Effects of chlortetracycline alone or in combination with direct fed microbials on nursery pig growth performance and antimicrobial resistance of fecal *Escherichia coli*[1]

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ABSTRACT: A total of 300 nursery pigs (initially 5.9 ± 0.05 kg BW) were used in a 42-d growth trial to evaluate the effects of feeding a therapeutic level of chlortetracycline (**CTC**) with or without direct fed microbials (**DFM**) on growth performance and antimicrobial resistance (**AMR**) of fecal *Escherichia coli*. CTC is a broad-spectrum in-feed antibiotic commonly used in the swine industry. 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 days 0 to 42) and DFM (0 vs. 0.05% DFM 1 vs. 0.05% DFM 2). Fecal samples were collected from three randomly selected pigs from each pen on days 0, 21, and 42 for *E. coli* isolation and AMR determination. 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 DFM 1 or DFM 2. Regardless of CTC, inclusion of DFM 2 in diets improved ($P < 0.05$) ADFI from days 0 to 14 and on day 14 BW compared to diets that did not include DFM 2. The addition of CTC with or without DFMs 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 AMR to ampicillin and tetracycline (**TET**) throughout the trial was observed, while resistance to ceftriaxone decreased ($P < 0.020$) from days 0 to 21 and increased from days 21 to 42 amongst dietary treatments regardless of CTC or DFM inclusion in the diet. A CTC × DFM × day interaction ($P < 0.015$) was observed for streptomycin, whereby from days 21 to 42 AMR increased in diets containing either CTC or DFM 1 alone, but the combination decreased resistance. There was no evidence for any effect of DFMs on AMR of fecal *E. coli* isolates to any other antibiotics evaluated. In conclusion, therapeutic levels of added CTC with or without DFM inclusion improved nursery pig performance, but increased AMR of fecal *E. coli* isolates to TET and ceftiofur. A moderate improvement in intake and day 14 BW was observed when DFM 2 was included in the diet with or without CTC, but, except for streptomycin, there was no evidence that added dietary DFMs affected resistance of fecal *E. coli* to antibiotics.

Key words: antimicrobial resistance, chlortetracycline, direct fed microbial, *E. coli*, growth performance, nursery pigs

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INTRODUCTION

Emergence of in-feed antibiotics in the 1950s improved efficiency of growth and overall health of nursery pigs. A review by Cromwell (2002) summarized that including antibiotics in nursery diets 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 antimicrobial resistance (**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 et al., 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 direct fed microbials (**DFM**), are desired to reduce the use of in-feed antibiotics in nursery diets. In addition to growth performance benefits (Kritas and Morrison, 2005), DFMs may have a favorable impact on the development and persistence of AMR in gut bacteria (Amachawadi et al., 2018). DFM 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 DFMs with antibiotics in humans enhances the resilience of gut bacterial flora to antibiotics-induced alterations (Plummer et al., 2005; McFarland et al., 2006). Therefore, the objective of this study was to determine the effects of therapeutic levels of CTC with or without DFMs on nursery pig performance and on AMR 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 five 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 d, after which pens were blocked by initial BW and allotted to one of six dietary treatments in a completely randomized block design.

The six dietary treatments were arranged in a 2 × 3 factorial consisting of combinations of CTC (0 vs. 400 mg/kg from days 0 to 42; Zoetis Services, LLC., Florham Park, NJ), DFM 1 (0 vs. 0.05% Bioplus 2B; Chr. Hansen USA, Inc., Milwaukee, WI) or DFM 2 (0 vs. 0.05% Poultry Star; Biomin America, Inc., San Antonio, TX) added at manufacturer's recommendations. Experimental diets were fed throughout two study phases (phase 1: days 0 to 14 and phase 2: days 14 to 42) in meal form. On days 14 and 28, CTC was removed from the diet to comply with FDA regulations; when appropriate to the experimental diets, CTC was resumed on days 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

