0.25	0.25
Trace mineral premix[5]	0.15	0.15	0.15
CombiAcid[6]	0.20	0.20	---
Choline chloride, 60% liquid	0.04	---	---
Phytase[7]	---	---	---
Elarom SES[6,8]	+/-	+/-	+/-
Zinc oxide[8]	+/-	+/-	---
Denagard[8]	+/-	---	---
CTC-50[8]	+/-	---	---
Mecadox-2.5[8]	---	+/-	+/-
Total	100	100	100
Calculated analysis			
Standardized ileal digestible (SID) amino acids, %			
Lys	1.40	1.35	1.25
Met:Lys	33	37	34
Met and Cys:Lys	57	58	57
Thr:Lys	62	63	62
Trp:Lys	19.3	17.8	18.1
Val:Lys	68	69	66
Total Lys, %	1.58	1.51	1.40
ME, kcal/kg	3,353	3,298	3,306

NE, kcal/kg	2,479	2,435	2,446
CP, %	22.8	22.2	21.2
Ca, %	0.80	0.74	0.70
P, %	0.75	0.67	0.65
Available P, %	0.51	0.47	0.42

[1]Phase 1 diet was fed from d 0 to 7 (~5.23 to 6.10 kg BW), Phase 2 diets from d 7 to 21 (~6.1 to 10.2 kg BW) and Phase 3 diets from d 21 to 42 (~ 10.2 to 21.1 kg BW).
[2]International Ingredients, Inc., St. Louis, MO
[3]Dried distillers grains with solubles
[4]Hamlet Protein, Inc., Findlay, OH
[5]Trace mineral premix containing 17 mg/kg Cu and 110 mg/kg Zn.
[6]Trouw Nutrition USA, LLC., Highland, IL
[7]HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), provided 406.3 phytase units (FTU)/kg and an estimated release of 0.10% available P.
[8]Treatment diets contained zinc oxide added at 0 or 3,000 mg/kg from d 0 to 7 and at 0 or 2,000 mg/kg from d 7 to 21, Elarom (Trouw Nutrition USA, LLC., Highland, IL) added at either 0 or 0.20%. Antibiotic regimen with 400 mg/kg CTC-50 (Zoetis Services, LLC., Florham Park, NJ) and 35 mg/kg Denagard (Elanco Animal Health, Greenfield, IN) added from d 0 to 7. From d 7 to 42, 50 mg/kg Mecadox-2.5 (Phibro Animal Health, Teaneck, NJ). Additions of treatment ingredients were made in place of an equivalent amount of corn in respective experimental diets.

Table 1-2. Diet composition, Exp. 2 (as-fed basis)[1]

Ingredient, %	Phase 1	Phase 2	Phase 3
Corn	36.02	51.10	62.81
Soybean meal	20.67	27.30	34.99
Dairylac 80[2]	15.00	---	---
Corn DDGS, 6-9% oil[3]	5.00	---	---
HP 300[4]	5.00	5.00	---
Spray dried whey	1.25	1.25	---
Fish meal	5.00	---	---
Monocalcium phosphate, 21% P	1.13	1.10	1.18
Limestone	1.03	1.00	1.08
Sodium chloride	0.30	0.60	0.35
Choice white grease	1.00	1.00	1.00
Sodium chloride	0.30	0.30	0.35
L-Lys HCl	0.30	0.38	0.28
DL-Met	0.17	0.20	0.14
L-Thr	0.10	0.16	0.13
L-Val	---	0.06	---
L-Trp	---	0.02	---
Vitamin premix	0.25	0.25	0.25
Trace mineral premix[5]	0.15	0.15	0.15
Zinc oxide	0.42	0.28	---
CombiAcid[6]	0.20	0.20	0.20
Choline chloride, 60% liquid	0.04	---	---
Phytase[7]	---	---	---
Elarom SES[6,8]	+/-	+/-	+/-
TBCC[8,9]	---	---	+/-
Total	100	100	100
Calculated analysis			
Standardized ileal digestible (SID) amino acids, %			
Lys	1.40	1.35	1.25
Met:Lys	33	37	35
Met and Cys:Lys	57	58	58
Thr:Lys	62	63	65
Trp:Lys	19.3	19.3	19.1
Val:Lys	68	69	69
Total Lys, %	1.58	1.50	1.40
ME, kcal/kg	3,395	3,338	3,306

NE, kcal/kg	2,508	2,468	2,433
CP, %	22.7	22.2	22.1
Ca, %	0.80	0.76	0.71
P, %	0.75	0.69	0.66
Available P, %	0.51	0.49	0.42

[1]Phase 1 diet was fed from d 0 to 7 (~5.6 to 6.7 kg BW), Phase 2 diets from d 7 to 21 (~6.7 to 11.2 kg BW) and Phase 3 diets from d 21 to 42 (~11.2 to 24.3 kg BW).
[2]International Ingredients, St. Louis, MO
[3]Dried distillers grains with solubles
[4]Hamley Protein, Inc., Findlay, OH
[5]Trace mineral premix containing 17 ppm Cu and 110 pm Zn.
[6]Trouw Nutrition USA, LLC., Highland, IL
[7]HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), provided 406.3 phytase units (FTU)/kg and an estimated release of 0.10% available P.
[8]Treatment diets contained Elarom SES (Trouw Nutrition USA, LLC., Highland IL) added at either 0 or 0.2% from d 0 to 42 and TBBC (Intellibond C; Micronutrients USA, LLC., Indianapolis, IN) added at 0, 108, or 183 mg/kg from d 21 to 42.
[9]Micronutrients USA, LLC., Indianapolis, IN.

Table 1-3. Chemical analysis of diets, Exp. 1, % (as-fed basis)[1,2]

Elarom-SES	-	+	-	-	+	+	-	+
Added ZnO	-	-	+	-	+	-	+	+
Antimicrobial	-	-	-	+	-	+	+	+
Phase 1 Diets								
DM	91.0	91.2	91.4	91.2	91.0	91.1	91.0	91.4
CP	22.3	21.9	22.4	22.6	22.6	22.5	22.4	22.6
Ca	1.07	0.98	0.94	1.01	1.11	1.06	1.04	1.11
P	0.82	0.78	0.76	0.76	0.83	0.78	0.79	0.81
Zn, mg/kg	122	113	2,998	165	2,263	148	3,109	2,921
Phase 2 Diets								
DM	89.0	88.4	89.7	88.9	89.5	89.3	90.0	88.8
CP	20.3	20.7	21.9	21.1	21.5	20.9	21.8	21.7
Ca	0.93	0.96	0.87	1.07	0.96	1.00	1.04	0.96
P	0.67	0.69	0.64	0.64	0.65	0.69	0.67	0.65
Zn, mg/kg	101	120	1,627	237	1,603	314	1,503	1,551
Phase 3 Diets								
DM	88.1	88.1	88.1	87.6	88.1	88.2	87.6	88.2
CP	21.7	20.5	21.7	20.7	20.5	21.0	20.7	21.0
Ca	0.83	0.91	0.83	0.97	0.91	0.96	0.97	0.96
P	0.63	0.62	0.63	0.61	0.62	0.62	0.61	0.62
Zn, mg/kg	135	136	135	100	136	95	100	136

[1]Complete diet samples were obtained from each dietary treatment during manufacturing. Samples of diets were then submitted for analysis of DM, CP, Ca, P, and Zn (Ward Laboratories, Inc., Kearney, NE).

[2]Phase 1 diet was fed from d 0 to 7 (~5.23 to 6.10 kg BW), Phase 2 diets from d 7 to 21 (~6.10 to 10.18 kg BW) and Phase 3 diets from d 21 to 42 (~ 10.18 to 21.07 kg BW).

Table 1-4. Chemical analysis of diets, Exp. 2, % (as-fed basis)[1,2]

	- Elarom SES			+ Elarom SES		
	TBCC, mg/kg[2]			TBCC, mg/kg[2]		
	0	108	183	0	108	183
Phase 1 Diets						
DM	92.1	---	---	91.5	---	---
CP	22.1	---	---	21.5	---	---
Ca	1.18	---	---	1.02	---	---
P	0.77	---	---	0.74	---	---
Cu, mg/kg	28	---	---	26	---	---
Phase 2 Diets						
DM	90.8	---	---	91.8	---	---
CP	22.4	---	---	23.2	---	---
Ca	0.83	---	---	0.97	---	---
P	0.67	---	---	0.78	---	---
Cu, mg/kg	20	---	---	33	---	---
Phase 3 Diets						
DM	89.3	89.2	89.4	89.6	89.1	88.1
CP	21.6	22.1	23.1	22.1	22.9	22.8
Ca	0.78	0.67	0.88	0.79	0.83	0.89
P	0.59	0.58	0.70	0.65	0.68	0.72
Cu, mg/kg	18	111	169	27	87	202

[1]Complete diet samples were obtained from each dietary treatment during manufacturing. Samples of diets were then submitted for analysis of DM, CP, Ca, P, and Cu (Ward Laboratories, Inc., Kearney, NE).
[2]Tribasic copper chloride (Intellibond C; Micronutrients USA, LLC., Indianapolis, IN) added at 0, 108, or 183 mg/kg from d 21 to 42.

Table 1-5. Effects of Elarom-SES, ZnO, or in-feed antibiotics on nursery pig performance (Exp. 1)[1]

Elarom-SES[2]	-	+	-	-	+	+	-	+	
Added ZnO[3]	-	-	+	-	+	-	+	+	
Antibiotics[4]	-	-	-	+	-	+	+	+	SEM
BW, kg									
d 0	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	0.04
d 7	6.1	6.0	6.1	6.2	6.1	6.0	6.2	6.1	0.07
d 21	9.6^{ef}	9.3^{f}	9.9^{ed}	10.4^{bc}	10.3^{bcd}	10.3^{cd}	10.7^{ab}	10.9^{a}	0.15
d 42	20.7^{cd}	20.3^{d}	20.1^{d}	22.0^{ab}	21.5^{abc}	21.0^{abcd}	22.1^{a}	20.9^{bcd}	0.40
d 0 to 7									
ADG, g	119	110	122	141	127	117	137	129	8.99
ADFI, g	130	132	148	143	131	136	143	142	6.89
G:F	0.915^{ab}	0.851^{a}	0.836^{a}	0.995^{b}	0.957^{b}	0.853^{ab}	0.954^{a}	0.906^{ab}	0.047
d 7 to 21									
ADG, g	250	238	276	290	300	305	320	338	10.06
ADFI, g	311	297	339	336	357	356	367	380	10.19
G:F	0.806	0.802	0.814	0.863	0.839	0.858	0.869	0.886	0.016
d 0 to 21									
ADG, g	206^{de}	195^{e}	224^{cd}	240^{c}	242^{bc}	243^{bc}	258^{ab}	268^{a}	7.03
ADFI, g	250	242	275	270	282	283	292	301	7.40
G:F	0.826^{ab}	0.808^{a}	0.816^{a}	0.886^{c}	0.859^{bc}	0.858^{bc}	0.885^{c}	0.891^{c}	0.014
d 21 to 42									
ADG, g	532^{a}	526^{a}	478^{b}	549^{a}	533^{a}	507^{ab}	539^{a}	475^{b}	15.01
ADFI, g	750^{bc}	744^{bc}	704^{c}	811^{a}	764^{ab}	760^{ab}	787^{ab}	753^{bc}	19.28
G:F	0.709^{d}	0.706^{d}	0.676^{bc}	0.676^{bc}	0.698^{cd}	0.668^{b}	0.684^{bcd}	0.631^{a}	0.013
d 0 to 42									
ADG, g	369^{bc}	361^{c}	351^{c}	391^{ab}	388^{ab}	373^{abc}	397^{a}	372^{abc}	9.05
ADFI, g	500	493	499	535	523	519	537	527	11.74
G:F	0.738^{c}	0.731^{bc}	0.716^{ab}	0.730^{bc}	0.741^{c}	0.720^{abc}	0.739^{c}	0.705^{a}	0.007
Fecal consistency[5]									
	2.81	2.80	2.80	2.76	2.79	2.83	2.74	2.74	0.043

[a,b,c] Means within the same row with different superscripts differ ($P \leq 0.05$).

[1]A total of 360 pigs (DNA 200 × 400) were used in a 3-phase nursery trial with 5 pigs per pen and 9 replications per treatment.

[2]Elarom SES (Trouw Nutrition USA, LLC, Highland, IL) added at 0.20% of the diet.

[3]Zinc oxide fed at 3,000 ppm in phase 1 (d 0 to 7) and 2,000 ppm in Phase 2 (d 7 to 21).

[4]Phase 1: (400 mg/kg CTC and 35 mg/kg Denagard); Phases 2 and 3: (Mecadox 50 mg/kg) (Phibro Animal Health, Teaneck, NJ).

[5]Fecal consistency was categorized through scoring of feces from each pen (fecal scoring occurred on d 0, 4, 7, 14, 21, 28, 35, and 42). Pens were scored by 3 trained individuals; those 3 scores were then averaged and reported as pen means for overall and each collection day fecal consistency. Scoring scale guidelines: 1 = dry, firm pellet; 2 = firmly formed stool; 3 = soft stool that retains shape; 4 = soft, unformed stool; and 5 = watery liquid stool. There was no overall or individual treatment effect ($P > 0.100$).

