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Impact of added copper, alone or in combination with chlortetracycline, on growth performance and antimicrobial resistance of fecal enterococci of weaned piglets

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Abstract

Studies suggest a link between added copper (**Cu**) and co-selection of antimicrobial resistance (**AMR**) in *Enterococcus* spp., but data are inconsistent. This study aimed to assess the impact of added Cu, alone or with a feed-grade antimicrobial, on growth performance, transferable Cu resistance gene (***tcrB***) prevalence, abundance of *tcrB* in fecal community DNA, and AMR in fecal enterococci in weaned piglets. A total of 320 barrows (DNA 200 × 400, DNA Genetics) weaned at approximately 21 d of age with 7.4 kg (7.4 ± 0.06 kg) BW were used in a 28-d study. Piglets were fed a common non-medicated diet for 7 d of acclimation. Treatments were arranged in a 2 × 2 factorial design with main effects of added Cu (0 vs. 200 mg/kg Cu from Cu sulfate) and chlortetracycline (0 vs. 440 mg/kg **CTC**). Growth performance and fecal samples were obtained on days 0, 14, and 28. There was no evidence ($P > 0.05$) for Cu and CTC interaction in growth performance. Pigs fed diets with added Cu had increased ($P < 0.05$) ADG and ADFI from days 0 to 14, with no evidence for differences ($P > 0.05$) from days 15 to 28 and 0 to 28. Pigs fed diets with CTC had improved ($P < 0.01$) ADG, ADFI, and G:F from days 0 to 28. Prevalence of *tcrB*-positive enterococci was not affected by the addition of Cu and/or CTC ($P > 0.05$). Prevalence of *tcrB*-positive enterococci was higher on day 14 than other sampling days ($P = 0.002$). Prevalence of tetracycline resistance gene [***tet*(M)**]-positive enterococci was not affected by treatments or day ($P > 0.05$). Prevalence of macrolide resistance gene [***erm*(B)**]-positive enterococci had a significant treatment and sampling day interaction ($P = 0.021$). The abundance of the *tcrB* gene in feces, quantified by PCR, was not affected by Cu treatment. The median Cu minimum inhibitory concentrations (**MIC**) of *tcrB*-negative and -positive isolates were 3 and 20 mM, respectively ($P < 0.001$). For day 0 and day 28, all *Enterococcus* isolates were susceptible to gentamicin, kanamycin, streptomycin, daptomycin, and tigecycline, with a majority of isolates resistant to chloramphenicol, erythromycin, lincomycin, linezolid, tetracycline, tylosin tartrate, and Synercid. In conclusion,

200 mg/kg added Cu or 440 mg/kg CTC in nursery diets improved growth performance of nursery pigs. Added Cu, with or without a selection pressure of CTC, did not increase Cu-resistant enterococci and did not co-select resistance to antibiotics.

Key words: antimicrobial resistance, copper addition, *Enterococcus*, swine

Abbreviations

AMR	antimicrobial
BLAST	Biological Local Alignment Search Tool
CLSI	Clinical and Laboratory Standard Institute
CTC	chlortetracycline
Cu	copper
erm(B)	macrolide gene
ME	M-Enterococcus
MIC	minimum inhibitory concentrations
NARMS	National Antimicrobial Monitoring System
NCBI	National Center for Biotechnology Information
qPCR	quantitative PCR
sodA	superoxide dismutase
tcrB	transferable Cu gene
tet(M)	tetracycline gene
vanA	vancomycin gene

Introduction

Copper (**Cu**) is an essential microelement required for basic cellular metabolism in eukaryotic and prokaryotic cells. In swine, Cu is included in trace amounts in the diets (5 to 20 mg/kg), as part of a multi-mineral supplement to meet nutritional requirements (NRC, 2012). At elevated concentrations in the diet, generally at 100 to 250 mg/kg, Cu has growth promotional benefits which are attributed to alteration of gut microflora, retention of nutrients, reduction in gut fermentation losses, and reduced morbidity and mortality, particularly in piglets (Højberg et al., 2005). High concentrations of Cu have antimicrobial activities because of the production of intracellular superoxides, which inhibit basic cellular processes that utilize lipids, nucleic acids, and proteins (Macomber and Imlay, 2009). Acquired Cu resistance attributed to a plasmid-borne transferrable Cu-resistance gene (***tcrB***), homologous to copper efflux pump (*copB*), has been reported in fecal enterococci of swine and cattle (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2010, 2011). The *tcrB*-carrying plasmid has also been shown to carry macrolide [***erm*(B)**] and glycopeptide (***vanA***) resistance genes in fecal enterococci of swine and cattle in Europe (Hasman and Aarestrup, 2002; Hasman et al., 2006), and *erm*(B) and tetracycline [***tet*(M)**] resistance gene in fecal enterococci of swine in the United States (Amachawadi et al., 2010, 2011, 2015). This genetic link between Cu and antimicrobial resistance (**AMR**) genes suggests the potential association of added Cu and AMR. The concern is heightened because of the propensity of *Enterococcus* spp. to become multidrug resistant and its ability to horizontally transfer resistance genes to other bacteria in the gut, including *Salmonella* spp. and *Campylobacter* spp. (Winokur et al., 2001; Carattoli, 2009).