Table 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]	—	—
Direct fed microbial 1[7]	—	—
Direct fed microbial 2[8]	—	—
Total	100	100
Calculated analysis		
Standardized ileal digestible (SID) amino acids, %		
Lys	1.35	1.35
Met:Lys	36	36
Met andCys: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 days 0 to 14 (~5.9 to 8.5 kg BW) and phase 2 diets from days 14 to 42 (8.5 to 25.0 kg BW). A common starter diet was fed to all pigs for 4 d 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 per kilogram 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 (CTC) 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 2. Diet analysis, % (as-fed basis)[1,2]

CTC	−	−	−	+	+	+
Direct fed microbial 1	−	+	−	−	+	−
Direct fed microbial 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 days 0 to 14 (~5.9 to 8.5 kg BW) and phase 2 diets from days 14 to 42 (8.5 to 25.0 kg BW). A common starter diet was fed to all pigs for 4 d 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.

from the 5th, 10th, 15th, 20th, 25th, 30th, 35th, and 40th 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 days 0, 21, and 42, fecal samples were collected by gentle rectal massage from three 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 of phosphate-buffered saline. Fifty microliters of the fecal suspension were 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 days 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 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. The antimicrobials evaluated included: amoxicillin/clavulanic acid 2:1 ratio, ampicillin (**AMP**), azithromycin, ceftiofur, ceftriaxone, ciprofloxacin (**CIP**), gentamicin (**GEN**), nalidixic acid (**NAL**), streptomycin, cefoxitin, chloramphenicol (**CHL**), sulfisoxazole, TET, and trimethoprim/sulfamethoxazole.

Statistical Analysis

Growth data. Growth data for ADG, ADFI, G:F on intervals days 0 to 14, 14 to 28, 28 to 42, 0 to 42, as well as BW at days 0, 14, 28 and 42, all measured at the pen level were analyzed using general linear-mixed models with pen as the experimental unit. Growth performance was evaluated using five preplanned contrasts. The two interactions of CTC × DFM 1 or DFM 2 were evaluated as the response of CTC in the diets without DFM 1 or DFM 2 compared to the CTC response in the diet with either DFM 1 or DFM 2. The main effect of CTC was evaluated as a comparison of diets with CTC and those without. The DFM 1 or DFM 2 effect was evaluated using two contrasts evaluating the comparison of the two diets with DFM 1 or the two diets with DFM 2 compared to the two diets without DFM. The model also included the random effect block. Residual assumptions were checked using studentized residuals and were found to be reasonably met.

Antimicrobial susceptibility. Initially, frequency tables of resistant vs. susceptible isolates for each antibiotic were evaluated. Frequency tables were further broken down by CTC, DFM treatment, sampling day and their combinations, in order to anticipate potential extreme categorical problems during model fitting. Subcategories with all resistant or nonresistant isolates or frequencies close to these extremes can lead to model fitting problems due to lack of variability. For Azithromycin and Sulfisoxazole, all fecal isolates (100%) were categorized as susceptible and thus no further statistical analyses were conducted.

For each of the remaining antibiotics, antimicrobial susceptibility was analyzed separately using a generalized linear mixed model assuming a Bernoulli distribution and a logit link function. The linear predictor included the fixed effects of CTC antibiotic (present or absent), inclusion of DFMs (none, 1, or 2) and sampling days (days 0, 21, and 42), as well as their interactions. In addition, the random effects block and block by CTC and DFM treatments used to identify the pen as the level of replication for treatment due to the repeated measure over time.

Extreme category problems in the data prevented fitting the interaction between CTC, DFM, and sampling day for the statistical models fitted for cefoxitin, amoxicillin/clavulanic acid 2:1 ratio (AUG2), AMP, TET, NAL, GEN, CIP, and CHL. In addition, extreme category problems also prevented fitting any interaction with sampling day for NAL, GEN, CIP, and CHL. Over dispersion was assessed using the maximum-likelihood-based fit statistic Pearson chi-square over degrees of freedom. In all cases, the final models used for inference showed no evidence for over dispersion.