Table 1-6. Main and interactive effects of Elarom SES, added ZnO, and in-feed antibiotics on nursery pig growth performance (Exp. 1)[1,2]

	Probability, *P* <						
	Elarom SES	ZnO	Antibiotic	Elarom SES × ZnO	Elarom SES × Antibiotic	ZnO × Antibiotic	Elarom SES × ZnO × Antibiotic
BW, kg							
d 0	0.944	0.742	0.888	0.832	0.655	0.814	0.906
d 7	0.183	0.219	0.076	0.224	0.201	0.773	0.890
d 21	0.687	0.001	0.001	0.043	0.700	0.240	0.407
d 42	0.302	0.638	0.001	0.171	0.005	0.648	0.090
d 0 to 7							
ADG, g	0.112	0.210	0.047	0.192	0.211	0.642	0.922
ADFI, g	0.238	0.164	0.244	0.451	0.666	0.580	0.199
G:F	0.249	0.533	0.122	0.016	0.165	0.909	0.169
d 7 to 21							
ADG, g	0.091	0.001	0.001	0.139	0.428	0.338	0.218
ADFI, g	0.170	0.001	0.001	0.347	0.322	0.234	0.168
G:F	0.448	0.07	0.001	0.339	0.911	0.718	0.858
d 0 to 21							
ADG, g	0.283	0.001	0.001	0.053	0.744	0.273	0.240
ADFI, g	0.318	0.001	0.001	0.546	0.256	0.217	0.357
G:F	0.950	0.071	0.001	0.026	0.299	0.760	0.434
d 21 to 42							
ADG, g	0.186	0.04	0.968	0.352	0.001	0.885	0.055
ADFI, g	0.573	0.30	0.001	0.133	0.013	0.934	0.373
G:F	0.070	0.01	0.001	0.309	0.002	0.689	0.006
d 0 to 42							
ADG, g	0.560	0.599	0.013	0.145	0.007	0.871	0.043
ADFI, g	0.996	0.376	0.001	0.160	0.117	0.774	0.304
G:F	0.221	0.356	0.136	0.718	0.004	0.754	0.009

[1]A total of 360 pigs (DNA 200 x 400) were used in a 3-phase nursery trial with 5 pigs per pen and 9 replications per treatment.
[2]All experimental diets were fed in three phases (d 0 to 7, d 7 to 21, and d 21 to 42). All diets contained 110 ppm of Zn from the trace mineral premix.

Table 1-7. Effect of Elarom SES and tribasic copper chloride level on nursery pig performance (Exp. 2)[1,2]

	- Elarom SES			+ Elarom SES[3]			Probability, $P <$			
	TBCC mg/kg[4]			TBCC mg/kg[4]					TBCC	
	0	108	183	0	108	183	SEM	Elarom SES	Linear	Quadratic
BW, kg										
d 0	6.0	6.0	6.0	6.0	6.0	6.0	0.13	0.521	---	---
d 21	11.1	11.5	11.4	11.2	11.4	11.2	0.35	0.555	---	---
d 42	23.9	24.7	24.2	23.8	24.6	24.6	0.53	0.834	0.140	0.204
d 0 to 21										
ADG, g	241	262	256	245	253	248	12.26	0.595	---	---
ADFI, g	311	323	322	308	323	308	12.75	0.509	---	---
G:F	0.773	0.810	0.793	0.799	0.780	0.802	0.015	0.896	---	---
d 21 to 42										
ADG, g	610	621	612	604	631	638	13.29	0.346	0.129	0.460
ADFI, g	915	930	937	906	939	939	20.78	0.970	0.101	0.620
G:F	0.667	0.670	0.655	0.667	0.673	0.680	0.008	0.120	0.891	0.562
d 0 to 42										
ADG, g	425	441	433	424	440	443	10.69	0.729	0.114	0.329
ADFI, g	612	625	628	605	628	624	15.01	0.798	0.128	0.440
G:F[5]	0.694	0.706	0.691	0.700	0.701	0.710	0.006	0.161	0.504	0.402
Fecal Consistency[6]										
	3.02	3.08	3.02	3.07	3.01	3.00	0.031	0.740	0.869	0.115

[1]A total of 360 pigs (DNA 200 × 400) were used in a 3-phase nursery trial with 5 pigs per pen and 12 replications per treatment. 17 mg/kg of Cu from $CuSO_4$ was added to each diet from the trace mineral premix.
[2]Indicates analysis was not conducted due to tribasic copper chloride not being included in the diet during these phases. ,
[3]Elarom SES (Trouw Nutrition USA, LLC, Highland, IL) added at 0.20% of the diet in all phases (d 0-42).
[4]Tribasic copper chloride (Intellibond C; Micronutrients USA, LLC, Indianapolis, IN) added at 0, 108, or 183 mg/kg in phase 3 (d 21-42).
[5]A tendency for an Elarom SES × TBCC interaction ($P < 0.058$) was observed.
[6]Fecal consistency was categorized through scoring of feces from each pen (fecal scoring occurred on d 0, 4, 7, 14, 21, 28, 35, and 42). Pens were scored by 3 trained individuals; those 3 scores were then averaged and reported as pen means for overall and each collection day

fecal consistency. Scoring scale guidelines: 1 = dry, firm pellet; 2 = firmly formed stool; 3 = soft stool that retains shape; 4 = soft, unformed stool; and 5 = watery liquid stool.

Table 1-8. Nursery pig fecal consistency (Exp. 1)

Day	Fecal score[1]
0	3.4
4	3.4
7	3.3
14	2.3
21	2.1
28	2.4
35	2.6
42	2.8

[1]Fecal consistency scores were categorized by the consistency of feces per pen (fecal scores collected on d 0, 4, 7, 14, 21, 28, 35, and 42). Pens were scored by 3 trained individuals; those scores were then averaged and reported as pen means for each collection day. Scoring scale guidelines: 1 = dry, firm pellet; 2 = firmly formed stool; 3 = soft stool that retains shape; 4 = soft, unformed stool; and 5 = watery liquid. Treatment × Day interaction (P = 0.53, SEM = 0.04) and day effect (P < 0.01, SEM = 0.04).

Table 1-9. Nursery pig fecal consistency (Exp. 2)

Day	Fecal score[1]
0	2.9
4	3.1
7	3.2
14	2.9
21	3.2
28	2.9
35	3.0
42	3.1

[1]Fecal consistency scores were categorized by the consistency of feces per pen (fecal scores collected on d 0, 4, 7, 14, 21, 28, 35, and 42). Pens were scored by 3 trained individuals; those scores were then averaged and reported as pen means for each collection day. Scoring scale guidelines: 1 = dry, firm pellet; 2 = firmly formed stool; 3 = soft stool that retains shape; 4 = soft, unformed stool; and 5 = watery liquid. Treatment × Day interaction (P = 0.230, SEM = 0.03) and day effect (P < 0.001, SEM = 0.03).

Chapter 2 - Effects of dietary lysine concentration and crystalline amino acid concentration, with or without formaldehyde-treatment of diets on growth performance and fecal microbiota in nursery pigs

ABSTRACT

Two experiments were conducted to determine the effects of Lys concentration and crystalline AA addition in formaldehyde-treated diets on nursery pig performance. In Exp.1, 299 barrows (initially 15.2 ± 0.26 kg) were used to compare the effects of formaldehyde (Termin-8; Anitox Corp, Lawrenceville, GA or Sal CURB; Kemin Industries, Inc., Des Moines, IA) and Lys levels. Dietary treatments were arranged in a 3 × 2 factorial with 3 formaldehyde treatments (no formaldehyde; 3.2 kg/t Sal CURB, and 3.0 kg/t Termin-8) and 2 dietary Lys concentrations (1.25% standardized ileal digestible [SID] Lys: Adequate, or 1.10% SID Lys, Low). Pens of pigs were balanced by initial BW with 5 pigs per pen and 10 pens per treatment in a 14-d study. Overall, there was a marginally significant ($P < 0.10$) formaldehyde source × Lys concentration interaction for ADG and G:F. Regardless of formaldehyde source or Lys concentration, pigs fed formaldehyde-treated diets marginally reduced ($P < 0.10$) ADG and decreased ($P < 0.05$) G:F with pigs fed Termin-8 having a greater decrease in performance than pigs fed other diets. In Exp. 2, 1,235 pigs (initially 12.2 ± 0.12 kg) were used in a 28-d study to compare formaldehyde-treatment (Sal CURB) on growth performance, crystalline AA utilization, feed bacterial microflora, and fecal microbiota. Dietary treatments were arranged in a (2 × 2) + 1 factorial with formaldehyde treatment (none vs. 3.2 kg/t Sal CURB) and crystalline AA inclusion (low vs. high) plus a positive control. The positive control represented Lys requirement estimate whereas treatment diets were formulated to be 80% of the positive control. Pens of pigs were allotted

based on average BW with 19 to 22 pigs per pen and 12 replications per treatment. Treating diets with formaldehyde reduced ADG ($P = 0.001$). Crystalline AA × formaldehyde interactions ($P < 0.05$) were observed for ADFI and G:F with the reduced ADG due to decreased ADFI in the high crystalline AA diets and lower G:F in the low crystalline AA diets. These studies show pig growth is negatively influenced with the inclusion of formaldehyde in diets formulated below the pigs AA requirement. The inclusion level of crystalline AA had no impact on performance while formaldehyde had a negative impact on AA utilization and altered fecal microbial communities, it reduced bacterial microflora of complete feeds.

Key words: amino acids**,** formaldehyde**,** nursery, growth performance

INTRODUCTION

Two commercial formaldehyde products (Termin-8, Anitox Corp, Lawrenceville, GA and Sal CURB, Kemin Industries, Inc., Des Moines, IA) are commonly used in the poultry industry for *Salmonella* control in feed. According to the Food and Drug Administration's federal register (FDA #21 CFR 573.460, 2015), the food additive formaldehyde can be included in animal feed or ingredients to maintain complete feed and ingredients as *Salmonella* negative for up to 21 d. Since the emergence of porcine epidemic diarrhea virus (PEDV) in the United States, formaldehyde products have received attention as a potential method to reduce the risk of PEDV transmission due to the ability of complete feed serving as a vector for the transmission of the disease (Dee et al., 2015). Previous research demonstrating formaldehyde use in reducing PEDV infectivity in contaminated feed and ingredients has been successful (Dee et al., 2015, Cochrane et al., 2015).

However, formaldehyde is known to produce reactions with numerous groups of amino acid residues of proteins that can lead to the formation of methylol groups, Schiff-bases, and methylene bridges amongst these residues (Metz et al., 2004). Thus, inclusion in diets may reduce the availability of dietary AA for pigs, which may influence growth performance and nutrient utilization. Ochoa et al. (2017) evaluated the effects of treating complete feed with formaldehyde on grow-finishing pig performance, but found no evidence of difference on performance. Therefore, two nursery pig studies were conducted to determine if formaldehyde treatment would interact with dietary Lys level and crystalline AA concentration and affect nursery pig growth performance, feed bacteria concentration, and fecal microbiota.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee (IACUC # 3529) approved the protocols used in these experiments. Experiment 1 was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS. Each pen (1.22 × 1.22 m) was equipped with a 4-hole, dry-self feeder and a nipple waterer to provide ad libitum access to feed and water. Experiment 2 was conducted at a commercial wean-to-finish facility in Webster City, IA. Each pen (2.44 × 5.64 m) was equipped with a 4-hole, dry-self feeder and a pan waterer to provide ad libitum access to feed and water.

Experiment 1

Animals

A total of 299 pigs (PIC 327 × 1050, initially 15.2 ± 0.26 kg) were used in a 14-d study. Pens of pigs were allotted by initial BW and then randomly allotted to 1 of 6 treatments with 5 pigs per pen and 10 replications per treatment. Dietary treatments were arranged in a 2 × 3

factorial with 2 Lys levels (1.10 vs. 1.25% SID Lys) and 3 formaldehyde sources (no formaldehyde, 3.2 kg/tonne Sal CURB, or 3.0 kg/tonne Termin-8). Pigs and feeders were weighed on d 0, 7, and 14 of the trial to determine ADG, ADFI, and G:F.

Feed Manufacturing

Two corn-soybean meal basal diets (low Lys and adequate Lys, Table 1) were manufactured at a commercial feed mill (Mound City, SD). Each diet was divided into three batches and either left untreated, treated with 3.0 kg/tonne source 1 (Termin-8, Anitox Corp., Lawrenceville, GA) in the same mill or transported to a separate commercial mill (Hastings, NE) and treated with 3.2 kg/tonne source 2 (Sal CURB, Kemin Industries, Inc., Des Moines, IA). Adequate Lys diets were formulated to meet the assumed SID Lys requirement for pigs during this phase of growth according to the NRC (NRC, 2012). Low Lys diets were formulated to 90% of the SID Lys requirement of pigs according to the NRC (2012). Source 1 is a premix of 33% aqueous formaldehyde and propionic acid. Source 2 is a premix of 37% aqueous formaldehyde and propionic acid. The inclusion of either formaldehyde treatment provided 300 mg/kg of propionic acid that was analyzed to ensure correct inclusion rates, respectively. Formaldehyde application methods were conducted according to manufacturers' recommendations, with inclusion occurring in the mixer (Anitox Corp., Lawrenceville, GA manufacturing procedures; Kemin Industries, Inc., Des Moines, IA manufacturing procedures). All diets were then transported to the O.H. Kruse Feed Technology Innovation Center in Manhattan, KS where they were bagged and transported to the Kansas State University Swine Teaching and Research Farm. Diets were sampled immediately following manufacturing and analyzed for DM (AOAC 935.29, 2012), CP (AOAC 990.03, 2006), crude fiber (AOAC 978.10, 2006), ether extract (AOAC

method 920.39 A; 2012), ash (AOAC 942.05; 2012) and AA concentration including total and available Lys (Table 3).

Experiment 2

Animals

A total of 1,235 pigs (PIC 359 × PIC 1050, initially 12.2 ± 0.12 kg) were used in a 28-d study. Pens of pigs were allotted to 1 of 5 dietary treatments based on average BW and location within barn with 19 to 22 pigs per pen and 12 replications per treatment in a randomized complete block design. Dietary treatments were arranged in a (2 × 2) + 1 factorial with formaldehyde treatment (none vs. 3.2 kg/tonne (Sal CURB, Kemin Industries, Inc., Des Moines, IA) and crystalline AA inclusion (low vs. high). A positive control was used in the experiment to represent diets that met the assumed SID Lys requirement estimate for pigs used in this study. Treatment diets were formulated to be 80% of the SID Lys level in the positive control. Pigs and feeders were weighed on d 0, 12, and 28 to determine ADG, ADFI, and G:F.