In previous studies, data on the effects of Cu addition on the acquisition of resistance to Cu and co-selection of Cu and AMR have been inconsistent. Although a dose-related *tcrB* prevalence in enterococci in weaned piglets to Cu addition has been reported (Amachawadi et al., 2011), in other studies (Amachawadi et al., 2010, 2015), elevated Cu did not increase the prevalence of *tcrB*-positive fecal enterococci. Because of this inconsistency, there is a need to reevaluate the impact of Cu on the selection of Cu resistance and co-selection of AMR in gut bacteria of swine. Therefore, the objective of this study was to evaluate the impact of added Cu, alone or in combination with an antibiotic selection pressure in the form of a feed-grade antimicrobial (chlortetracycline; **CTC**), on growth performance, Cu resistance gene (*tcrB*) prevalence, and AMR of fecal enterococci in weaned piglets. Also, to assess the impact of added Cu and CTC on *tcrB*, a real-time, quantitative PCR (**qPCR**) assay was developed, validated, and utilized to quantify the abundance of *tcrB* in fecal community DNA.

Materials and methods

Animals, experimental design, and treatments

The Kansas State University Animal Care and Use Committee approved the use of animals and the experimental procedures for this research (KSU IACUC# 4036). The study was conducted in the University Segregated Early Weaning Swine Facility. A total of 320 weaned barrow piglets (DNA 200 × 400; DNA Genetics, Columbus, NE) were used. Piglets at 21 d of age with an average initial BW of 7.4 kg (7.4 ± 0.06 kg) were allocated into 64 pens with five piglets per pen (0.30 m^2 pen floor space per piglet) distributed into two barns. Specifically, each barn housed 32 pens oriented in four rows with 8 pens in each row. Row one adjoined row two and row three adjoined row four creating two 16-pen blocks per barn, which were separated by a mid-way. Per 16-pen block, there were 8 pen-pairs that allowed contact between rows and restricted contact within-row due to solid polyvinyl boards. The availability of contact between rows resulted in the experimental unit being expressed by a pair of pens. Therefore, each barn housed 16-pen pairs, equaling a total of 16 experimental units per barn and totaling 32 experimental units in the study.

Treatments were arranged in a 2 × 2 factorial design with main effects of added Cu (0 vs. 200 mg/kg from copper sulfate) and CTC (0 vs. 440 mg/kg). Pen-pairs were randomly assigned to one of four treatments with a total of eight replications per treatment. Treatment allocation followed a block design to ensure all treatments had equal adjacent contact with other treatments. The treatment groups consisted of: a control group fed a basal diet with Cu level (17 mg/kg of feed) to meet the National Research Council (NRC, 2012) requirements of nursery pigs and no antibiotic supplementation, a Cu-supplemented group fed the basal diet with 200 mg/kg of feed added Cu from copper sulfate, an antibiotic-supplemented group fed the basal diet with CTC at 440 mg/kg of feed (22 mg/kg of BW), and a combination of high Cu and CTC.

Diets were corn–-soybean meal-based formulated to meet or exceed the nutrient requirements of nursery pigs according to the NRC (2012) and fed in meal form. Composition of dietary treatments is presented in Table 1. Ad libitum access to feed and water was provided in each pen with a four-hole, dry self-feeder and a nipple waterer. The study period was 35 d with days –7 to –1 as acclimation period and days 0 to 28 as treatment period. During acclimation, pigs were fed a common non-medicated pelleted diet. Treatments containing CTC were administered with the first fed from days 0 to 14 and the second fed from days 15 to 28. Representative diet samples were obtained from all feeders and pooled by treatment in a composite sample. Samples were analyzed (Cumberland Valley Analytical Services, Inc., Waynesboro, PA) for dry matter (DM; method 935.29), crude protein (CP; method 990.03), Ca (method 985.01), P (method 985.01), and Cu (method 925.56) AOAC International (1990). Additionally, samples were submitted for CTC analysis (Midwest Laboratories, Inc., Omaha, NE; method 4438; FDA Laboratory Information Bulletin).

Growth performance and fecal sampling

Piglets and feeders were weighed on days 0, 14, and 28 to determine average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F). Fecal samples were collected by gentle rectal massage randomly from three of the five piglets in each pen on days 0, 14, and 28. Fecal samples from each pen were pooled and transported in a cooler with ice to Kansas State University's Pre-harvest Food Safety Laboratory for the analysis. Laboratory and farm personnel were blinded to treatment groups.