Overall. Pairwise comparisons were conducted using Bonferroni adjustment. Statistical models were fitted using the PROC GLIMMIX procedure of SAS (Version 9.4; SAS Institute, Inc., Cary, NC). The model used for inference was fit using residual pseudo-likelihood implemented using Newton–Raphson optimization with ridging. Results were considered significant with $P \leq 0.05$.

RESULTS

Chemical Analysis

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

Growth Performance

No evidence for CTC × DFM interactions were observed either from days 0 to 14 or from days 14 to 28. From days 0 to 14, pigs fed diets with CTC increased ($P < 0.05$) ADG, ADFI, G:F, and day 14 BW compared to those fed diets without CTC (Table 3), regardless of whether the diet included a DFM or not. In addition, pigs fed diets with DFM 2 had improved ($P < 0.05$) ADFI and d 14 BW compared to those fed diets without any DFM.

From days 14 to 28, the inclusion of CTC in diets increased ($P < 0.05$) ADG, ADFI, and day 28 BW compared to those pigs fed diets without CTC, regardless of DFM. Also, amongst those pigs fed no CTC, the inclusion of DFM 2 showed marginally greater ($P = 0.052$) ADFI than those fed no DFM.

From days 28 to 42, a CTC × DFM 2 interaction ($P = 0.05$) was observed for ADFI whereby addition of dietary CTC in diets without the inclusion of DFM 1 or DFM 2 increased ($P < 0.05$) ADFI compared to diets that included DFM 1 or DFM 2 without CTC, or to diets that included DFM 1 without CTC; all remaining dietary treatments

Table 3. Effects of in-feed CTC and direct fed microbials (DFM) on growth performance (least square means and SEM) of nursery pigs[1]

CTC[2]	−	−	−	+	+	+						
DFM 1[3]	−	+	−	−	+	−		Probability, $P <$				
DFM 2[4]	−	−	+	−	−	+	SEM	CTC	DFM 1	DFM 2	CTC × DFM 1	CTC × DFM 2
Days 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
Days 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
Days 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	0.582	0.667	0.083	0.573
Days 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												
Day 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
Day 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
Day 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
Day 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] 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 five pigs per pen and 10 pens per treatment. On days 14 and 28, antibiotics were removed from the diet according to FDA regulations. Experimental diets containing antibiotics resumed feeding on days 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.

showed intermediate ADFI that was not significantly different from each other.

For the overall study (days 0 to 42), no evidence for any CTC × DFM interactions were observed for any of the responses on growth performance. 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 DFM had been added to the diet. No evidence for any effects of the addition of either DFM to the diet was observed for growth performance.

Antimicrobial Susceptibility Testing

Table 4 shows the estimated probability of AMR of fecal *E. coli* isolates in response to in-feed CTC and DFMs, to antibiotics of critical importance to human medicine, namely amoxicillin/clavulanic acid, AMP, azithromycin, ceftiofur, ceftriaxone, CIP, GEN, NAL, and streptomycin. Specifically, for amoxicillin/clavulanic acid, there was no evidence for any effect of CTC or DFM on AMR over the study period ($P \geq 0.22$ for all two-way interactions and $P \geq 0.13$ for all main effects). For AMP, only the main effect of day significantly contributed to explain AMR (P = 0.002), whereby most isolates were found to be resistant on day 0 but the probability of resistance decreased during the study, regardless of whether the diets contained CTC and/or DFMs. For azithromycin, no fecal isolates showed any resistance for the duration of the study. For ceftiofur, only the main effect of CTC significantly contributed to explain AMR ($P < 0.011$), whereby inclusion of CTC in fed diets yielded greater probability of resistance of fecal *E. coli* throughout the study period and regardless of DFM in diet. There was no evidence for any additional source of differences in AMR to ceftiofur. For ceftriaxone, only the main effect of day was significant ($P = 0.02$), whereby the overall probability of resistance decreased from days 0 to 21, and then increased from days 21 to 42 regardless of whether the diets fed contained CTC and/or DFMs. There was no evidence for any effect of CTC or DFM on AMR over the study period for CIP, GEN and NAL ($P \geq 0.29$ for all two-way interactions and $P \geq 0.14$ for all main effects). For streptomycin, there was evidence for day × CTC × DFM interaction (P = 0.0151). The overall probability of resistance to streptomycin increased over time when diets fed contained DFM 1 and no addition of CTC ($P < 0.05$). There was no evidence for differences in AMR to streptomycin in any other treatment combination.