Feed Manufacturing

Experimental diets were fed in two phases from d 0 to 12 and d 12 to 28. Within each phase, three individual diets (Table 2) were manufactured at a commercial feed mill (Altoona, IA). The positive control diet in each phase was not treated with formaldehyde. The diets with low or high amounts of crystalline AA were divided into two equal batches with 50% of each diet treated with 3.2 kg/tonne formaldehyde. Formaldehyde amount and application methods were conducted according to manufacturers' recommendations, with inclusion occurring in the mixer (Kemin Industries, Inc., Des Moines, IA manufacturing procedures). Diets in each phase were sampled from the mill and 6 random feeders, pooled, and analyzed for DM (AOAC 935.29, 2012), CP (AOAC 990.03, 2012), Ca (AOAC 965.14/985.01, 2012), P (AOAC 965.17/985.01,

2012), propionic acid, and Lys content, specifically total Lys, free Lys, and available Lys (Table 4). Propionic acid was analyzed according to manufacturer's procedures (Kemin Industries Inc., Des Moines, IA analysis procedures) to confirm correct inclusion rates of formaldehyde to treatment diets.

Feed collection

Feed samples were collected directly from each individual batch of feed in 5 spaced sub-samples by passing sterile Whirl-Pak (Nasco, Ft. Atkinson, WI) through the stream of feed as it was emptied from load-out bin into the feed delivery truck. Feed samples were also collected directly from 6 different feeders for each dietary treatment and placed in sterile Whirl-Pak bags to represent farm samples. Both mill and farm samples were pooled within collection location and transported to the Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University for feed bacterial enumeration analysis.

Enumeration of feed bacteria

Feed samples were tested using 3M Petrifilm plates (3M Microbiology, St. Paul, MN) with each of these plates selecting for certain organisms. The specific organisms being detected for this experiment were: total coliforms (TC), aerobic plate counts (APC), and *Enterobacteriaceae* (EB).One gram of feed sample was diluted in 10 mL of phosphate buffered saline (PBS) tube and vortexed to make a uniform suspension before serially diluting the feed suspension to achieve 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} concentrations. With a sterile pipette, 1 mL of feed sample at each dilution was placed in the center of the Petrifilm in triplicates for each plate type. Dilutions were vortexed before plating to ensure equal distribution of feed inoculum. A 3M Petrifilm spreader was placed on top of film over inoculum which distributed the sample inoculum over a circular area of 20 cm^2 on the bottom film. Total coliform, *Enterobacteriaceae,*

and Aerobic plates were incubated for 48, 24, and 48 hrs, respectively. After the incubation period, a plate reader (3M Petrifilm, St. Paul, MN) was used to enumerate each plate for specific ranges, colony morphology, gas production, and acidification. Counting ranges were 50 to150 colonies for coliform plates, 15 to 100 colonies for *Enterobacteriaceae* plates, and 25 to 250 colonies aerobic count plates. Colony counts were expressed as colony forming units per g of feed sample (cfu/g) and bacterial counts were expressed as an average of 2 separate runs ran in duplicate with a different feed sample.

Fecal collection

Fecal samples were collected into individual Whirl-Pak bags via rectal massage from 6 randomly selected pigs on d 0 and from 3 randomly selected pigs per pen on d 28. Samples were stored at 4°C and then transported to Kansas State University where d 28 samples were pooled within pen into individual samples to represent 6 baseline samples and 12 samples per treatment. Samples were stored at -80°C until transportation to the University of Nebraska-Lincoln for bacterial community analysis.

Fecal Microbiological Procedures

DNA Extraction and PCR Amplification

Fecal DNA (6 from baseline, 12 per treatment) were isolated from approximately 100 mg of samples using Mag-Bind Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA) according to the manufacturer's protocol with the following modifications: the raw sample was transferred to a 2 mL sterile Safe-Lock tube (Eppendorf, Hauppauge, NY) with 0.3 g of acid washed beads (Scientific Asset Management, Basking Ridge, NJ) and 300 µL of SLX-Mlus Buffer, followed by bead-beating at frequency of 20 for 10 min (QIAGEN Inc., Valencia, CA); after samples were mixed with RNase A and DS Buffer, samples were incubated in a 90˚C water bath for 8 min and

occasionally vortexed; the samples were centrifuged at a speed of 5,000 × *g* for 10 min and the supernatant was transferred; finally, DNA was eluted with 130 µL of elution buffer at the last step.

The V4 region of the 16S rRNA gene specific to the eubacterial communities was amplified from the extracted DNA samples. A PCR reaction (20 µL) consisted of 2 µL of template DNA, 0.5 µL each of both forward and reverse 16S rRNA V4 primers (final concentration 10.0 µM; Integrated DNA Technologies, Coralville, IA), 0.5 µL of Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA), 10 µL of 2X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA) and 6.5 µL of nuclease-free water (Hoefer Inc., Holliston, MA). Using a Veriti 96-well thermocycler (Life Technologies, Carlsbad, CA), the amplification was performed at 98˚C for 3 min, followed by 25 cycles of 30 s at 98˚C, 30 s at 55˚C, and 45 s at 68˚C, with a final elongation stage of 4 min at 68˚C. The amplified PCR products were tested by agarose gel electrophoresis (5 µL PCR product, 1.5% agarose gel) at 100 V for 60 min for size verification and to confirm amplification.

Preparation of 16S rRNA Library and sequencing

From each sample, 10 µL of the PCR product was pooled together and mixed. The pooled 16S rRNA gene library was column purified using PCR cleanup procedure (DNA, RNA, and protein purification; Clontech Laboratories, Mountain View, CA) and eluted into 40 µL. Subsequently, the Pippin Prep (Sage Science, Beverly, MA) was used to remove any spurious PCR fragments from the purified concentrated library. Finally, sequencing was performed using the Illumina Miseq platform (Illumina, Santa Clara, CA) according to the manufacturer's protocol.

Bacterial Community Analysis

The raw reads from pyrosequencing were demultiplexed using the Quantitative Insights Into Microbial Ecology program (QIIME; Caporaso et al., 2012) and run through a quality control described by Anderson et al. (2016). Reads were trimmed to a fixed length of 251 bp using Mothur (Schloss et al., 2009) and the FASTX-TOOLKIT (Edgar, 2013), followed by identification of operational taxonomic unit (OTU) based on 97% similarity using UPARSE pipeline (Edgar, 2013). The generated OTU sequences were aligned using Ribosomal Database Project (http://pyro.cme.msu.edu). Taxonomic classification was performed using QIIME and the GreenGenes database (version 13_8; McDonald et al., 2012). The OTUs belonging to the phylum *Cyanobacteria* were removed as these are from dietary source. Single sequences that may be generated from sequencing error, were removed from the data. A core set of 854 OTUs (42.3% of the original OTU table), presenting in at least 75% of the samples, was identified for further analysis.

Statistical analysis

Experiment 1

Data were analyzed as a completely randomized block design with pen as the experimental unit using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC). Treatment was considered the fixed effect and a random effect of block was used in analysis. Pre-planned contrasts were utilized to compare the interaction between formaldehyde source and Lys level, the main effects of formaldehyde or Lys level, and formaldehyde inclusion, regardless of source, compared to none. Results were considered significant at $P \leq 0.05$ and marginally significant at $P > 0.05$ and $P \leq 0.10$.

Experiment 2

Growth performance data were analyzed as a completely randomized design with pen as the experimental unit using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC). Treatment was considered the fixed effect and a random effect of block was used in analysis. Pre-planned contrasts were utilized to compare the interaction between Sal CURB and Lys level, the main effects of Sal CURB or Lys level, and crystalline AA inclusion compared to the positive control. Results were considered significant at $P \leq 0.05$ and marginally significant at $P > 0.05$ and $P \leq 0.10$.

Bacterial Community Analysis

All data were analyzed as a completely randomized design (CRD) and the responses were presented as least-squares means (± SEM). Pen was the experimental unit and considered a random effect. Additionally, OTU abundances at family level in the bacterial communities were analyzed using the GLIMMIX procedure of SAS. Results were considered significant at $P \leq 0.05$ and marginally significant at $P > 0.05$ and $P \leq 0.10$.

Fecal Microbial Diversity

The observed core OTU, Chao 1 and Shannon index (α-diversity) for the 10 subsampling events were analyzed using the GLIMMIX procedure of SAS. Global changes in bacterial community structure (β-diversity) were evaluated using normalized unweighted UniFrac distance matrices, which were computed from subsampled core OTU tables at the minimum depth of sequence reads (14,163). The unweighted UniFrac distance matrices were used as input for a multivariate analysis using the Fathom Toolbox for MATLAB (Jones, 2015). Additionally, OTU abundances at family level in the bacterial communities were analyzed using the GLIMMIX procedure of SAS.

RESULTS

Chemical Analysis

In Exp. 1, analysis of DM, crude fiber, fat, ash, and CP closely matched formulated values (Table 3). However, analyzed total AA % for the adequate Lys diets were higher than formulated values. Other analyzed total AA % were similar to formulated values. In Exp. 2, DM, CP, Ca, and P closely resembled formulated values (Table 4). Propionic acid analysis confirmed that formaldehyde was included at the correct levels in respective dietary treatments.

Experiment 1

There were no formaldehyde source × Lys level interactions detected for BW on d 0, 7, and overall (Table 7).

From d 0 to 14, there was marginal significance ($P = 0.053$) for a formaldehyde source × Lys level interaction where ADG was not influenced by formaldehyde treatment for pigs fed the adequate Lys diets, but in the low Lys diets pigs fed the source 2 treatment had decreased ADG compared with pigs fed the control diet. Pigs fed diets treated with source 2 had decreased ($P < 0.05$) ADG compared with pigs fed the non-treated and source 1 treated feed. Formaldehyde inclusion decreased ($P = 0.0007$) G:F with pigs fed the diet with source 2 treatment having the poorest ($P < 0.05$) G:F compared to the non-treated and source 1 treated feed. Pigs fed adequate Lys diets had greater ($P < 0.05$) ADG and G:F compared to those fed the low Lys diets.

Experiment 2

Analysis of total Lys in the positive control and treatment diets without formaldehyde inclusion were similar to formulated values (Table 5). However, treating the low crystalline AA formulated diets with formaldehyde reduced (P < ???) total and available Lys by 8.7 and 10.4%, respectively in phase 1 and 12.6% and 13.1% in phase 2. In high crystalline AA diets,

formaldehyde treatment marginally reduced total and available Lys by 3.2% in phase 1 with little to no effect in phase 2. Formaldehyde treatment of feed had no observed effect on free Lys which is an indicator of the amount of crystalline AA added in the diets.

For diet bacterial concentrations, as anticipated, analysis of phase 1 feed samples collected at both the feed mill and farm formaldehyde treatment of diets reduced (P < ???) the bacterial concentration compared to diets not treated with Sal CURB (Table 6). However, in phase 2, data were not as clear. Only the diets with high crystalline AA collected at the feed mill had reduced bacterial concentration with formaldehyde.

No evidence of difference ($P > 0.10$) was observed for Crystalline AA concentration × formaldehyde interactions for growth criteria measured from d 0 to 12 (Table 8). Pigs fed formaldehyde-treated diets had decreased ($P < 0.001$) ADG and G:F compared with pigs fed diets that were not treated with formaldehyde. Pigs fed the control diet had greater ($P < 0.05$) ADG and G:F compared with pigs fed the other diets containing reduced Lys, with no evidence of difference between diets containing low and high levels of crystalline AA.

From d 12 to 28, a Crystalline AA × formaldehyde interaction ($P < 0.05$) was observed for ADFI and G:F. These interactions were a result of reduced ADG from pigs with decreased ADFI in the high crystalline AA diets and lower G:F in the lower crystalline AA. The G:F interaction was observed because pigs fed low crystalline AA diets without treatment of formaldehyde resulted in greater G:F than pigs fed diets with formaldehyde treatment but the inverse was observed in high crystalline AA diets. Marginal significance ($P = 0.073$) for a Crystalline AA × formaldehyde interaction was observed for ADG with a greater reduction in ADG when formaldehyde was added to the low crystalline AA than when included in the high crystalline AA diets. Pigs fed formaldehyde-treated feed had reduced ($P < 0.05$) ADG and d 12

BW and marginal significance ($P = 0.052$) for reduced ADFI compared with pigs fed diets not treated with formaldehyde. Pigs fed the control diet had greater ($P < 0.05$) ADG and G:F compared with pigs fed the treatment diets with lower Lys levels, but there was no evidence of difference between diets containing low and high levels of crystalline AA.

Overall (d 0 to 28), pigs fed the control diet had improved ($P < 0.05$) ADG, ending BW, and G:F compared to those fed other diets containing reduced Lys, but there was no evidence of difference between diets containing low and high levels of crystalline AA. The application of formaldehyde to diets resulted in reduced ($P < 0.05$) ADG and ending BW compared to not treating diets with formaldehyde. A Crystalline AA × formaldehyde interaction ($P < 0.05$) was observed for ADFI and G:F. The interaction for ADFI occurred because treating diets with formaldehyde decreased ADFI for pigs fed diets with high crystalline AA inclusions, but did not influence ADFI for pigs fed low crystalline AA diets. The interaction for G:F was observed because treating diets with formaldehyde decreased G:F for pigs fed low crystalline AA diets but did not influence G:F for pigs fed high crystalline AA diets.

For bacterial community abundance, no evidence of difference ($P > 0.10$) existed in bacterial abundances amongst the dietary treatments for Methanobacteriaceae, Prevotellaceae, Lachnospiraceae, or Spirochaetaceae (Table 9). A crystalline AA × formaldehyde interaction ($P = 0.003$) was observed for Streptococcaceae abundances in the bacterial community of the gut, because pigs fed low crystalline AA diets had a more dramatic reduction in abundance when treated with formaldehyde compared to the high crystalline AA diets. The treatment of diets with formaldehyde decreased ($P < 0.05$) bacterial abundance for Paraprevotellaceae and Lactobacillaceae species, while formaldehyde treatment increased ($P < 0.05$) Clostridiaceae and Erysipelotrichaceae species within the bacterial community of the gut. Pigs fed formaldehyde-

treated diets had marginal significance ($P = 0.074$) for lower percentages of S24-7 bacteria species than pigs fed non-formaldehyde treated diets. Pigs fed low crystalline AA diets had increased ($P < 0.05$) abundance of Paraprevotellaceae, Lactobacilliaceae, Ruminococcaceae, and Veillonellaceae bacterial species compared to high crystalline AA diets. Pigs fed high crystalline AA diets had increased ($P = 0.007$) Clostridiaceae and marginally ($P = 0.080$) increased Erysipelotrichaceae bacterial species compared to pigs fed low crystalline AA diets. Treatment diets fed to lower lysine levels than the control had increased ($P = 0.009$) Clostridiaceae bacterial species, while Paraprevotellaceae species were marginally ($P = 0.091$) lower in these diets compared to the positive control.