Quantification, isolation, and speciation of enterococci

Difco (Becton and Dickson, Sparks, MD) was the source of all culture media used in this study unless otherwise mentioned. One gram of pooled fecal sample was suspended in 9 mL of phosphate-buffered saline (1:10) and thoroughly vortexed, then 100 µl of the fecal suspension was plated using a spiral plater (Eddy Jet version 1.23, IUL S. A., Barcelona, Spain) onto M-*Enterococcus* (**ME**) agar, ME agar with 8 mM of Cu as Cu sulfate (Fisher Scientific, Pittsburgh, PA; MECu), and ME agar with 16 µg/mL of CTC (Sigma-Aldrich, St. Louis, MO; MECTC) to determine the concentrations of total enterococci and enterococci resistant to Cu or CTC, respectively. Plates were incubated for 24 to 48 h at a minimum of 37 °C and colonies were counted using a counting grid, which relates colonies on the spiral plate to the volume deposited in the area, as per the manufacturer's instructions and guidelines outlined in the FDA Bacteriological Analytical Manual (Maturin and Peeler, 2001). Two putative enterococcal colonies (pin-point, red, pink, or metallic pink) were picked from the spiral plated ME agar plates, replated onto blood agar, and then incubated for 24 h at 37 °C. Isolates were then subjected to preliminary genus confirmation by performing an esculin hydrolysis test. One colony from the blood agar plate was suspended in 100 µl of Enterococcosel broth in a 96-well microtiter plate (Falcon 96-well U-bottom plate, Corning, Inc., Corning, NY) and incubated for 4 h at 37 °C. Two esculin hydrolysis positive isolates per fecal sample were suspended in tryptic soy broth with 15% glycerol and stored at –80 °C until used.

Speciation of enterococci was performed by a duplex PCR assay that identifies *E. faecalis* and *E. faecium* (Jackson et al., 2004). Bacterial DNA was prepared by suspending colonies

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

Item	Control	Cu	CTC	Cu + CTC
Ingredients, %				
Corn	56.05	55.97	55.62	55.54
Soybean meal, 47% crude protein	24.76	24.77	24.79	24.80
Dried whey	10.00	10.00	10.00	10.00
Enzyme-treated soybean meal[2]	5.00	5.00	5.00	5.00
Monocalcium phosphate, 21.5% phosphorus	1.20	1.20	1.20	1.20
Calcium carbonate	1.05	1.05	1.05	1.05
Sodium chloride	0.60	0.60	0.60	0.60
L-Lysine HCl	0.45	0.45	0.45	0.45
DL-Methionine	0.20	0.20	0.20	0.20
L-Threonine	0.20	0.20	0.20	0.20
L-Tryptophan	0.02	0.02	0.02	0.02
L-Valine	0.05	0.05	0.05	0.05
Trace mineral premix[3]	0.15	0.15	0.15	0.15
Vitamin premix[4]	0.25	0.25	0.25	0.25
Phytase[5]	0.02	0.02	0.02	0.02
Copper sulfate[6]	—	0.08	—	0.08
Chlortetracycline[7]	—	—	0.40	0.40
Total	100	100	100	100
Calculated analysis				
SID[8] amino acids, %				
Lysine	1.35	1.35	1.35	1.35
Isoleucine:lysine	57	57	57	57
Leucine:lysine	115	115	115	115
Methionine:lysine	36	36	35	35
Methionine and cysteine:lysine	57	57	57	57
Threonine:lysine	64	64	64	64
Tryptophan:lysine	18.5	18.5	18.5	18.5
Valine:lysine	65	65	65	65
NE, kcal/kg	2436	2434	2425	2423
Crude protein, %	21.1	21.1	21.1	21.1
Total calcium, %	0.79	0.79	0.79	0.79
STTD[9] phosphorus, %	0.53	0.53	0.53	0.53
Analyzed composition				
Dry matter, %	88.2	88.1	88.1	88.2
Crude protein, %	20.6	20.7	20.9	21.1
Calcium, %	0.85	0.91	0.93	0.91
Phosphorus, %	0.63	0.65	0.69	0.66
Copper, mg/kg	24	202	31	194
Chlortetracycline, mg/kg	ND[10]	0.06	539	309

[1]Diets were fed in meal form from 7.4 to 19.7 kg BW. Copper (Cu) sulfate and chlortetracycline (CTC) were included in the diet at the expense of corn.
[2]HP 300 (Hamlet Protein, Inc., Findlay, OH).
[3]Provided per kg of premix: 73 g Zn from Zn sulfate; 73 g Fe from iron sulfate; 22 g Mn from manganese oxide; 11 g Cu from copper sulfate; 0.2 g I from calcium iodate; 0.2 g Se from sodium selenite.
[4]Provided per kg of premix: 3,527,399 IU vitamin A; 881,850 IU vitamin D; 17,637 IU vitamin E; 1,764 mg vitamin K; 15.4 mg vitamin B12; 33,069 mg niacin; 11,023 mg pantothenic acid; 3,307 mg riboflavin.
[5]Ronozyme Hiphos 2700 (DSM Nutritional Products, Inc., Basel, Switzerland) provided 476 phytase units per kg of feed.
[6]Copper sulfate provided 200 mg/kg of Cu.
[7]Aureomycin 50 (Zoetis Services, LLC., Parsippany, NJ) provided 440 mg/kg CTC.
[8]SID, standardized ileal digestible.
[9]STTD, standardized total tract digestible.
[10]ND, not detected above the detection limit of 0.05 mg/kg.

in nuclease-free water with 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) followed by 10 min of incubation at 95 °C in a thermal cycler (Mastercycler nexus GSX1 flex lid, Eppendorf, Hamburg, Germany). The running conditions, primers, and master mixes for the PCR are described by Kariyama et al. (2000). Positive controls for the PCR assay were ATCC strains 29212 and 19434 of *E. faecalis* and *E. faecium*, respectively (American Type Culture Collection, Manassas, VA). Isolates that were not *E. faecium* or *E. faecalis* were subjected to superoxide dismutase (***sodA***) gene sequence analysis for speciation, as described by Poyart et al. (2000). The *sodA* gene products were purified (Geneclean Turbo Kit, MP Biomedicals, Santa Ana, CA) and then sequenced (Sanger Sequencing, Genewiz, South Plainfield, NJ). Sequences were analyzed using the National Center for Biotechnology Information (**NCBI**) GenBank database Biological Local Alignment Search Tool (**BLAST**) search.