Table 5 shows the estimated probability of AMR of fecal *E. coli* isolates in response to in-feed CTC and DFMs, to antibiotics of high importance to human medicine, namely cefoxitin, chloramphenical, sulfsioxazole, TET, and trimethoprim/sulfamethoxazole. Specifically, for both cefotoxin and CHL, there was no evidence for any effect of CTC or DFM on AMR over the study period ($P \geq 0.56$ for all two-way interactions and $P \geq 0.17$ for all main effects). For sulfisoxazole, none of the fecal isolates showed resistance, regardless of dietary CTC or DFM. For TET, only the main effects of CTC and day significantly contributed to explain AMR ($P < 0.05$ and $P < 0.001$, respectively). Overall, addition of CTC to diets resulted in greater probability of fecal *E. coli* isolates being resistant to TET, regardless of whether the diets included DFMs during the study. Moreover, regardless of dietary treatment, the probability of resistance to TET increased during the study ($P < 0.05$). For trimethoprim/sulfamethoxazole, there was no evidence for any effect of CTC or DFM on AMR over the trial period (P = 0.46 for three-way interaction, $P \geq 0.2$ for all two-way interactions and $P \geq 0.22$ for all main effects).

DISCUSSION

Early research has observed that the inclusion of sub-therapeutic levels 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 sub-therapeutic levels of CTC were included in the diet. Additionally, the inclusion of in-feed antibiotics in multi-site modern commercial pig production systems has been shown to be efficacious at improving rate of gain in weaned pigs but is less effective at improving efficiency of growth (Dritz et al., 2002). The study herein observed that the inclusion of therapeutic levels of CTC improved rate of gain by 5.0% with no evidence of difference for G:F. More recent research conducted by Feldpausch et al. (2016) indicated that the inclusion of CTC up to 441 ppm tended to increase feed intake, which resulted in a tendency for a linear increase in BW gain. They also observed no evidence of differences in G:F with the inclusion of CTC. The results from our study agree with the results of Dritz et al. (2002) that the inclusion of CTC in nursery pig diets improved gain and feed intake, which resulted in increased BW gain but no evidence of an improved efficiency of gain.

DFM 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

Table 4. Effects of in-feed cCTC and DFMs on the probability of AMR of fecal *E. coli* to antibiotics of critical importance to human medicine[1,2]