DISCUSSION

The use of formaldehyde for the treatment of complete feeds or individual feed ingredients to reduce bacterial concentrations and the subsequent effect on animal performance has been investigated previously. The addition of formaldehyde to poultry diets reduces contamination from bacterial pathogens such as *Salmonella* (Carrique-Mas et. al 2007). This reduction in bacterial contamination could result in performance benefits that have been observed with the addition of formaldehyde to poultry diets. Rowghani et. al (2007) evaluated the addition of Formycine (Novus International, Saint Charles, MO), a commercially available formaldehyde and propionic acid additive to broiler diets and its effects on growth performance. When fed to broiler chicks, Formycine improved ($P < 0.05$) feed conversion ratio by 4.8% compared to the control. In a performance study, chickens received 1 of 4 diets treated with either 0, 630, 1580 or 6,300 mg formaldehyde/kg feed to test zootechnical performance (EFSA, 2014). A reduction in BW was observed for chickens fed formaldehyde treated diets, regardless

of level, compared to the untreated feed, although, there was not a significant difference between the lowest concentration of formaldehyde inclusion and the control

In swine, more limited research has been conducted evaluating the effects of formaldehyde on single ingredients or the complete diet on bacterial concentrations and performance. DeRouchey et. al (2004) evaluated the application of Termin-8 to spray-dried animal plasma prior to complete diet manufacturing. Pigs fed diets with formaldehyde-treated animal plasma had improved ($P < 0.05$) ADG and ADFI compared to pigs fed control diet or pigs fed whole diets treated with formaldehyde. However, there was no differences in G:F ($P > 0.11$) observed amongst the dietary treatments. These results differed from Exp. 1, as the pigs consuming the source 2 reduced ADG and G:F compared to the pigs fed non-treated and source 1 treated feed. Although, the pigs fed diets treated with source 2 had improved ADFI compared to the source 1 treated feed and the non-treated feed. Furthermore, the results from DeRouchey et al. (2004) also differed from Exp. 2 where pigs consuming diets treated with formaldehyde had decreased ADG and d 28 BW compared to pigs fed diets not treated with formaldehyde. This suggests that the treatment of whole diets formulated below the pigs AA requirement with formaldehyde has a larger negative impact on performance than the treatment of individual feed ingredients in diets formulated at or above the pigs AA requirement.

Formaldehyde has the ability to produce reactions with numerous groups of AA, including Lys (French and Edsall 1945, Metz et al. 2004). The reactions between formaldehyde and AA, especially Lys, could render these AA unavailable which could possibly alter growth performance. Rude et al (2016) studied the effects of Sal CURB application to corn with varying levels of L-Lys HCL, which is an inclusion indicator for free Lys. Sal CURB was applied to treatments at levels of 4.09 to 4.24 kg/tonne to increase the formaldehyde challenge. An

interaction was not observed ($P = 0.688$) between Sal CURB inclusion and L-Lys level on analyzed free Lys in the treatments. These results would agree with the analyzed free Lys values in Exp. 2 being similar amongst dietary treatments regardless of Sal CURB inclusion at required level. Although, the current study reveals that Sal CURB inclusion may have a larger impact on total and available Lys. The reactions that occur between formaldehyde and Lys residues in proteins may explain why Sal CURB is reducing the amount of total and available Lys in the current studies and could alter protein utilization, thus explaining the reduction in performance observed in Exp. 2.

Ochoa et. al (2017) evaluated the effects of feeding Sal CURB- treated feed to pigs throughout the growing period to study its effects on AA utilization from crystalline Lys or protein sources. A (2 × 2) + 1 factorial was used with main effects of Sal CURB inclusion (none vs. 3.2 kg/tonne Sal CURB) and crystalline AA inclusion (none vs. high). A control was used in this study to include Sal CURB with no crystalline AA that was formulated to 90% of the pigs' Lys requirement. Overall, ADG and ADFI were not impacted across treatments, but G:F was negatively affected with the inclusion of Sal CURB regardless of crystalline AA inclusion. These results would differ from observations in Exp. 2, as pigs fed diets containing Sal CURB had decreased ADG regardless of crystalline AA inclusion and pigs fed diets containing high crystalline AA with Sal CURB had decreased ADFI. Also, pigs fed diets containing Sal CURB with low crystalline AA inclusion had lower G:F and pigs fed diets with high crystalline AA had similar G:F regardless of Sal CURB inclusion. A possible reason for the varied results is the difference in BW and Lys level used between the two studies. The previous study used early-finisher pigs that require a lower Lys requirement while the current study used mid- to late nursery pigs that require a higher Lys requirement. This could suggest that the inclusion of

formaldehyde in finisher diets may have less of an effect on performance than in nursery diets. Another possibility is that formaldehyde inclusion in diets formulated at or above the pigs AA requirement does not have as large of a negative impact as with pigs fed diets below their AA requirement.

From a dietary bacteria concentration prospective, the use of formaldehyde to reduce bacterial load and aid in the prevention of recontamination in complete feeds for poultry (Carrique-Mas et al. 2007) and swine (DeRouchey et al., 2004; Sbardella et al., 2015) has been evaluated. Inclusion of formaldehyde was effective at reducing bacterial loads in the phase 1 dietary treatments collected at either the feed mill or the farm compared to diets not containing formaldehyde. This also was the case in high crystalline AA diets treated with formladehyde in phase 2 of the study. However, formaldehyde inclusion did not affect bacterial counts in the phase 2 low crystalline AA diets at either the feed mill or the farm. This could be due to contamination within manufacturing of the diets in the mixer and load-out bins or contamination within load-out of the diets onto the feed delivery truck to the feed bin into the feeder. Another possibility is contamination from sample handling or cross-contamination within the lab during analysis. Diet analysis confirmed formaldehyde inclusion and analyzed total and available Lys confirmed similar results to what was observed in Exp. 1. It is not known why the Phase 2 diets did not have a greater reduction in microbial load similar to Phase 1.

The gastrointestinal tract of pigs is comprised of beneficial commensal bacteria that play a role in regulating gene expression that can influence numerous gut integrity and immune responses (Brestoff and Artis, 2013). Advancements in sequencing techniques have allowed researchers to focus on sequencing specific regions within the 16S rRNA gene to reveal patterns in composition of the pig gastrointestinal tract (Adams et al., 2015). Holman et al. (2017) utilized

a meta-analysis to determine the major types of commensal bacteria species that compose the gastrointestinal tract of pigs. The researchers observed that *Prevotella, Clostridium, Ruminococcus,* and *Lactobacillus* species were found in greater than 90% of fecal samples collected in those studies, which would be similar to the findings of the current study. Furthermore, swine researchers are beginning to utilize this sequencing technology to determine the effects of diet composition on gut microbiota of pigs. Levesque et al. (2014) utilized high and low complexity diets to determine the effect of diet on weaned pigs. The researchers observed that species specific changes in mucosal bacteria does occur with the feeding of two different diet compositions, which shows the effect diet can have on affecting gut commensal bacteria. Although other researchers have looked at different feed ingredients and their effects on gut microbiota changes (Looft et al., 2014; Mann et al., 2014), this study is the first to observe the effects of formaldehyde treatment of diets on nursery gut microflora. The decrease of lactic acid bacteria species, specifically *Lactobacillaceae,* and increase of *Clostridiaceae* species in the gut microflora of pigs fed formaldehyde-treated diets warrants further investigation to determine the short- and long-term effects this shift in commensal bacterial species has on gut integrity and health.

In summary, these studies have provided evidence that in ~12 to 15 kg BW nursery pigs the inclusion of formaldehyde in complete feeds has a negative impact on ADG, ADFI, G:F, and ending BW when diets are fed below the lysine requirement of the pigs. Furthermore, it can be observed that the inclusion of formaldehyde in complete nursery diets reduced the amount of total and available Lys within the diet, which suggests formaldehyde is affecting AA availability of the diet. Formaldehyde also negatively impacted fecal microbial diversity with a reduction in

Lactobacillaceae species but increase in *Clostridiaceae* species. As desired, the inclusion of formaldehyde within the diet reduces bacterial concentration within complete diets.

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Table 2-1. Diet composition, Exp. 1 (as-fed basis)[1]

Item	Low Lys[2]	Adequate Lys[3]
Ingredient, %		
Corn	68.80	63.74
Soybean meal	26.80	31.73
Monocalcium phosphate	0.93	0.83
Limestone	1.23	1.20
Basemix[4]	2.35	2.50
Total	100.00	100.00
Calculated analysis		
Standard ileal digestible (SID) AA, %		
Lys	1.10	1.25
Ile:Lys	61	60
Leu:Lys	130	124
Met:Lys	32	31
Met & Cys:Lys	56	54
Thr:Lys	62	61
Trp:Lys	17.7	17.7
Val:Lys	67	65
SID Lys:ME, g/Mcal	3.37	3.84
ME, kcal/kg	3,256	3,250
Total Lys, %	1.23	1.39
CP, %	19.0	20.9
Ca, %	0.73	0.72
P, %	0.61	0.61
Available P, %	0.41	0.41

[1]Treatment diets were fed from ~15.2 to 25.6 kg BW with diets either untreated or treated with 3.2kg/tonne Sal CURB (Kemin Industries, Inc., Des Moines, IA) or 3.0 kg/tonne Termin-8 (Anitox Corp., Lawrenceville, GA) according to manufacturers' recommendations.
[2]Indicates diets were formulated to 90% of the SID Lys requirement (NRC, 2012).
[3]Indicates diets were formulated to meet the SID Lys requirement (NRC, 2012).
[4]Base mix was formulated to contain 25.72% corn, 7.30% monocalcium phosphate, 20% salt, 14.88% L-Lys HCl, 4.24% DL-Met 4.72% L-Thr, 1.40% choline chloride 60%, 0.74% Phytase (HiPhos 2700, DSM Nutritional Products, Inc., Parsippany, NJ,which provided 4,091 FYT/kg of basemix), 1% tribasic copper chloride, and 20% vitamin and trace mineral premix.

Table 2-2. Diet composition, Exp. 2 (as-fed basis)[1]

	Phase 1			Phase 2		
Ingredient, %	Control[2]	Low Crystalline AA[3]	High Crystalline AA[3]	Control[2]	Low Crystalline AA[3]	High Crystalline AA[3]
Corn	45.61	46.10	56.19	43.48	43.90	58.70
Soybean Meal (46.5% CP)	47.64	37.60	28.21	30.36	30.33	16.58
Corn DDGS, 6-9% oil[4]	10.00	10.00	10.00	20.00	20.00	20.00
Choice white grease	3.20	3.25	2.00	3.40	3.45	1.65
Limestone	1.08	1.08	1.13	1.15	1.15	1.25
Monocalcium phosphate, 21% P	0.80	0.80	0.85	0.40	0.40	0.47
Sodium chloride	0.46	0.46	0.46	0.41	0.41	0.41
L-Lys-HCL	0.41	0.05	0.34	0.33	---	0.43
L-Thr	0.13	---	0.13	0.08	---	0.10
L-Trp	---	---	0.01	---	---	0.03
Phytase[5]	0.02	0.02	0.02	0.02	0.02	0.02
Trace mineral and vitamin premix	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin E[6]	0.05	0.05	0.05	0.05	0.05	0.05
Zinc oxide	0.15	0.15	0.15	0.06	0.06	0.06
Copper sulfate	0.13	0.13	0.13	0.13	0.13	0.13
CTC-100[7]	0.20	0.20	0.20	---	---	---
Formaldehyde[8]	---	---	---	---	---	---
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated analysis						
Standardized ileal digestable (SID) AA, %						
Lys	1.45	1.[17]	1.17	1.25	0.99	0.99
Met:Lys	38	48	44	38	49	43
Met and Cys:Lys	61	75	68	63	80	68
Thr:Lys	61	65	65	63	72	63
Trp:Lys	18.1	22.4	18.8	18.5	23.4	18.8
Val:Lys	67	84	71	75	94	72
Total Lys, %	1.64	1.36	1.33	1.45	1.19	1.15

ME, kcal/kg	3,421	3,149	3,370	3,450	3,447	3,377
NE, kcal/kg	2,484	2,484	2,484	2,515	2,515	2,515
CP, %	25.1	24.7	21.4	24.0	23.6	18.8
Ca, %	0.66	0.66	0.66	0.61	0.61	0.61
P, %	0.62	0.62	0.59	0.53	0.54	0.49
Available P, %	0.40	0.40	0.40	0.35	0.35	0.35

[1]Phase 1 diets fed from ~12.22 to 17.56 kg BW and Phase 2 diets from ~17.56 to 27.50 kg BW.
[2]Control diets were formulated to exceed the SID Lys requirement (NRC, 2012).
[3]Treatment diets were formulated to 80% of the control diet and contained low or high levels of crystalline AA.
[4]Dried distillers grains with solubles.
[5]Optiphos 2000 (Huvepharma LLC., Sofia, Bulgaria), provided 406.3 phytase units (FTU)/kg and an estimated release of 0.10% available P.
[6]20,000 IU.
[7]Chlortetracycline-100 (Zoetis Services, LLC., Florham Park, NJ) added at 400 mg/kg.
[8]Sal CURB (Kemin Industries, Inc., Des Moines, IA) added 3.2 kg/tonne diet in all phases (d 0-28) according to manufacturer's recommendations.