PCR detection of *tcrB*, *erm*(B), and *tet*(M) genes

Bacterial DNA was extracted as described previously and used for the detection of *tcrB* (Hasman et al., 2006), *erm*(B), and *tet*(M) genes (Jacob et al., 2010). *Enterococcus faecium* (7430272-6) served as the positive control for *tcrB*, and field strains of *E. faecium* (AGR 15) and *E. faecalis* (AGR 329) served as positive controls for *erm*(B) and *tet*(M), respectively.

Development and validation of a real-time qPCR for the relative abundance of *tcrB* in fecal DNA

A qPCR assay was developed and validated to determine the relative abundance of *tcrB* gene using a 16S rRNA gene as an indicator of total enterococci DNA in the feces. The NCBI BLAST database was used to analyze the *tcrB* gene target. All sequences available at the time were downloaded and aligned using CLC Main Workbench 7.6 (QIAGEN Bioinformatics, Valencia, CA). Primers and probes were designed from the conserved regions of the alignment result. Heterodimer analysis was evaluated in silico using the OligoAnalyzer tool (Integrated DNA Technologies, Inc., Skokie, IL). To test interactions of the assay, *tcrB* and 16S rRNA (Yang et al., 2002) targets were amplified individually and concurrently from serially diluted DNA extracted from a strain of *tcrB*-carrying *E. faecium* (A17Sv1) and from a swine fecal sample positive for *tcrB*. Primer and probe sequences were as follows: *tcrB*-F-Primer: 5′-AGTAGCATTGGATTCAGCCGA-3′; *tcrB*-R-Primer: 5′-CATCTTACGTGTGGTCTTCTGAG-3′; *tcrB*-Probe: 5′-/5MAXN/TCTTGACACAGTCAGACCCTGG/ BHQ_2/-3′. Primer and probe sequences of the 16S rRNA were used according to Yang et al. (2002). To optimize multiplex reaction chemistry, Cy5 channel and BHQ-3 quencher were selected, 16S-F: 5′-TGGAGCA TGTGGTTTAATTCGA-3′; 16S-R: 5′-TGCGGGACTTAACCCAACA-3′; 16S-Probe 5′-/5Cy5/CACGACCTGACGACARCCATGCA/3BHQ_2/-3′.

The 20 μL qPCR reaction mixture was comprised of 5 μL of DNA template, 1 μL of 10 μM *tcrB* primer mix (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA), 0.5 μL of 10 μM *tcrB* probe (Integrated DNA Technologies, Inc., Skokie, IL), 1 μL of 10 μM 16S primer mix (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA), 0.5 μL of 10 μM 16S probe (Integrated DNA Technologies, Inc., Skokie, IL), 10 μL of 2X Bio-Rad power mix (Bio-Rad Laboratories, Hercules, CA), and nuclease-free water to make up the total reaction volume of 20 μL. The qPCR running conditions consisted of an initial denaturation for 10 min at 95 °C, followed by 45 cycles for 15 s at 95 °C and 45 s at 60 °C using a Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA). The raw data were analyzed using the Bio-Rad CFX Manager software (Bio-Rad Laboratories, Hercules, CA). Initially, the assay was tested with *tcrB*-positive (*E. faecium* A17Sv1) and *tcrB*-negative (*E. faecalis* ATCC 29212) strains of *Enterococcus*. DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen, Germantown, MA).

The analytical sensitivity of the assay was determined using a *tcrB*-positive strain (*E. faecium* A17Sv1). The *tcrB*-positive strain was grown on blood agar and a single colony was inoculated into 10 mL Luria–Bertani (**LB**) broth and incubated overnight. One-hundred microliters of the culture were inoculated into 10 mL LB broth and grown to an absorbance of 0.45 at 600 nm (approximately 3.5 to 6 h at 37 °C and 10^8 CFU/mL). Serial 10-fold dilutions of the culture were prepared. To determine viable cell counts, 100 μL of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spread-plated onto blood agar in triplicate and incubated at 37 °C overnight. The DNA was extracted from the 10^8 CFU/mL culture using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MA) and was serially diluted. The DNA was tested in quintuplicates with the qPCR assay. Standard curves were generated from the resulting data, and PCR efficiency and correlation coefficient (R^2) values were calculated from the standard curves. Fecal samples for qPCR assay were processed using the QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD). The DNA from days 0 and 28 were used in the qPCR assay to determine the relative abundance of *tcrB* as a percentage of total fecal bacterial DNA.