CTC[3]	−	−	−	+	+	+
DFM 1[4]	−	+	−	−	+	−
DFM 2[5]	−	−	+	−	−	+
Amoxicillin/clavulanic acid 2:1 ratio						
Day 0	0 [.][6]	7 [2, 24]	3 [0.46, 20]	3 [0.46, 20]	3 [0.46, 20]	3 [0.46, 20]
Day 21	3 [0.46, 20]	7 [2, 23]	7 [2, 23]	7 [2, 23]	7 [2, 23]	7 [2, 23]
Day 42	0 [.]	3 [0.5, 19]	10 [3, 27]	17 [7, 35]	17 [7, 35]	10 [3, 27]
Ampicillin[7]						
Day 0	100 [.]	93 [76, 98]	97 [80, 99]	100 [.]	100 [.]	100 [.]
Day 21	70 [52, 84]	73 [55, 86]	80 [62, 91]	83 [66, 93]	87 [70, 95]	93 [78, 98]
Day 42	60 [42, 76]	60 [42, 76]	73 [55, 86]	70 [52, 84]	57 [39, 73]	73 [55, 86]
Azithromycin						
Day 0	N/A[8]	N/A	N/A	N/A	N/A	N/A
Day 21	N/A	N/A	N/A	N/A	N/A	N/A
Day 42	N/A	N/A	N/A	N/A	N/A	N/A
Ceftiofur[9]						
Day 0	47 [30, 64]	37 [22, 55]	27 [14, 45]	43 [27, 61]	47 [30, 64]	30 [16, 48]
Day 21	30 [16, 48]	33 [19, 52]	27 [14, 45]	40 [24, 58]	53 [36, 70]	50 [33, 67]
Day 42	17 [7, 34]	23 [11, 42]	37 [22, 55]	43 [27, 61]	33 [19, 52]	33 [19, 52]
Ceftriaxone[10]						
Day 0	67 [48, 81]	63 [45, 78]	37 [22, 55]	60 [42, 76]	60 [42, 76]	50 [33, 67]
Day 21	33 [19, 52]	43 [27, 61]	37 [22, 55]	43 [27, 61]	37 [22, 55]	60 [42, 76]
Day 42	50 [33, 67]	43 [27, 61]	63 [45, 78]	63 [45, 78]	63 [45, 78]	60 [42, 76]
Ciprofloxacin						
Day 0	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]
Day 21	0 [.]	0 [.]	0 [.]	7 [1, 25]	0 [.]	10 [3, 30]
Day 42	3 [0.4, 21]	10 [3, 30]	7 [1, 25]	3 [0.4, 21]	3 [0.4, 21]	7 [1, 25]
Gentamicin						
Day 0	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]
Day 21	0 [.]	0 [.]	0 [.]	3 [0.4, 23]	0 [.]	0 [.]
Day 42	7 [1, 28]	3 [0.4, 23]	0 [.]	3 [0.4, 23]	3 [0.4, 23]	0 [.]
Nalidixic acid						
Day 0	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]	0 [.]
Day 21	10 [3, 27]	13 [5, 31]	0 [.]	7 [2, 23]	0 [.]	7 [2, 23]
Day 42	7 [2, 23]	3 [0.5, 20]	3 [0.5, 20]	3 [0.5, 20]	3 [0.5, 20]	0 [.]
Streptomycin[11]						
Day 0	60 [42, 76]	53 [36, 70]	37 [22, 55]	37 [22, 55]	70 [52, 84]	47 [30, 64]
Day 21	57 [39, 73]	77 [58, 88]	70 [52, 84]	53 [36, 70]	67 [48, 81]	60 [42, 76]
Day 42	57 [39, 73]	83 [66, 93]	63 [45, 78]	63 [45, 78]	43 [27, 61]	57 [39, 73]

[1]Values represent the estimated probability of AMR (and 95% confidence intervals) of 30 *E. coli* isolates per sampling day (day 0, day 21, or day 42); three 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 five 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]Values in brackets indicate 95% confidence intervals.

[7]Day ($P < 0.003$).

[8]N/A indicates that no estimates are available because none of the fecal isolates were categorized as resistant to the specified antimicrobial.

[9]CTC ($P < 0.011$).

[10]Day ($P < 0.020$).

[11]Day × CTC × DFM ($P < 0.015$).

Table 5. Effects of in-feed CTC and DFMs on the probability of AMR of fecal *E. coli* to antibiotics of high importance to human medicine[1,2]