Table 2-3. Chemical analysis of diets, Exp. 1, % (as-fed-basis)[1]

	Low Lys[2]			Adequate Lys[3]		
	None	Source 1[4]	Source 2[5]	None	Source 1[4]	Source 2[5]
Proximate analysis, %						
DM	86.1	86.1	86.3	86.7	86.7	86.4
CP	21.0	23.8	22.3	22.2	24.4	23.8
Crude fiber	2.6	2.7	4.4	2.7	2.6	2.4
ether extract	1.9	4.0	1.7	3.6	3.7	2.6
Ash	5.7	5.9	6.8	6.2	6.4	6.9
Total AA, %						
Lys	1.38	1.27	1.42	1.58	1.59	1.59
Available Lys[6]	1.35	1.21	1.37	1.54	1.55	1.56

[1]Complete diet samples were obtained from each dietary treatment during manufacturing. Samples of diets were then submitted for analysis of DM, CP, Crude Fiber, Fat, Ash, and Amino Acid profile (Experiment Station Chemical Laboratories, University of Missouri-Columbia, Columbia, Missouri).
[2]Indicates diets were formulated to 90% of the SID Lys requirement (NRC, 2012).
[3]Indicates diets were formulated to meet the SID Lys requirement (NRC, 2012).
[4]Sal CURB (Kemin Industries Inc., Des Moines, IA) added at 3.2 kg/t.
[5]Termin-8 (Anitox Corp., Lawrenceville, GA) added at 3.0 kg/t.
[6]Available Lys represents the difference in amount of Lys residues in proteins pre- and post-reaction.

Table 2-4. Chemical analysis of diets, Exp. 2 (as-fed basis)[1,2]

		Low crystalline AA[4]		High crystalline AA[4]	
	Control[3]	No Formaldehyde	Formaldehyde[5]	No Formaldehyde	Formaldehyde[5]
Phase 1 Diets					
DM, %	91.0	91.0	89.9	90.3	90.0
CP, %	25.3	25.9	24.1	21.7	21.0
Ca, %	0.68	0.77	0.81	0.76	0.98
P, %	0.62	0.69	0.60	0.60	0.65
Propionic acid, ppm[6]	<LOQ[7]	<LOQ	295	<LOQ	300
Phase 2 Diets					
DM, %	90.2	90.4	89.9	90.2	89.6
CP, %	23.8	23.7	22.7	18.9	19.6
Ca, %	0.53	0.71	0.63	0.73	0.52
P, %	0.58	0.62	0.55	0.60	0.57
Propionic acid, ppm	<LOQ	<LOQ	305	<LOQ	300

[1]Phase 1 diets fed from ~12.22 to 17.56 kg BW and Phase 2 diets from ~17.56 to 27.50 kg BW.
[2]Complete diet samples were obtained from each dietary treatment and phase during manufacturing and from the farm feeder. Samples of diets were pooled and then submitted for analysis of DM, CP, Ca, and P (Ward Laboratories, Inc., Kearney, NE).
[3]Control diets were formulated to exceed the SID Lys requirement (NRC, 2012).
[4]Treatment diets were formulated to 80% of the control diet and contained low or high levels of crystalline AA.
[5]Sal CURB (Kemin Industries, Inc., Des Moines, IA) added at 3.2 kg/t of the diet in all phases (d 0-28).
[6]Propionic acid testing conducted according to Kemin Industries, Inc. sampling methods.
[7]Below level of quantification.

Table 2-5. Effect of formaldehyde-treated diets and crystalline amino acid level on lysine content, % (Exp. 2)[1,2]

		Low Crystalline AA		High Crystalline AA	
	Control	No Formaldehyde	Formaldehyde[3]	No Formaldehyde	Formaldehyde[3]
Phase 1					
Calculated					
Total Lys	1.64	1.36	1.36	1.33	1.33
Free Lys	0.41	0.05	0.05	0.34	0.34
Analyzed					
Total Lys	1.59	1.32	1.21	1.28	1.24
Available Lys	1.56	1.32	1.19	1.29	1.25
Free Lys	0.30	0.06	0.06	0.25	0.26
Phase 2					
Calculated					
Total Lys	1.45	1.19	1.19	1.15	1.15
Free Lys	0.33	0	0	0.43	0.43
Analyzed					
Total Lys	1.38	1.18	1.04	1.11	1.10
Available Lys	1.37	1.14	1.00	1.08	1.07
Free Lys	0.23	0.02	0.02	0.27	0.33

[1]Phase 1 diets fed from ~12.22 to 17.56 kg BW and Phase 2 diets from ~17.56 to 27.50 kg BW.
[2]Complete diet samples were obtained from each dietary treatment during manufacturing and from the farm feeder. Samples of diets were pooled and then submitted for analysis of total lysine, available lysine, and free lysine (Experiment Station Chemical Laboratories, University of Missouri-Columbia, Columbia, Missouri). Values represent average of duplicate analyses on pooled samples.
[3]Kemin Industries Inc., Des Moines, IA.

Table 2-6. Effect of lys level and formaldehyde source on nursery pig growth performance (Exp. 1)[1]

	Low Lys[2]			Adequate Lys[3]				Probability, *P* <		
	None	Source 1[4]	Source 2[5]	None	Source 1[4]	Source 2[5]	SEM	Formaldehyde × Lys	Lys Level	Formaldehyde
BW, kg										
d 0	15.3	15.2	15.2	15.2	15.3	15.3	0.257	0.882	0.975	0.967
d 7	20.6	20.7	20.3	20.2	20.1	19.9	0.308	0.936	0.088	0.521
d 14	25.9	26.2	25.8	25.6	25.3	24.9	0.358	0.652	0.023	0.399
d 0 to 14										
ADG, g	759[a]	783[a]	754[a]	749[ab]	713[bc]	688[c]	13.30	0.053	0.001	0.037
ADFI, g	1,247	1,206	1,265	1,204	1,226	1,259	19.74	0.286	0.561	0.054
G:F	0.601	0.592	0.544	0.630	0.639	0.599	0.007	0.189	0.001	0.001

[1]A total of 299 pigs (PIC 327 × 1050, initially 15.2 ± 0.26 kg) were used in a 14-d study with 5 pigs per pen and 10 replications per treatment. Pigs were weaned at approximately 21 d, fed a common starter diet for 21 d post-weaning, and then fed experimental diets.
[2]Indicates diets were formulated to 90% of the SID Lys requirement (NRC, 2012).
[3]Indicates diets were formulated to meet the SID Lys requirement (NRC, 2012).
[4]Sal CURB (Kemin Industries Inc., Des Moines, IA) added at 3.2 kg/t.
[5]Termin-8 (Anitox Corp., Lawrenceville, GA) added at 3.0 kg/t.

Table 2-7. Effect of formaldehyde-treated diets and crystalline amino acid level on complete feed bacterial concentration (Exp. 2)[1,2,3,4]

		Low crystalline AA		High crystalline AA	
	Control	No Formaldehyde	Formaldehyde[5]	No Formaldehyde	Formaldehyde[5]
Phase 1 feed mill[6]					
Aerobic plate count	1.7×10^5	5.3×10^4	6.1×10^4	7.9×10^4	5×10^3
Enterobacteriaceae count	3.2×10^3	1.5×10^3	0	4.6×10^3	0
Total coliform count	3.5×10^3	1.2×10^4	0	9.0×10^3	0
Phase 1 farm[7]					
Aerobic plate count	2.2×10^5	8.6×10^4	8.0×10^4	1.3×10^5	8×10^3
Enterobacteriaceae count	6.7×10^3	2.9×10^3	0	3.4×10^4	0
Total coliform count	5.9×10^4	1.5×10^4	0	6.5×10^4	0
Phase 2 feed Mill[6]					
Aerobic plate count	2.6×10^5	4.5×10^4	2.3×10^5	4.8×10^4	3.8×10^4
Enterobacteriaceae count	2.0×10^4	5.5×10^3	1.0×10^4	1.0×10^4	0
Total coliform count	4.2×10^4	5.5×10^3	1.5×10^4	4.4×10^4	0
Phase 2 farm[7]					
Aerobic plate count	1.1×10^6	4.7×10^5	3.5×10^4	1.3×10^5	4.6×10^5
Enterobacteriaceae count	7.0×10^4	2.8×10^4	3.1×10^3	2.7×10^4	6.9×10^4
Total coliform count	3.6×10^5	5.5×10^4	3.7×10^4	4.9×10^4	2.5×10^5

[1]Phase 1 diets fed from ~12.22 to 17.56 kg BW and Phase 2 diets from ~17.56 to 27.50 kg BW.

[2]Complete feed samples from each dietary treatment and phase were collected during manufacturing and from the farm for enumeration of feed bacterial concentration (Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS).

[3]1 g of each composite sample from the feed mill and farm were diluted in PBS and serially diluted onto 3M Petrifilm plates that selected for *Escherichia coli* (EC), coliforms (EC), aerobic plate counts (APC), and *Enterobacteriaceae* (EB).Samples were incubated at respective times for each selected organism and a 3M plate reader was used to enumerate feed bacterial concentration (Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS).

[4]Feed bacterial concentrations are expressed as colony forming units per gram of feed sample (cfu/g).

[5]Kemin Industries Inc., Des Moines, IA.

[6]Indiciates feed samples were collected directly from each individual batch of feed for each dietary treatment in each phase during manufacturing. 5 equally spaced sub-samples were collected by passing sterile Whirl-Pak through stream of lot during manufacturing and pooled to create one composite sample for each dietary treatment in each phase to represent feed mill sample.

[7]Indicates feed samples were collected from 6 randomly chosen feeders from each dietary treatment in each phase. The 6 sub-samples were then pooled into one composite sample for each dietary treatment in each phase to represent farm sample.

. **Table 2-8.** Effect of formaldehyde-treated diets and crystalline amino acid level on nursery pig performance (Exp. 2)[1]

		Low crystalline AA[3]		High crystalline AA[3]			Probability, *P*<			
	Control[2]	No formaldehyde	Formaldehyde[4]	No formaldehyde	Formaldehyde	SEM	Control vs. Others	Crys AA × formaldehyde	Low vs. High crystalline	Formaldehyde
d 0 to 12										
ADG, g	487	443	426	459	412	9.23	0.002	0.103	0.910	0.001
ADFI, g	695	688	688	713	680	11.20	0.665	0.105	0.402	0.110
G:F	0.703	0.644	0.620	0.647	0.600	0.009	0.001	0.197	0.349	0.001
d 12 to 28										
ADG, g	686	617	579	607	600	8.74	0.001	0.073	0.526	0.009
ADFI, g	1,133[a,b]	1,106[b,c]	1,112[a,b,c]	1,148[a]	1,086[c]	15.60	0.713	0.023	0.594	0.052
G:F	0.606[a]	0.558[b]	0.522[c]	0.528[c]	0.551[b]	0.004	0.001	0.001	0.948	0.114
d 0 to 28										
ADG, g	601	542	513	543	519	7.30	0.001	0.757	0.637	0.001
ADFI, g	945[a,b]	927[b,c]	930[b,c]	960[a]	911[c]	12.41	0.921	0.020	0.526	0.036
G:F	0.636[a]	0.585[b]	0.553[d]	0.566[c]	0.567[c]	0.003	0.001	0.001	0.478	0.001
BW, kg										
d 0	12.2	12.2	12.3	12.2	12.2	0.120	0.530	0.278	0.142	0.139
d 12	18.1	17.5	17.4	17.7	17.1	0.183	0.002	0.055	0.626	0.009
d 28	29.1	27.4	26.8	27.5	26.7	0.280	0.001	0.713	0.931	0.001

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

[1]A total of 1,235 pigs (PIC 359 × Genetiporc F25, initially 12.20 ± 0.12 kg) were used in a 2-phase nursery study with 19 to 22 pigs per pen and 12 replications per treatment.

[2]Control diets were formulated to exceed the SID Lys requirement (NRC, 2012).

[3]Treatment diets were formulated to 80% of the control diet and contained low or high levels of crystalline AA.

[4]Sal CURB (Kemin Industries Inc., Des Moines, IA) added at 3.2 kg/t.

Table 2-9. Effect of formaldehyde-treated diets and crystalline amino acid level on fecal bacterial abundances at family level[1,2]

		Low crystalline[4]		High crystalline[4]			Probability $P <$			
	Control[3]	No formaldehyde	Formaldehyde[5]	No formaldehyde	Formaldehyde[5]	SEM	Control vs. others	Crys AA × formaldehyde	Low vs. High crystalline	Formaldehyde
Abundances, %[6]										
Clostridiaceae	19.0	19.2	27.5	25.9	35.5	2.65	0.009	0.796	0.007	0.001
Erysipelotrichaceae	2.12	1.97	2.59	2.51	3.19	0.33	0.210	0.918	0.08	0.047
Lachnospiraceae	8.27	7.95	10.6	9.50	10.2	1.21	0.338	0.440	0.639	0.169
Lactobacillaceae	17.3	11.9	0.60	9.90	1.04	1.88	0.682	0.532	0.001	0.001
Methanobacteriaceae	3.71	4.74	5.83	4.98	5.11	1.08	0.224	0.661	0.824	0.578
Paraprevotellaceae	1.16	1.33	0.69	0.81	0.48	0.17	0.091	0.371	0.041	0.008
Prevotellaceae	10.5	11.9	10.6	8.57	9.07	1.56	0.796	0.568	0.129	0.802
Ruminococcaceae	11.7	11.1	13.2	10.0	10.6	0.88	0.661	0.410	0.038	0.136
Spirochaetaceae	1.04	0.78	0.67	0.59	0.51	0.30	0.211	0.953	0.550	0.760
Streptococcaceae	4.52	6.19	0.02	3.30	0.31	0.53	0.001	0.003	0.011	0.001
S24-7	3.38	4.22	3.75	4.41	2.94	0.53	0.458	0.352	0.557	0.074
Veillonellaceae	1.53	1.83	2.03	1.55	0.98	0.25	0.790	0.126	0.010	0.471

[1]A total of 1,235 pigs (PIC 359 × PIC 1050, initially 12.20 ± 0.12 kg) were used in a 2-phase nursery study with 19 to 22 pigs per pen and 12 replications per treatment. Pigs were weaned at approximately 21 d, fed a common starter diet for 10 d post-weaning, and then fed experimental diets.
[2]3 random fecal samples were collected per pen on d 28 of the trial and pooled to form 1 composite sample for each pen on each dietary treatment, DNA was isolated, and each composited sample was assessed.
[3]Control diets were formulated to exceed the SID Lys requirement (NRC, 2012).
[4]Treatment diets were formulated to 80% of the control diet and contained low or high levels of crystalline AA.
[5]Sal-CRUB (Kemin Industries Inc., Des Moines, IA) added at 3.2 kg/t.
[6]Bacterial species that composed at least 1% of total bacterial population in an individual treatment.