Copper susceptibility determinations

Copper susceptibilities of enterococcal isolates were determined by an agar dilution method (Hasman et al., 2006; Amachawadi et al., 2010). All *tcrB*-positive isolates ($n = 39$), including two Denmark strains (7430162-6 and 7430275-4) as positive control and an equal number of *tcrB*-negative strains ($n = 39$) were included. Mueller–Hinton agar plates containing 0, 2, 4, 8, 12, 16, 20, 24, 24, 28, 32, 36, and 40 mM of Cu sulfate (Fisher Scientific, Pittsburgh, PA), adjusted to pH 7.0, were used. All isolates were grown to 0.5 McFarland standard and spot inoculated (20 μL) on each plate, in duplicate, and incubated for 48 h at 37 °C to determine growth or no growth.

Antibiotic susceptibility determinations

Minimum inhibitory concentrations (**MIC**) were determined by a micro-broth dilution method (CLSI, 2018). All enterococcal isolates from days 0 ($n = 64$) and 28 ($n = 63$) were included. The *E. faecalis* ATCC29212 strain was used as a reference for quality control. Stock solutions of all antibiotics, sourced from either Sigma-Aldrich (St. Louis, MO) or TOKU-E (Bellingham, WA), were prepared with sterile distilled water to a final concentration of 1,000 μg/mL based on their individual potencies. Antibiotics tested were chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin, and vancomycin similar to the National Antimicrobial Resistance Monitoring System (**NARMS**; CMV3AGPF) Sensititre panel. All antibiotics except tigecycline were tested at concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL. Tigecycline was tested at 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0125, and 0.003125 μg/mL. A single colony of each isolate was inoculated into 10 mL Muller–Hinton broth and grown to 0.5 McFarland standard, followed by a 1:100 dilution of the culture. The antimicrobial susceptibility tests were performed in 96-well microtiter plates (Becton, Dickinson and Company, Franklin Lakes, NJ). Plates were incubated at 37 °C for 20 to 24 h and results were recorded as growth or no growth. Susceptible breakpoints for enterococci have only been established for daptomycin (≤4 μg/mL) and tigecycline (≤0.25 μg/mL; CLSI, 2018).

Statistical analysis

Statistical analysis was conducted with STATA MP (version 14.2; StataCorp, LLC, College Station, TX). The pair of pens segregated by solid pen dividers served as the experimental unit. Growth performance data were analyzed using a linear mixed model with treatment as the main effect and block as the random effect. Preplanned contrast statements were built to evaluate the main effects and interactions of Cu and CTC. Prevalence outcomes were statistically described before multivariable analysis. Poisson-model (log-linear regression) with the log-rank assumption of normality for bacterial counts and gene copies was used to analyze quantification data. The main effects of Cu, CTC, and their combination were analyzed first on prevalence, then, interaction terms. Descriptive, bivariate, and multivariate analyses were used in a multilevel mixed model framework for each outcome. From the final model, marginal predicted probabilities were obtained for the proportion of enterococci positive for *tcrB*, *erm*(B), and *tet*(M) genes along with their 95% confidence intervals. Due to the clustering of pens within the treatment, the clustering of animals in pens, and within-animal dependency (due to resampling of animals), multilevel and longitudinal data were considered. Both Cu and CTC susceptibility data were $\log_2$-transformed before analyzing by survival analysis. Results were considered statistically significant at a *P*-value of < 0.05.

Results

Analyzed Cu and CTC levels in diets reflected the addition of 200 mg/kg Cu and 440 mg/kg CTC to respective treatment diets (Table 1). Analyzed levels of DM, CP, Ca, and P were consistent with calculated values.

Growth performance

There was no evidence of Cu by CTC interaction in nursery growth performance throughout the study ($P > 0.05$). Thus, only the main effects of Cu and CTC are detailed in Table 2. In the period of 0 to 14 d, pigs fed diets with added Cu had increased ($P < 0.05$) ADG and ADFI, and pigs fed diets with CTC had improved ($P < 0.01$) ADG, ADFI, and G:F. In the period of 15 to 28 d, there was no evidence ($P > 0.05$) for an effect of added Cu on the growth performance. Pigs fed diets with CTC had increased ($P < 0.001$) ADG and ADFI. For the overall treatment period (0 to 28 d), there was no statistical evidence ($P > 0.05$) that added Cu improved ADG, ADFI, or G:F. However, pigs fed 200 mg/kg Cu did have higher BW ($P < 0.05$) when measured on days 15 and 28. Pigs fed diets with CTC had improved ($P < 0.01$) ADG, ADFI, and G:F, resulting in heavier ($P < 0.05$) pigs on days 14 and 28 compared with pigs fed the CTC-free diet.

Concentrations of total, Cu-, and tetracycline-resistant enterococci

The average concentration of total fecal enterococci on ME agar for the control, Cu, CTC, and Cu with CTC treatment groups across all days were 9.5×10^5, 9.3×10^5, 6.1×10^5, and 1.0×10^6, respectively. There was a significant sampling day by treatment interaction ($P = 0.008$) in concentrations of total enterococci. There was a log reduction in the concentration of total enterococci on ME agar in day 28 compared to day 0 in the Cu with CTC group. No growth of Cu- or CTC-resistant enterococci was observed in MECu or MECTC plates, respectively, in any sampling day or treatment group (data not shown).