CTC[3]	−	−	−	+	+	+
DFM 1[4]	−	+	−	−	+	−
DFM 2[5]	−	−	+	−	−	+
Cefoxitin						
Day 0	67 [48, 81][6]	63 [45, 78]	37 [22, 55]	60 [42, 76]	60 [42, 76]	50 [33, 67]
Day 21	33 [19, 52]	43 [27, 61]	37 [22, 55]	43 [27, 61]	47 [30, 64]	60 [42, 76]
Day 42	50 [33, 67]	47 [30, 64]	63 [45, 78]	63 [45, 78]	60 [42, 76]	60 [42, 76]
Chloramphenicol						
Day 0	20 [9, 38]	3 [0.5, 21]	3 [0.5, 21]	0 [.]	13 [5, 30]	13 [5, 30]
Day 21	13 [5, 30]	7 [2, 23]	7 [2, 23]	7 [2, 23]	7 [2, 23]	13 [5, 30]
Day 42	7 [2, 23]	10 [3, 28]	13 [5, 30]	13 [5, 30]	10 [3, 28]	7 [2, 23]
Sulfisoxazole						
Day 0	N/A[7]	N/A	N/A	N/A	N/A	N/A
Day 21	N/A	N/A	N/A	N/A	N/A	N/A
Day 42	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline[8]						
Day 0	93 [78, 98]	77 [59, 88]	80 [62, 61]	87 [68, 95]	90 [73, 97]	93 [77, 98]
Day 21	90 [71, 97]	97 [81, 99]	93 [76, 98]	100 [.]	97 [80, 97]	100 [.]
Day 42	97 [80, 99]	93 [77, 98]	97 [80, 99]	100 [.]	97 [80, 97]	100 [.]
Trimethoprim/sulfamethoxazole						
Day 0	17 [7, 34]	3 [0.5, 20]	13 [5, 31]	23 [12, 42]	10 [3, 27]	10 [3, 27]
Day 21	10 [3, 27]	30 [16, 48]	17 [7, 34]	10 [3, 27]	10 [3, 27]	17 [7, 34]
Day 42	23 [12, 42]	13 [5, 31]	20 [9, 38]	20 [9, 38]	17 [7, 34]	17 [7, 34]

[1] Values represent the estimated probability of AMR (and 95% confidence intervals) 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]Values insides brackets indicate 95% confidence intervals.

[7]N/A indicates that no estimates are available because none of the fecal isolates were categorized as resistant to the specified antimicrobial.

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

(Adams and Marteau, 1995; Oelschlaeger, 2010). These proposed mechanisms include beneficially altering gut microbiome, regulating the immune system (Suda et al., 2014) and providing antipathogenic activity (Bomba et al., 2002) to reduce infections from enteric pathogens. DFM are suggested to promote gut health by colonizing the epithelial membrane of the gastrointestinal tract, producing fermentation products and bacteriocins, and enzymes that aid in nutrient uptake and absorption (Gaggia et al., 2010; Giannenas et al., 2012). To be effective and express these mechanisms, a DFM must survive in feed and be able to pass through the gastrointestinal tract of the pig (Jacela, 2010). Although the proposed health benefits of DFMs support their addition to nursery pig diets, the results have been inconsistent.

Direct fed microbial 1 is a dual-strain DFM-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 DFM 1 in diets on nursery pig performance. The antibiotic regimen used in their study included 400 mg/kg of neomycin for the first 7 d post-weaning, 100 mg/kg of neomycin and 100 mg/kg oxy-TET 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 DFM 1 compared to that of pigs fed an antimicrobial regimen. However, Keegan et al. (2005) conducted multiple experiments on the effects of DFM products and in-feed antibiotics on nursery pig performance. They observed in both a university and commercial setting the addition of DFM 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

DFM 1 diets. These results are consistent with the findings from this trial that the addition of DFM 1 alone did not have a significant effect on growth performance in nursery pigs. A multitude of reasons exist that may contribute to why DFMs are inconsistent in improving performance when added to nursery diets. These include the strain of bacteria not surviving the feed manufacturing process, although diets in this study were fed in meal form and not exposed to thermal processing. The dosage of DFM strain not to be high enough; however, we added the DFMs at manufacturers recommended inclusion rates. A third speculation is that the therapeutic level of CTC possibly had a negative effect on the DFM survival. The two species contained in DFM-1 are susceptible to CTC based in antimicrobial susceptibility testing (data not shown). The in vitro susceptibility does not necessarily translate into inhibition in in vivo because bacterial cells do get physically protected in the feed and in the in the gut contents. An improved response to DFMs in combination with an antibiotic has been shown (LeMieux et al., 2003). A marginal interaction between DFM 1 and CTC was observed during this trial with improved efficiency observed when CTC and DFM 1 were included in combination compared to alone. This proposes that the mode of action for both DFMs and antibiotics may exert a synergistic relationship towards certain pathogens present in the gut. DFMs, similar to antibiotics, also exert antibacterial activities because of the production of compounds, such as bacteriocins, hydrogen peroxide, that inhibit pathogens within the small intestine (Chopra et al., 1992; Cho et al., 2011). This could allow for a stable gut microflora contributing to improved growth in swine, but this needs to be researched.