Chapter 3 - Effects of chlortetracycline alone or in combination with probiotics on nursery pig growth performance and antimicrobial resistance of fecal *Escherichia coli*

ABSTRACT

A total of 300 pigs (200 × 400 DNA, Columbus, NE; initially 5.9 ± 0.05 kg BW) were used in a 42-d growth trial to evaluate the effects of feeding chlortetracycline with or without probiotics on growth performance and antimicrobial resistance (AMR) of fecal *Escherichia coli*. Chlortetracycline (CTC) is a broad-spectrum in-feed antibiotic commonly used in the swine industry. Probiotic 1 (Chr. Hansen USA, Inc., Milwaukee, WI) is a *Bacillus* strain based probiotic and Probiotic 2 (Biomin America, Inc., San Antonio, TX) is a multi-species based product. Weaned pigs (~21 d of age) were allotted to pens based on initial BW and fed a common starter diet for 4 d. Pens were then blocked by BW and allotted to dietary treatments in a completely randomized block design. Dietary treatments were arranged in a 2 × 3 factorial consisting of combinations of CTC (none vs. 400 mg/kg from d 0 to 42) and probiotic (0 vs. 0.05% probiotic 1 vs. 0.05% probiotic 2). Overall, pigs fed diets containing CTC had improved ($P < 0.001$) ADG, ADFI, and BW compared to those not fed CTC with no evidence for any effect of either probiotic on overall growth. Inclusion of probiotic 2 in diets improved ($P < 0.05$) ADFI from d 0 to 14 and d 14 BW. Fecal samples were collected from 3 randomly selected pigs from each pen on d 0, 21, and 42 for *E. coli* isolation and AMR determination. The addition of CTC with or without probiotic to nursery pig diets increased ($P < 0.05$) the probability of AMR to tetracycline and ceftiofur of fecal *E. coli* isolates, but this resistance generally decreased ($P < 0.05$) over time. A decrease ($P < 0.05$) in resistance to ampicillin and tetracycline throughout the

trial was observed, while resistant isolates to ceftriaxone decreased ($P < 0.020$) from d 0 to 21 and increased from d 21 to 42 amongst dietary treatments regardless of CTC or probiotic inclusion in the diet. A CTC×probiotic×day interaction ($P < 0.015$) was observed for streptomycin, whereby from d 21 to 42 AMR increased in diets containing either CTC or probiotic 1 alone, but the combination decreased resistance. There was no evidence for any effect of probiotics on AMR of fecal *E. coli* isolates. In conclusion, added CTC with or without probiotic inclusion improved nursery pig performance, but increased AMR of fecal *E. coli* isolates to tetracycline and ceftiofur. A moderate improvement in intake and d 14 BW was observed when probiotic 2 was included in the diet with or without CTC, but there was no evidence that added probiotic affected resistance of fecal *E. coli* to antibiotics.

Key words: antimicrobial resistance, chlortetracycline, *E. coli*, growth performance, nursery, probiotic

INTRODUCTION

Emergence of in-feed antibiotics in the 1950's improved efficiency of growth and overall health of nursery pigs. A review by Cromwell (2002) summarized that including antibiotics in feed improved growth by 16.4% and efficiency by 6.9% and reduced mortality from 4.3 to 2.0%. Also, the antibiotic chlortetracycline (CTC) is used in sow diets to treat respiratory disease and has been shown to improve litter size, litter growth, and reproductive performance (Soma and Speer, 1975; Maxwell et al. 1994).

Questions have arisen over inclusion of in-feed antibiotics contribution to AMR within food animal production (WHO, 2014). Addition of in-feed antibiotics to nursery pig diets has been associated with increased resistance of *E. coli* to antibiotics (Funk et al., 2006; Agga, 2014). Furthermore, addition of CTC to sow diets at sub-therapeutic and therapeutic levels has shown to

increase antibiotic-resistant coliforms compared to sows fed a diet without antibiotics (Langlois et al., 1984). In addition, the potential for AMR genes to be transferred from the sow to the offspring is apparent and of concern.

Alternative technologies, such as probiotics, are desired to reduce use of in-feed antibiotics in nursery diets. In addition to growth performance benefits (Kritas and Morrison, 2005), probiotics may have a favorable impact on the development and persistence of AMR in gut bacteria. Probiotics promote growth and persistence of selective species or groups of bacteria in the gut and this may impact, directly or indirectly, the emergence, prevalence and persistence of AMR in gut commensals and pathogens. There is evidence that co-administration of probiotics with antibiotics in humans enhances the resilience of gut bacterial flora to antibiotics-induced alterations (Plummer et al., 2005; McFarland, 2006). Therefore, the objective of this study was to determine the effects of CTC with or without probiotics on nursery pig performance and on antimicrobial resistance in *E. coli* isolated from feces.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee approved the protocol for this experiment. The study was conducted at the Kansas State University Segregated Early Weaning Facility in Manhattan, KS. Each pen (1.22 × 1.22 m) had metal tri-bar flooring, one 4-hole self-feeder and a cup waterer to provide ad libitum access to feed and water.

Animals

A total of 300 nursery pigs (DNA 200 × 400, Columbus, NE; initially 5.9 ± 0.05 kg BW) were used in a 42-d study with 5 pigs per pen and 10 pens per treatment. Pigs were weaned at approximately 21 d of age and allotted to pens based on initial BW. Pigs were fed a common

starter diet that did not contain in-feed antimicrobials for 4 days, after which pens were blocked by initial BW and allotted to 1 of 6 dietary treatments in a completely randomized block design.

The 6 dietary treatments were arranged in a 2 × 3 factorial consisting of combinations of chlortetracycline (0 vs. 400 mg/kg from d 0 to 42; Zoetis Services, LLC., Florham Park, NJ) and probiotics (0 vs. probiotic 1 vs. probiotic 2). Probiotic 1 consisted of 0.05% Bioplus 2B (Chr. Hansen USA, Inc., Milwaukee, WI) whereas probiotic 2 consisted of 0.05% Poultry Star (Biomin America, Inc., San Antonio, TX). Experimental diets were fed throughout 2 study phases (Phase 1: d 0 to 14 and Phase 2: d 14 to 42) in meal form. On d 14 and 28, CTC was removed from the diet to comply with FDA regulations; when appropriate to the experimental diets, CTC was resumed on d 15 and 29. Pens and feeders were weighed every 7 d to determine ADG, ADFI, and G:F.

Diet Preparation

All diets were prepared at the O.H. Kruse Feed Technology and Innovation Center located in Manhattan, KS. Phase 1 diets contained specialty protein ingredients and all treatment diets were formulated according to the Nutrient Requirements of Swine (NRC, 2012) to be at or above the pigs' daily nutrient requirements as not to limit growth performance. The treatment ingredients were substituted for an equivalent amount of corn in the respective diets to form the experimental diets (Table 1). During feed manufacturing, when bagging the experimental diets, feed samples were collected from the 5^{th}, 10^{th}, 15^{th}, 20^{th}, 25^{th}, 30^{th}, 35^{th}, and 40^{th} bags, and these samples were pooled and used for nutrient analysis.

Chemical Analysis

One sample of mixed ingredients per dietary treatment from the pooled feed samples was sent to a commercial laboratory (Ward Laboratories, Kearney, NE) for analysis of DM (AOAC

935.29, 2012), CP (AOAC 990.03, 2012), Ca (AOAC 965.14/985.01, 2012), and P (AOAC 965.17/985.01, 2012; Table 2).

Fecal collection

On d 0, 21, and 42, fecal samples were collected by gentle rectal massage from 3 randomly selected pigs per pen and placed into individual plastic bags (Whirl-Pak, Nasco, Ft. Atkinson, WI), for a total of 30 samples per treatment for each sampling day. Samples were immediately transported to the Pre-Harvest Food Safety Laboratory, Department of Diagnostic Medicine/Pathobiology at the College of Veterinary Medicine, Kansas State University, for bacterial isolation and further characterization.

E. coli Isolation

Approximately 1 g of fecal sample was suspended in 9 mL phosphate-buffered saline. Fifty μL of the fecal suspension was then spread-plated onto a MacConkey agar (Becton Dickinson, Sparks, MD) for the isolation of *E. coli*. Two lactose fermenting colonies were picked from each MacConkey agar; each colony was individually streaked onto a blood agar plate (Remel, Lenexa, KS) and incubated at 37°C for 24 h. Indole test was done and indole-positive isolates were stored in to cryo-protect beads (Cryocare®, Key Scientific Products, Round Rock, TX) at -80°C.

Antimicrobial Susceptibility Testing of E. coli Isolates

Antimicrobial susceptibility testing was done on *E. coli* isolates recovered on d 0, 21, and 42. The microbroth dilution method as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2013) was used to determine the minimal inhibitory concentrations (MIC) of several antibiotics. Each isolate, stored in cryo-protect beads, was streaked onto a blood agar plate and incubated at 37°C for 24 h. Individual colonies were suspended in demineralized water

(Trek Diagnostic Systems, Cleveland, OH) and turbidity was adjusted to 0.5 McFarland turbidity standards. Then, 10 μL of the bacterial inoculum was added to Mueller-Hinton broth and vortexed to mix. A Sensititre® automated inoculation delivery system (Trek Diagnostics Systems) was used to dispense 100 μL of the culture into National Antimicrobial Resistance Monitoring System (NARMS) panel plates designed for Gram-negative (CMV3AGNF, Trek Diagnostic Systems) bacteria. *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) strains were included as quality controls for *E. coli* susceptibility testing. Plates were incubated at 37°C for 18 h and bacterial growth was assessed using Sensititre ARIS® and Vizion™ systems (Trek Diagnostic Systems). Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines were used to classify each isolate as resistant or susceptible (intermediate and susceptible) according to the breakpoints established for each antimicrobial.

Statistical Analysis

Growth Data

Growth data were analyzed using general linear mixed models implemented using the PROC GLIMMIX procedure of SAS (SAS Institute, Inc., Cary, NC) with pen as the experimental unit. The linear predictor included the fixed effect of treatment and the random effect of BW block. The main effects of CTC and probiotics as well as their interactions, were evaluated using preplanned CONTRAST statements. Differences between treatments were determined by using least squares means. Results were considered to be significant with *P*-values ≤ 0.05 and were considered marginally significant with *P*-values ≤ 0.10.

Antimicrobial Susceptibility

Antimicrobial susceptibility data were analyzed using generalized linear mixed models that assumed a Bernoulli distribution of the response and link function? . Models were implemented using the PROC GLMM procedure of SAS (SAS Institute, Inc., Cary, NC) with pen as the experimental unit and 3 sample observations per pen per sampling day. Treatment was considered the fixed effect and random effects of block and blockxTrt in order to recognize pen as the experimental unit for treatment.. For each antimicrobial evaluated, a series of frequency tables of resistant/susceptible as functions of treatment, day and their combination were created in order to anticipate potential extreme categorical problems (i.e quasi-complete separation of datapoints) during model fitting. Over dispersion was assessed using the maximum-likelihood-based fit statistic Pearson Chi-Square over degrees of freedom.. The final model used for inference was fitted using residual pseudo- likelihood implemented with a Newton-Raphson optimization with ridging. Treatment main effects of CTC, BioPlus 2B, and Poultry Star, day of sampling (d 0, 21, or 42), and their interactions were evaluated. Results were considered to be significant with *P*-values ≤ 0.05 and were considered marginally significant with *P*-values ≤ 0.10.

RESULTS

Chemical Analysis

Results of DM, CP, and P analysis closely matched formulated values (Table 2).

Growth Performance

No evidence existed for significant interactions between CTC and probiotic 1 or probiotic 2 either from d 0 to d 14 or from d 14 to 28. From d 0 to 14, pigs fed diets with CTC increased

($P < 0.05$) ADG, ADFI, G:F, and d 14 BW compared to those fed diets without CTC (Table 3), regardless of whether the diet included a probiotic or not. Regardless of CTC inclusion, pigs fed diets with probiotic 2 had improved ($P < 0.05$) ADFI and d 14 BW compared to those fed diets without any probiotic. The inclusion of CTC in diets increased ($P < 0.05$) ADG, ADFI, and d 28 BW compared to those pigs fed diets without CTC. Also, pigs fed probiotic 2 had marginal significance for greater ($P = 0.052$) ADFI than those not fed probiotic 2.

From d 28 to 42, a CTC × Probiotic 2 interaction ($P = 0.05$) was observed for ADFI. The interaction occurred because no evidence of difference existed between diets including probiotic 2 alone or in combination with CTC compared to the control, but inclusion of CTC alone resulted in improved ADFI compared to the control. Furthermore, a marginally significant interaction of CTC × probiotic 1 ($P = 0.082$) was observed for G:F as a result of increased G:F when pigs were fed diets containing CTC and probiotic 1, but poorer G:F when pigs were fed diets containing either CTC or probiotic 1 alone.

For the overall study (d 0 to 42), no evidence for CTC by probiotic interactions were observed for any of the responses on growth performance. On average, pigs fed diets containing CTC had greater ($P = 0.001$) ADG, ADFI, and overall BW compared to those not fed CTC, regardless of whether a probiotic had been added to the diet. There was no evidence for any effects of the addition of either probiotic to the diet on growth performance.

Antimicrobial Susceptibility

A day effect was observed for *E. coli* resistance to ampicillin with resistance decreasing ($P < 0.003$; Table 4) for each dietary treatment as the trial progressed. A CTC effect ($P < 0.011$) was observed for susceptibility to ceftiofur with the addition of CTC to diets resulting in increased resistance. A day effect ($P < 0.020$) was observed for ceftriaxone with percentage

resistance amongst fecal isolates decreasing from d 0 to 21 but increasing from d 21 to 42 for all dietary treatments. A day × CTC × probiotic interaction ($P < 0.015$) was observed for susceptibility to streptomycin. This interaction occurred because the variation in resistance on d 0 resulted in resistance increasing when diets fed contained CTC or probiotic 2 alone from d 0 to 42, while feeding other diets resulted in similar resistance over time.