Species distribution of enterococci isolates

A total of 384 enterococcal isolates (64 samples; two isolates per fecal sample) were obtained from samples collected from four treatment groups on days 0, 14, and 28. The isolates from each pen were randomly picked for speciation ($n = 192$; Table 3). Only one isolate from the CTC group on sample day 14 did not grow, therefore, it was not speciated ($n = 191$). The predominant species of *Enterococcus* in all treatment groups were *E. faecalis* (97/191, 50.8%), *E. hirae* (39/191, 20.4%), and *E. faecium* (33/191, 17.3%). The other species identified were *E. casseliflavus* (14/191, 7.3%), *E. villorum* (5/191, 2.6%), *E. gallinarum* (2/191, 1.0%), and *E. mundtii* (1/191, 0.5%). The species distribution was not affected ($P > 0.05$) by treatment, except for *E. faecalis,* which was detected in fewer samples in the Cu group compared with the Cu with CTC group ($P < 0.05$; Table 3).

Prevalence of the *tcrB*, *erm*(B), and *tet*(M) genes

All *Enterococcus* spp. isolates ($n = 384$) were tested for *tcrB*, *erm*(B), and *tet*(M) genes. The overall prevalence of *tcrB* gene was 10.2% (39/384; Table 4). The prevalence of *tcrB*-positive enterococcal isolates was not significantly affected ($P > 0.05$) by treatment group. However, the prevalence of *tcrB*-positive enterococcal isolates was higher ($P = 0.002$) in samples from day 14 than from day 0. Twelve of the 39 *tcrB*-positive isolates were among the 192 isolates speciated. The 12 isolates belonged to *E. casseliflavus* (5), *E. faecalis* (3), *E. faecium* (3), and *E. gallinarum* (1). Of the 39 *tcrB*-positive isolates, 32 (82.1%) and 37 (94.9%) were positive for *erm*(B) and *tet*(M) genes, respectively. Of the remaining *tcrB*-negative isolates ($n = 345$), 270 (78.3%) and 166 (48.1%) were positive for *erm*(B) and *tet*(M), respectively.

The overall prevalence of the *erm*(B) gene in enterococcal isolates was 78.6% (302/384; Table 4). The prevalence of the *erm*(B) gene was significantly affected by treatment by sample day interaction ($P = 0.02$). A total of 150 out of 302 *erm*(B)-positive isolates were among the 192 isolates speciated. The species distribution included *E. faecalis* (88/150, 58.7%), *E. faecium* (27/150, 18%), *E. hirae* (21/150, 14%), *E. casseliflavus* (9/150, 6%), *E. vilorum* (3/150, 2%), *E. gallinarum* (1/150, 0.67%), and *E. mundtii* (1/150, 0.67%). The species distribution was not affected by treatment and sample day interaction ($P > 0.05$). The overall prevalence of the *tet*(M) gene in enterococcal isolates was 52.9% (203/384; Table 4). The total prevalence of *tet*(M)-positive enterococcal isolates was not significantly affected ($P > 0.05$) by treatment group or sample day. A total of 176 out of 203 *tet*(M)-positive isolates were among the 192 isolates speciated. The species distribution included *E. faecalis* (94/176, 53.4%), *E. hirae* (37/176, 21%), *E. faecium* (29/176, 16.4%), *E. casseliflavus* (8/176, 4.5%), *E. vilorum* (5/176, 2.8%), *E. gallinarum* (2/176, 1.1%), and *E. mundtii* (1/176, 0.5%).

Relative abundance of *tcrB* in fecal DNA by real-time qPCR

For analytical sensitivity, both *tcrB*-positive (*E. faecium* A17Sv1) and *tcrB*-negative (*E. faecalis* ATCC 29212) strains of *Enterococcus* spp. were initially tested. Results indicated that the gene target was amplified in *tcrB*-positive *Enterococcus* spp. and not in *tcrB*-negative *Enterococcus*. The detection limit of the qPCR assay for serially diluted *tcrB*-positive pure culture was 4×10^2 per mL (data not shown). The analytical sensitivity of the assay was tested using *E. faecium* A17Sv1 DNA to determine the cycle threshold (**Ct**) difference between 16S and *tcrB* to calculate the mean copy-number difference of the two genes, which was used to normalize the calculation of the percentage of bacteria

Table 2. Effects of diets with or without added Cu and/or CTC on the growth performance of nursery pigs[1,2]

Cu, mg/kg[3]	0	200	0	200		Probability, *P* <		
CTC, mg/kg[4,5]	0	0	440	440	SEM	Cu × CTC	Cu	CTC
Days 0 to 14								
ADG, g	249	275	294	313	10.41	0.702	0.029	0.001
ADFI, g	345	374	378	401	10.22	0.755	0.013	0.005
G:F	722	734	777	779	12.10	0.668	0.559	0.001
Days 15 to 28								
ADG, g	575	574	617	627	10.54	0.586	0.697	0.001
ADFI, g	857	856	910	934	14.68	0.332	0.386	0.001
G:F	671	670	678	671	5.75	0.596	0.523	0.491
Days 0 to 28								
ADG, g	412	424	457	470	8.93	0.946	0.171	0.001
ADFI, g	602	614	647	668	10.97	0.686	0.122	0.001
G:F	684	690	706	703	5.10	0.435	0.793	0.002
BW, kg								
Day 0	7.4	7.4	7.4	7.4	0.06	0.865	0.717	0.262
Day 14	10.9	11.3	11.5	11.8	0.15	0.702	0.011	0.001
Day 28	18.9	19.4	20.1	20.5	0.25	0.868	0.035	0.001