DFM 2 is a multi-strain DFM-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; Mountzouris et al., 2007). Of the four bacterial species, only *Pediococcus acidilactici* was resistant to CTC and the other three species were susceptible (data not shown). As stated before, DFM bacterial cells are not likely to be negatively affected by the CTC in the feed or in the gut contents. To our knowledge, ours is the first published trial that evaluated DFM 2 in a swine diet. In this study, the addition of DFM 2 resulted in increased ADFI and BW through the first 14 d of the study. This finding suggests DFM 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.

AMR 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, 2010). Thus, it is important to understand what dietary factors, if any, may contribute to increased AMR 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 NARMS (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 Hillen, 1996). CTC is one of the most commonly used in-feed antibiotics in 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 AMR (Dawson et al., 1984). *Tet* and *otr* genes confer resistance to TETs (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 therapeutic levels of CTC to diets increased the proportion of *E. coli* isolates resistant to TET, although the proportion of fecal *E. coli* isolates in diets supplemented with CTC decreased as the trial progressed. These findings suggest that TET 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 AMP, but no evidence of any 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 AMP, but none of the swine isolates exhibited resistance to amoxicillin/clavulanic acid. Boerlin et al. (2005) observed *E. coli* isolates had increased resistance to AMP and all isolates were resistant to amoxicillin. Cavaco et al. (2008) found that pigs inoculated with a NAL 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 AMP/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 days 0 to 42 of the current study suggests that the use of AMP/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 therapeutic levels 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 TET resistance with the supplementation of therapeutic levels 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 TET resistance (Agga et al., 2014). *Escherichia coli* resistance to ceftriaxone decreased from days 0 to 21, but resistance increased back to baseline levels on day 42. Funk et al. (2006) observed that the supplementation of sub-therapeutic levels 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 therapeutic levels of CTC or DFM did not have an effect on *E. coli* resistance to cefoxitin during this experiment. Agga et al. (2014) reported that supplementation of therapeutic levels 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 therapeutic levels of CTC and the length at which CTC is administered in the feed do play a part in affecting *E. coli* resistance to cefoxitin in fecal isolates of nursery pigs.

Streptomycin and GEN 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 (Courvalin, 1994; Yamane et al., 2005). In the current study, an antibiotic × DFM × day interaction was observed for *E. coli* resistance to streptomycin. This interaction occurred because the variation in resistance on day 0 resulted in resistance increasing when diets fed contained therapeutic levels of CTC or DFM 2 alone from days 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 GEN. 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 therapeutic levels of CTC, DFM 1, DFM 2, or a combination of CTC and the individual DFM products on the proportions of fecal *E. coli* to azithromycin, CIP, NAL, sulfisoxazole, CHL, 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 therapeutic levels of CTC to nursery diets on *E. coli* resistance to azithromycin, CIP, NAL, or sulfisoxazole. The researchers also found that feeding therapeutic levels of CTC to nursery pigs decreased resistance of *E. coli* to CHL 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 therapeutic levels 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 therapeutic levels of CTC in nursery diets improves growth performance of nursery pigs. The addition of DFM 2 to nursery diets resulted in improvements in ADFI and day 14 BW, thus indicating that DFM 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 DFM 2 are observed during a health challenge, similar to results observed in poultry trials. No evidence for differences in performance was observed with the addition of DFM 1 to nursery diets and this coincides with previous research that shows inconclusive results on the effect of addition of DFM 1 to nursery pig performance (Keegan et al., 2005). In general, the addition of therapeutic levels of CTC to nursery pig diets increased the proportion of fecal *E. coli* isolates resistant to TET and ceftiofur. Although, the resistance towards TET and other antibiotics tested against decreased or indicated no evidence of difference over time. In this trial, no evidence of difference on AMR of *E. coli* was observed with the inclusion of DFMs in diets.

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