A CTC ($P < 0.050$; Table 5) and day effects ($P < 0.001$) were observed for *E. coli* susceptibility to tetracycline. Addition of CTC to diets resulted in higher percentages of fecal *E. coli* being resistant to tetracycline. The day effect was the result of percentage *E. coli* isolates resistant to tetracycline decreasing on sampling days as the trial progressed. There was no evidence for difference of main effects of day, antibiotic, or probiotic and their interactions observed for fecal isolates grown in the presence of cefoxitin, chloramphenicol, or trimethoprim/sulfamethoxazole.

DISCUSSION

Early research has observed that the inclusion of CTC in nursery diets improved ADG and G:F compared to pigs fed diets not containing CTC (NCR-89, 1984). The studies found that rate and efficiency of gain improved by 13.2% and 4.7%, respectively, when CTC was included in the diet. Additionally, the inclusion of in-feed antibiotics in commercial production systems have been shown to be efficacious at improving rate of gain in weaned pigs but are less effective at improving efficiency of growth (Dritz et al., 2002). The study herein observed that the inclusion of in-feed antibiotics improved rate of gain by upwards of 5.0% with no evidence of difference for G:F. More recent research conducted by Feldpausch et al. (2015) indicated that the inclusion of CTC up to 441 ppm increased feed intake, which resulted in linear increases in BW gain. They also observed no evidence of differences in G:F with the inclusion of in-feed

antibiotics. The results from our study agree with the previously mentioned study that the inclusion of CTC in nursery pig diets improved gain and feed intake, which resulted in increased BW gain.

Probiotics from bacterial species such as *Lactobacillus* and *Enterococcus* are suggested to have the ability to improve gastrointestinal function and prevent infections through a multitude of mechanisms (Oelschlaeger, 2010). These proposed mechanisms include beneficially altering gut microbiome, regulating the immune system (Suda et al, 2014) and providing anti-pathogenic activity (Bomba et al, 2002) to reduce infection from enteric pathogens. These gastrointestinal and health benefits are a reason why probiotics are being considered as an alternative to antibiotics. Probiotics are suggested to promote gut health and gut microbiome by stabilizing the epithelial membrane of the gastrointestinal tract, producing fermentation products and bacteriocins, and enzymes that aid in nutrient uptake and absorption (Gaggia, 2010). To be effective and express these mechanisms, a probiotic must survive in feed and be able to pass through the gastrointestinal tract of the pig (Jacela, 2010). Although the proposed health benefits of probiotics support their addition to nursery pig diets, the results have been inconsistent.

Probiotic 1 is a dual-strain probiotic based feed supplement containing *Bacillus licheniformis* and *Bacillus subtilus* bacterial species. Kritas and Morrison (2005) conducted a field study to compare the effects of antibiotic regimen or added probiotic 1 in diets on nursery pig performance. The antibiotic regimen used in the study included 400 mg/kg of neomycin for the first 7 days post-weaning, 100 mg/kg of neomycin and 100 mg/kg oxytetracycline the next 7 d, and 20 mg/kg tylosin to 70 d of age post-weaning. The researchers observed that in high-health herds no evidence for differences existed between pigs fed diets including probiotic 1 compared to that of pigs fed an antimicrobial regimen. However, Keegan et al. (2005) conducted

multiple experiments on the effects of probiotic products and in-feed antibiotics on nursery pig performance. They observed in both a university and commercial setting the addition of probiotic 1 had no evidence for differences ($P > 0.10$) on ADG, ADFI, or G:F compared to the control, and pigs fed diets containing antibiotics had improved ($P < 0.05$) ADG, ADFI, and G:F compared to pigs fed the control or probiotic 1 diets. These results are consistent with the findings from this trial that the addition of probiotic 1 alone did not have a significant effect on growth performance in nursery pigs. A multitude of reasons exist that may contribute to why probiotics are inconsistent in improving performance when added to nursery diets. These include the strain of bacteria not surviving the feed manufacturing process, although diets were fed in meal form in the current study, or the dietary concentration of probiotic strain not high enough, but no definitive evidence exists to support these claims. Furthermore, the reduced bacterial concentration of the environment through biosecurity measures may reduce the efficacy of probiotics. However, a marginal interaction between probiotic 1 and CTC was observed during this trial with improved efficiency observed when CTC and probiotic 1 were included in combination compared to alone. This proposes that the mode of action for both probiotics and antibiotics may exert a synergistic relationship towards certain pathogens present in the gut but no evidence in the literature supports these findings.

Probiotic 2 is a multi-strain probiotic based feed supplement containing a blend of *Enterococcus faecium, Lactobacillus reuteri, Lactobacillus salivarius,* and *Pediococcus acidilactici* that is included at 10^9 CFU/kg (FAO, 2016). This product has been used in the poultry industry because of its potential to increase performance of broilers during a disease challenge and to increase activation of the immune system (Koenen et al., 2004; Chichlowski et al., 2007). To our knowledge, ours is the first published trial that evaluated probiotic 2 in a swine

diet. In the present study, the addition of probiotic 2 resulted in increased ADFI and BW through the first 14 d of the study. This finding suggests probiotic 2 may have an impact on performance in early phases of nursery pig production, but more research should be conducted with swine to confirm this response.

Antimicrobial resistance is a major public health challenge and a complex issue to address (WHO, 2014). The development of antimicrobial resistant bacteria can occur through mutation and selection or acquiring genes from other bacteria that encode for phenotypic resistance mechanisms. The acquisition of genes from other bacteria occurs through conjugative transposons that can transfer genes that code for resistance mechanisms to the plasmids of bacteria within the gastrointestinal tract (Scott, 2002). These mechanisms include acquiring genes encoding enzymes that inactivate antibiotics, development of efflux pumps that remove the antibiotic from the cell before reaching its target site, acquiring genes for metabolic pathways that alter binding site of antibiotics within cell walls, or acquiring mutations that down regulate binding of antibiotics to target sites within cells (Tenover, 2006). The emergence and development of antimicrobial resistant bacteria speculated to be from selective pressure that exists through the continuous use of antimicrobials in human therapies and animal food production (Davies and Davies, 2010). Thus, it is important to understand what dietary factors, if any, may contribute to increased antimicrobial resistance among fecal bacteria of nursery pigs.

The World Health Organization classifies antibiotics as critically and highly important to human medicine and resistance breakpoints for these antibiotics against Gram-negative bacteria are established by the National Antimicrobial Resistance Monitoring System (Feldpausch et al., 2016). Tetracyclines are a class of broad-spectrum antibiotics that display antimicrobial activity against many Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001).

Tetracyclines inhibit bacterial protein synthesis through binding of the 30s subunit of bacterial ribosomes and preventing aminoacyl-tRNA attachment (Schnappinger and Hilen, 1996). Chlortetracycline is one of the most commonly used in-feed antibiotics within the swine industry of the United States (Dewey et al., 1999; Apley et al., 2012). The continuous use of CTC at therapeutic levels for its enteric disease control properties and sub-therapeutic levels to capture its growth promotion benefits in nursery pigs have risen concerns for its potential to become a contributor for antimicrobial resistance. *Tet* and *otr* genes confer resistance to tetracyclines (Roberts, 2011) that encode for efflux proteins, ribosomal protection proteins, and inactivation of enzymes that allow for the development of resistance (Palm et al., 2008). Sub-therapeutic levels of feeding CTC have been shown to increase the prevalence of bacterial resistance genotypes and phenotypes (Funk et al, 2006; Agga et al., 2014). In our study, the addition of CTC to diets increased the proportion of *E coli* isolates resistant to tetracycline, although, the proportion of fecal *E coli.* isolates in diets supplemented with CTC decreased as the trial progressed. These findings suggest that tetracycline resistance may be increased in the early stages of the nursery due to its use upstream in the sow herd, but this resistance may decrease over time even with continual feeding of CTC as the pig grows. Because of this, withdrawal times of CTC during the nursery period must be considered when administering CTC in the feed as to control the amount of resistant *E. coli* bacteria within the pigs' microflora.

Fecal *E. coli* isolates collected over the three time points of the study had decreased resistance to ampicillin, but no evidence existed for a day or treatment effect was observed on *E coli.* resistance to amoxicillin/clavulanic acid. Ampicillin and amoxicillin are beta-lactam antibiotics of the penicillin family that offer antimicrobial activity against Gram-negative bacteria through an α-amino side chain that allows for improved uptake through bacterial porins

(Page, 1984). Amoxicillin and clavulanic acid are used in combination because of the acids ability to improve amoxicillin activity against Gram-negative bacteria. Schroeder et al. (2002) observed that over 20% of *E. coli* isolates derived from swine were resistant to ampicillin, but none of the swine isolates exhibited resistance to amoxicillin/clavulanic acid. Boerlin et al. (2005) observed *E. coli* isolates had increased resistance to ampicillin and all isolates were resistant to amoxicillin. Cavaco et al. (2008) found that pigs inoculated with a nalidixic acid resistant strain of *E. coli* treated with amoxicillin had greater resistant coliform counts than in control pigs not treated with antibiotics up to 22 d after treatment stoppage. This suggests that resistance to ampicillin/amoxicillin was high and that this resistance can remain within the pigs' bacterial flora over extended periods of time (Schroeder et al., 2002; Boerlin et al., 2005; Cavaco et al., 2008). Although, the reduction in resistance that occurred from d 0 to 42 of the current study suggests that the use of ampicillin/amoxicillin-based antibiotics has the potential to increase resistance early in the nursery, but declines over time as the pig grows.

Ceftiofur, ceftriaxone, and cefoxitin are β-lactam antibiotics in the cephalosporin family that have bactericidal activity against Gram-positive and -negative bacteria through inhibition of bacterial cell wall synthesis (Mason and Kietzmann, 1999). The addition of CTC in diets increased *E. coli* resistance to ceftiofur in fecal isolates of the current study. This supports findings of Agga et al. (2014) who reported strong associations with ceftiofur and tetracycline resistance with the supplementation of CTC in diets of nursery pigs. This association is also evident between the $bla_{\text{CMY-2}}$ genes that code for ceftiofur resistance and *tetA* genes that code for tetracycline resistance (Agga et al., 2014). *E coli.* resistance to ceftriaxone decreased from d 0 to 21, but resistance increased back to baseline levels on d 42. Funk et al. (2006) observed that the supplementation of CTC in swine diets increased the percentage of Gram-negative aerobic fecal

flora resistant to ceftriaxone, but days of feeding were not reported. The addition of CTC or probiotic did not have an effect on *E. coli* resistance to cefoxitin during this experiment. Agga et al. (2014) reported that supplementation of CTC did not affect the percentage of resistant *E. coli* isolates to cefoxitin during the CTC treatment period, but resistance decreased after CTC was withdrawn from the diet. These results suggest that the supplementation of CTC and the length at which CTC is administered in the feed does play a part in affecting *E. coli* resistance to cefoxitin in fecal isolates of nursery pigs.

Streptomycin and gentamicin are aminoglycoside antibiotics that exhibit bactericidal activity by targeting 16S rRNA of bacteria ribosomes which inhibits ribosomal function and causes lethal mutations that lead to misreading during RNA translation (Davis, 1987). Resistance to aminoglycosides can arise through bacteria producing methylases RmtA and RmtB that are coded for by plasmid borne genes which protect 16S rRNA from bactericidal activity (Yamane et al., 2005). In the current study, an antibiotic × probiotic × day interaction was observed for *E. coli* resistance to streptomycin. This interaction occurred because the variation in resistance on d 0 resulted in resistance increasing when diets fed contained CTC or probiotic 2 alone from d 0 to 42, while feeding other diets resulted in similar resistance over time. No evidence existed for dietary treatment or sampling day effects for *E. coli* susceptibility to gentamicin. The results from this study suggest that *E. coli* resistance to streptomycin is variable on entry into the nursery and these results must be further explored as to why this variability exists.

No evidence of differences existed with the addition of CTC, probiotic 1, probiotic 2, or a combination of CTC and the individual probiotic products on the proportions of fecal *E. coli* to azithromycin, ciprofloxacin, nalidixic acid, sulfisoxazole, chloramphenicol, or trimethoprim/sulfamethoxazole at any of the sampling points during the current study. Agga et

al. (2014) observed similar results in which no evidence of differences exited with the addition of CTC to nursery diets on *E. coli* resistance to azithromycin, ciprofloxacin, nalidixic acid, or sulfisoxazole. The researchers also found that feeding CTC to nursery pigs decreased resistance of *E. coli* to chloramphenicol and trimethoprim/sulfamethoxazole with an increase in resistance towards these antibiotics found before and after the CTC treatment period. The results from the current study and Agga et al. (2014) suggest that no evidence of differences exist with the feeding of CTC to nursery pigs on resistance to the macrolide, quinolone, phenicol, or folate pathway inhibitor families of antibiotics.

In summary, this study has provided further evidence that the addition of CTC in nursery diets improves growth performance of nursery pigs. The addition of probiotic 2 to nursery diets resulted in improvements in ADFI and d 14 BW, thus indicating that probiotic 2 could be considered as an alternative to improving growth when in diets during the early stages of the nursery period. Further research should be conducted to see if the early performance effects of probiotic 2 are observed during a health challenge, similar to results observed in poultry trials. No evidence for differences existed with the addition of probiotic 1 to nursery diets on performance and coincides with previous research that shows addition of probiotic 1 does not consistently affect nursery pig performance. In general, the addition of CTC to nursery pig diets increased the proportion of fecal *E. coli* isolates resistant to tetracycline and ceftiofur. Although, the resistance towards tetracycline and other antibiotics tested against decreased or indicated no evidence of difference over time. In this trial, no evidence of difference existed with the inclusion of probiotics in the diets on AMR of *E. coli*.