[1]A total of 320 weaned barrow piglets (DNA 200 × 400, DNA Genetics, Columbus, NE) were used in a nursery study with five piglets per pen and eight replications (pen-pair) per treatment.
[2]Piglets were weaned at 21 d of age and fed a common diet during an acclimation period from days −7 to −1 and treatment diets from days 0 to 28. Dietary treatments were arranged in a 2 × 2 factorial design with the main effects of added Cu (0 vs. 200 mg/kg from copper sulfate) and CTC (0 vs. 440 mg/kg).
[3]Cu from Cu sulfate.
[4]CTC from Aureomycin 50 (Zoetis Services, LLC., Parsippany, NJ).
[5]Treatments containing CTC were administered under two veterinary feed directives with the first fed from days 0 to 14 and the second fed from days 15 to 28 allowing for a one-day CTC withdrawal from day 14 to 15, thereby complying with the Food and Drug Administration guidelines.

Table 3. Species distribution of enterococci isolates from feces of weaned piglets fed diets with or without added Cu and/or CTC[1]

			Enterococcus spp., *n* (%)[4]						
Treatments[2]	Day	n[3]	*E. faecalis*	*E. faecium*	*E. hirae*	*E. casseliflavus*	*E. gallinarum*	*E. mundtii*	*E. vilorum*
Control	0	16	14	1	0	0	0	1	0
	14	16	6	3	3	4	0	0	0
	28	16	4	1	9	2	0	0	0
	0 to 28	48	24[ab] (50.0)	5 (10.4)	12 (25)	6 (12.5)	0	1 (2.1)	0
Cu	0	16	13	2	0	1	0	0	0
	14	16	5	9	0	1	0	0	1
	28	16	0	1	13	1	0	0	1
	0 to 28	48	18[a] (37.5)	12 (25)	13 (27.1)	3 (6.3)	0	0)	2 (4.2)
CTC	0	16	14	1	0	1	0	0	0
	14	15	6	3	2	2	1	0	1
	28	16	3	5	8	0	0	0	0
	0 to 28	47	23[ab] (48.9)	9 (19.1)	10 (21.3)	3 (6.4)	1 (2.1)	0	1 (2.1)
Cu + CTC	0	16	14	1	0	1	0	0	0
	14	16	8	4	1	1	1	0	1
	28	16	10	2	3	0	0	0	1
	0 to 28	48	32[b] (66.7)	7 (14.6)	4 (8.3)	2 (4.2)	1 (2.1)	0	2 (4.2)
Overall Total	0 to 28	191	97 (50.8)	33 (17.3)	39 (20.4)	14 (7.3)	2 (1.0)	1 (0.5)	5 (2.6)

[1]A total of 320 barrows (DNA 200 × 400, DNA Genetics, Columbus, NE) weaned at approximately 21 d of age with 7.4 kg BW were used in a 28-d nursery study. Piglets were fed a common diet during an acclimation period from days −7 to −1 and treatment diets from days 0 to 28.
[2]Dietary treatments were arranged in a 2 × 2 factorial design with main effects of added Cu (0 vs. 200 mg/kg from copper sulfate) and chlortetracycline (0 vs. 440 mg/kg CTC). Treatments consisted of control, with no addition of Cu or CTC in the diet; Cu, with 200 mg/kg added Cu from copper sulfate in the diet; CTC, with 440 mg/kg CTC in the diet; and Cu + CTC, with 200 mg/kg added Cu from copper sulfate and 440 mg/kg added CTC in the diet.
[3]Total enterococcal isolates.
[4]Direct rectal fecal samples were collected randomly from three piglets per pen on days 0, 14, and 28. Speciation of enterococci was performed by duplex PCR assay to identify *E. faecalis* and *E. faecium* and to superoxide dismutase gene sequence analysis for speciation of the other species.
[a,b]Column means not sharing the same superscript differ at $P < 0.05$.

Table 4. Prevalence of copper (*tcrB*), macrolide, (*erm*B), and tetracycline (*tet*M) resistance genes in *Enterococcus* spp. isolates from feces of weaned piglets fed diets with or without added Cu and/or CTC[1]

			Positive isolates, *n* (%)		
Treatments[2]	Day	n[3]	*tcrB*	*erm*(B)	*tet*(M)
Control	0	32	1	26	15
	14	32	6	23	19
	28	32	1	23	16
	0 to 28	96	8 (8.3)	72 (75)	50 (52.1)
Cu	0	32	0	24	13
	14	32	6	26	18
	28	32	1	17	15
	0 to 28	96	7 (7.3)	67 (69.8)	46 (47.9)
CTC	0	32	2	28	16
	14	32	4	24	18
	28	32	3	28	18
	0 to 28	96	9 (9.4)	80 (83.3)	52 (54.2)
Cu + CTC	0	32	3	28	17
	14	32	6	26	19
	28	32	6	29	19
	0 to 28	96	15 (15.6)	83 (86.5)	55 (57.3)
Overall Total	0 to 28	384	39 (10.2)	302 (78.6)	203 (52.9)