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Table 3-1. Ingredient composition of control diet (as-fed basis)[1]

Item	Phase 1	Phase 2
Ingredient, %		
Corn	55.75	62.50
Soybean meal, 46.5% CP	25.35	33.40
Spray dried whey	10.00	---
HP 300[2]	5.00	---
Limestone	1.05	1.18
Monocalcium phosphate, 21%	1.20	1.20
Sodium chloride	0.30	0.35
L-Lys HCl	0.45	0.45
DL-Met	0.20	0.20
L-Thr	0.20	0.20
L-Trp	0.03	0.03
L-Val	0.10	0.10
Phytase[3]	0.02	0.02
Trace mineral premix[4]	0.15	0.15
Vitamin premix[5]	0.25	0.25
CTC-50[6]	---	---
Probiotic 1[7]	---	---
Probiotic 2[8]	---	---
Total	100	100
Calculated analysis		
Standardized ileal digestible (SID) amino acids, %		
Lys	1.35	1.35
Met:Lys	36	36
Met&Cys:Lys	57	58
Thr:Lys	65	64
Trp:Lys	19.1	19.3
Val:Lys	70	70
Total Lys, %	1.49	1.50
ME, kcal/kg	3,291	3,260
NE, kcal/kg	2,431	2,396
CP, %	21.4	21.9
Ca, %	0.75	0.75
P, %	0.69	0.66
Available P, %	0.49	0.43

[1]Phase 1 diets were fed from d 0 to 14 (~5.9 to 8.5 kg BW) and Phase 2 diets from d 14 to 42 (8.5 to 25.0 kg BW). A common starter diet was fed to all pigs for 4 days after weaning and prior to the start of the experiment.

[2]Hamlet Protein, Inc., Findlay, OH.

[3] HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), providing 406.3 phytase units (FTU)/kg and an estimated release of 0.10% available P.

[4]Provided per kilogram of premix: 22 g Mn from manganese oxide; 73 g Fe from iron sulfate; 73 g Zn from zinc sulphate; 11 g Cu from copper sulfate; 198 mg I from calcium iodate; and 198 mg Se from sodium selenite.
[5]Provided per kilogram of premix: 3,527,360 IU vitamin A; 881,840 IU vitamin D3; 17,637 IU vitamin E; 3,307 mg riboflavin; 1,764 mg menadione; 11,023 mg pantothenic acid; 33,069 mg niacin; and 15.4 mg vitamin B12.
[6]Chlortetracycline provided at 400 mg/kg (Zoetis Services, LLC., Florham Park, NJ).
[7]Bioplus 2B (Chr. Hansen USA, Inc., Milwaukee, WI) added at 0.05% of the diet.
[8]Poultry Star (Biomin America, Inc., San Antonio, TX) added at 0.05% of the diet.

Table 3-2. Diet analysis, % (as-fed basis)[1,2]

CTC	-	-	-	+	+	+
Probiotic 1	-	+	-	-	+	-
Probiotic 2	-	-	+	-	-	+
Phase 1 diets						
DM	89.5	90.1	89.7	89.5	89.9	89.2
CP	21.1	21.3	21.8	21.4	21.8	21.1
Ca	0.85	0.93	0.86	0.91	1.05	0.94
P	0.74	0.72	0.73	0.70	0.70	0.69
Phase 2 diets						
DM	88.0	88.0	88.6	88.3	88.2	88.9
CP	21.7	21.5	21.0	20.7	20.8	21.8
Ca	0.85	0.96	0.95	0.99	1.05	1.08
P	0.66	0.67	0.69	0.69	0.68	0.70

[1]Phase 1 diets were fed from d 0 to 14 (~5.9 to 8.5 kg BW) and Phase 2 diets from d 14 to 42 (8.5 to 25.0 kg BW). A common starter diet was fed to all pigs for 4 days after weaning.
[2]Complete diet samples were obtained from each treatment during manufacturing and composited. Samples of diets were then submitted to Ward Laboratories, Inc. (Kearney, NE) for analysis.

Table 3-3. Effects of in-feed chlortetracycline and probiotic on growth performance (estimated least square means and SEM) of nursery pigs[1]

CTC[2]	-	-	-	+	+	+						
Probiotic 1[3]	-	+	-	-	+	-		Probability, $P <$				
Probiotic 2[4]	-	-	+	-	-	+	SEM	CTC	Probiotic 1	Probiotic 2	CTC × Probiotic 1	CTC × Probiotic 2
d 0 to 14												
ADG, g	159	162	176	196	212	212	10.59	0.001	0.356	0.108	0.505	0.976
ADFI, g	229	236	253	253	275	275	9.85	0.001	0.124	0.018	0.431	0.938
G:F	0.696	0.684	0.705	0.776	0.772	0.770	0.03	0.005	0.796	0.961	0.910	0.816
d 14 to 28												
ADG, g	451	425	472	507	522	534	20.15	0.001	0.795	0.242	0.310	0.868
ADFI, g	658	634	700	771	791	803	19.41	0.001	0.935	0.052	0.239	0.810
G:F	0.685	0.666	0.671	0.658	0.660	0.665	0.02	0.389	0.658	0.849	0.572	0.583
d 28 to 42												
ADG, g	678	655	701	703	716	674	19.85	0.227	0.788	0.860	0.361	0.195
ADFI, g	1069[b]	1053[b]	1127[ab]	1156[a]	1121[ab]	1106[ab]	26.57	0.045	0.350	0.872	0.738	0.050
G:F	0.634	0.620	0.621	0.609	0.640	0.611	0.01	0.614	0.521	0.668	0.082	0.573
d 0 to 42												
ADG, g	424	405	445	469	482	473	13.16	0.001	0.808	0.340	0.214	0.545
ADFI, g	644	625	687	726	727	728	16.22	0.001	0.573	0.173	0.531	0.215
G:F	0.659	0.645	0.648	0.645	0.664	0.650	0.01	0.795	0.839	0.803	0.185	0.517
BW, kg												
d 0	5.9	5.9	5.9	5.9	5.9	5.9	0.05	0.093	0.896	0.613	0.837	0.143
d 14	8.2	8.2	8.5	8.7	8.9	8.9	0.16	0.001	0.388	0.043	0.354	0.914
d 28	14.5	14.2	14.9	15.7	16.2	16.4	0.34	0.001	0.832	0.135	0.265	0.706
d 42	24.2	23.7	24.8	25.6	26.1	25.8	0.52	0.001	0.988	0.438	0.289	0.728

[a,b,c] Indicate differences within a row ($P \leq 0.05$).

[1]A total of 300 pigs (DNA 200 × 400) were used in a 42-d study with 5 pigs per pen and 10 pens per treatment. On d 14 and 28, antibiotics were removed from the diet according to FDA regulations. Experimental diets containing antibiotics resumed feeding on d 15 and 29.

[2]CTC-50 provided at 400 mg/kg (Zoetis Services, LLC., Florham Park, NJ) added at 400 mg/kg of the diet.

[3]Bioplus 2B (Chr. Hansen USA, Inc., Milwaukee, WI) added at 0.05% of the diet.

[4]Poultry Star (Biomin America, Inc., San Antonio, TX) added at 0.05% of the diet.

Table 3-4. Effects of in-feed chlortetracycline and probiotics on antimicrobial resistance of fecal *E. coli* to antibiotics of critical importance to human medicine[1,2]

CTC[3]	-	-	-	+	+	+
Probiotic 1[4]	-	+	-	-	+	-
Probiotic 2[5]	-	-	+	-	-	+
Amoxicillin/clavulanic acid 2:1 ratio						
d 0	0 (0)[6]	7 (4.50)	3 (3.28)	3 (3.28)	3 (3.28)	3 (3.28)
d 21	3 (3.28)	7 (4.50)	7 (4.50)	7 (4.50)	7 (4.50)	7 (4.50)
d 42	0 (0)	4 (3.28)	10 (5.51)	17 (7.07)	10 (5.51)	10 (5.66)
Ampicillin[7]						
d 0	100 (0)	93 (4.40)	97 (3.29)	100 (0)	97 (3.29)	100 (0)
d 21	70 (8.54)	73 (8.20)	80 (7.32)	83 (6.77)	80 (7.32)	93 (4.40)
d 42	60 (9.22)	60 (9.22)	73 (8.20)	70 (8.54)	73 (8.20)	73 (8.20)
Azithromycin						
d 0	N/A[8]	N/A	N/A	N/A	N/A	N/A
d 21	N/A	N/A	N/A	N/A	N/A	N/A
d 42	N/A	N/A	N/A	N/A	N/A	N/A
Ceftiofur[9]						
d 0	47 (9.11)	37 (8.80)	27 (8.07)	43 (9.05)	27 (8.07)	30 (8.37)
d 21	30 (8.37)	33 (8.61)	27 (8.07)	40 (8.94)	27 (8.07)	50 (9.13)
d 42	17 (6.80)	23 (7.72)	37 (8.80)	43 (9.05)	37 (8.80)	33 (8.61)
Ceftriaxone[10]						
d 0	67 (8.61)	63 (8.80)	37 (8.80)	60 (8.94)	37 (8.80)	50 (9.13)
d 21	33 (8.61)	43 (9.10)	37 (8.80)	43 (9.05)	37 (8.80)	60 (8.94)
d 42	50 (9.13)	43 (9.10)	63 (8.80)	63 (8.80)	63 (8.80)	60 (8.94)
Ciprofloxacin						
d 0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
d 21	0 (0)	0 (0)	0 (0)	7 (4.59)	0 (0)	10 (5.51)
d 42	3 (3.20)	10 (5.66)	7 (4.59)	3 (3.27)	7 (4.59)	7 (4.59)
Gentamicin						
d 0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
d 21	0 (0)	0 (0)	0 (0)	3 (3.27)	0 (0)	0 (0)
d 42	7 (4.540	3 (3.27)	0 (0)	3 (3.27)	0 (0)	0 (0)
Nalidixic Acid						
d 0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
d 21	10 (5.42)	13 (6.13)	0 (0)	7 (4.52)	0 (0)	7 (4.52)
d 42	7 (4.52)	3 (3.27)	3 (3.27)	3 (3.27)	3 (3.27)	0 (0)
Streptomycin[11]						
d 0	60 (8.94)	53 (9.11)	37 (8.80)	37 (8.80)	37 (8.80)	47 (9.11)
d 21	57 (9.05)	77 (7.72)	70 (8.37)	53 (9.11)	70 (8.37)	60 (9.22)
d 42	57 (9.05)	85 (6.80)	63 (8.80)	63 (8.80)	63 (8.80)	57 (9.05)

[1]Values represent the estimated probability of antimicrobial resistance of 30 *E. coli* isolates per sampling day (d 0, d 21, or d 42); 3 random fecal samples were collected per pen per day, *E. coli* isolated, and 1 *E. coli* isolate per fecal sample was assessed. There was a total of 300 pigs (DNA 200 × 400; initially 5.9 kg BW) housed with 5 pigs per pen and 10 pens per treatment.
[2]Critically important antibiotics according to World Health Organization categorization of human medicine antimicrobials.
[3]CTC-50 (Zoetis Services, LLC., Florham Park, NJ) added at 400 mg/kg of the diet.
[4]BioPlus 2B (Chr. Hansen USA, Inc., Milwaukee, WI) added at 0.05% of the diet.

[5]Poultry Star (Biomin America, Inc., San Antonio, TX.) added at 0.05% of the diet.
[6]Indicates SEM.
[7]Day ($P < 0.003$).
[8]N/A represents statistics were not performed because all fecal isolates were categorized as susceptible.
[9]CTC ($P < 0.011$).
[10]Day ($P < 0.020$).
[11]Day × CTC × Probiotic ($P < 0.015$).

Table 3-5. Effects of in-feed chlortetracycline and probiotics on antimicrobial resistance of fecal *E. coli* to antibiotics of high importance to human medicine[1,2]

CTC[3]	-	-	-	+	+	+
Probiotic 1[4]	-	+	-	-	+	-
Probiotic 2[5]	-	-	+	-	-	+
Cefoxitin						
d 0	0 (0)[6]	7 (4.27)	0 (0)	3 (3.25)	3 (3.25)	0 (0)
d 21	7 (4.72)	10 (5.36)	10 (5.36)	7 (4.72)	20 (7.03)	20 (7.03)
d 42	0 (0)	7 (4.72)	7 (4.72)	17 (7.15)	20 (7.03)	3 (3.25)
Chloramphenicol						
d 0	20 (7.33)	3 (3.28)	3 (3.28)	0 (0)	13 (6.32)	13 (6.22)
d 21	13 (6.22)	7 (4.50)	7 (4.56)	7 (4.56)	7 (4.50)	13 (6.22)
d 42	7 (4.56)	10 (5.49)	13 (6.22)	13 (6.22)	10 (5.49)	7 (4.56)
Sulfisoxazole						
d 0	N/A[7]	N/A	N/A	N/A	N/A	N/A
d 21	N/A	N/A	N/A	N/A	N/A	N/A
d 42	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline[8]						
d 0	100 (0)	93 (4.40)	97 (3.29)	100 (0)	93 (4.40)	100 (0)
d 21	70 (8.54)	73 (8.20)	80 (7.32)	83 (6.77)	73 (8.20)	93 (4.40)
d 42	60 (9.22)	60 (9.22)	73 (8.20)	70 (8.54)	60 (9.22)	73 (8.20)
Trimethoprim/sulfamethoxazole						
d 0	17 (6.80)	3 (3.27)	13 (6.21)	23 (7.72)	3 (3.27)	10 (5.48)
d 21	10 (5.48)	30 (8.37)	17 (6.80)	10 (5.48)	30 (8.37)	17 (6.80)
d 42	23 (7.72)	13 (6.21)	20 (9.07)	20 (9.07)	13 (6.21)	17 (6.80)

[1] Values represent the estimated probability of antimicrobial resistance of 30 *E. coli* isolates per sampling day (d 0, d 21, or d 42); 3 random fecal samples were collected per pen per day, *E. coli* isolated, and 1 *E. coli* isolate per fecal sample was assessed. There was a total of 300 pigs (DNA 200 × 400; initially 5.9 kg BW) housed with 5 pigs per pen and 10 pens per treatment.

[2]Highly important antibiotics according to World Health Organization categorization of human medicine antimicrobials.

[3]CTC-50 (Zoetis Services, LLC., Florham Park, NJ) added at 400 mg/kg of the diet.

[4]BioPlus 2B (Chr. Hansen USA, Inc., Milwaukee, WI) added at 0.05% of the diet.

[5]Poultry Star (Biomin America, Inc., San Antonio, TX) added at 0.05% of the diet.

[6]Indicates SEM.

[7]N/A represents statistics were not performed because all fecal isolates were categorized as susceptible.

[8]CTC ($P < 0.050$), Day ($P < 0.001$).