[1]A total of 320 barrows (DNA 200 × 400, DNA Genetics, Columbus, NE) weaned at approximately 21 d of age with 7.4 kg BW were used in a 28-d nursery study. Piglets were fed a common diet during an acclimation period from days −7 to −1 and treatment diets from days 0 to 28.
[2]Dietary treatments were arranged in a 2 × 2 factorial design with main effects of added Cu (0 vs. 200 mg/kg from copper sulfate) and chlortetracycline (0 vs. 440 mg/kg CTC). Treatments consisted of control, with no addition of Cu or CTC in the diet; Cu, with 200 mg/kg added Cu from copper sulfate in the diet; CTC, with 440 mg/kg CTC in the diet; and Cu + CTC, with 200 mg/kg added Cu from copper sulfate and 440 mg/kg added CTC in the diet.
[3]Total enterococcal isolates.
[4]Direct rectal fecal samples were collected randomly from three piglets per pen on days 0, 14, and 28. Prevalence of copper (*tcrB*), macrolide, (*erm*B), and tetracycline (*tet*M) resistance genes in *Enterococcus* spp. isolates was performed by PCR.

in a sample that carried the *tcrB* gene. Our results indicated a PCR efficiency of 94.0% and 107.1% for the *tcrB* and 16S targets, respectively. The R^2 values were 0.997 and 0.983 for the *tcrB* and 16S target, respectively. The average Ct of each serial dilution was used to generate the standard curve and calculate the 16S copy-number difference in fecal samples for five technical replicates (data not shown). For PCR optimization, the primer and probe interaction analysis, amplifications of *tcrB* alone, 16S alone, and the combination of *tcrB* and 16S for 1×, 10×, 100×, and 1,000× dilutions indicated no interactions when our *tcrB* and 16S reactions were duplexed. The average percentage of *tcrB* in total fecal bacterial DNA relative to the total bacterial load per experimental unit was not significantly affected ($P > 0.05$) by treatment group or sample day.

Minimum inhibitory concentrations of Cu

The MIC of Cu was not significantly affected by the treatment group within *tcrB*-positive and *tcrB*-negative isolates ($P > 0.05$; Table 5). However, the overall MIC of Cu was significantly greater for *tcrB*-positive isolates compared with *tcrB*-negative isolates ($P < 0.0001$; Table 5).

Minimum inhibitory concentrations of antibiotics

The MIC of antibiotics was determined by micro-broth dilution with slight modifications of Clinical and Laboratory Standard Institute (**CLSI**) standard methods (CLSI, 2018) guidelines for 16 antimicrobials defined by the NARMS Gram-positive panel plates (CMV3AGPF) on days 0 ($n = 64$) and 28 ($n = 63$).Susceptibilities of *Enterococcus* spp. isolates to antimicrobial compounds was not significantly affected ($P > 0.05$) by treatment group or sample day (Table 6). All isolates were susceptible to gentamicin, kanamycin, and streptomycin. As per the CLSI (2013), susceptible breakpoints for enterococci have been established only for daptomycin (≤4 μg/mL) and tigecycline (≤ 0.25 μg/mL). For macrolide antibiotics, 61 (95.3%) and 47 (74.6%) isolates were resistant to erythromycin, and 51 (79.7%) and 41 (65.1%) isolates were resistant to tylosin, with a similar distribution across all treatment groups, from days 0 and 28, respectively. There were 60 (93.8% and 95.2%) tetracycline-resistant isolates, distributed similarly across all treatment groups, from days 0 and 28. For lincomycin, there were 62 (96.9%) and 63 (100.0%) resistant isolates similarly distributed across all sample groups for days 0 and 28, respectively. For linezolid, there were 36 (56.3%) and 39 (61.9%) resistant isolates similarly distributed across all treatment groups from days 0 and 28, respectively. For chloramphenicol, there were 51 (79.7%) and 37 (58.7%) resistant isolates similarly distributed across all treatment groups from days 0 and 28, respectively. For penicillin, there were three (4.7%) resistant isolates from day 0. For nitrofurantoin, there were 2 (3.1%) and 18 (28.6%) resistant isolates from days 0 and 28, respectively. For ciprofloxacin, there were 9 (14.1%) and 19 (30.2%) resistant isolates from days 0 and 28, respectively. For Synercid, there were 63 (98.4%) and 37 (58.7%) resistant isolates from days 0 and 28, respectively. A comparison of *tcrB*-positive vs. *tcrB*-negative antibiotic susceptibilities can be found in Table 6.

Discussion

When fed at elevated levels of 100 to 250 mg/kg, Cu has growth promotional benefits, which are attributed to alteration of gut microflora, retention of nutrients, reduction in fermentation losses, and reduced morbidity and mortality, particularly in piglets (Højberg et al., 2005). Because of restrictions on the

Table 5. MIC of Cu towards *tcrB*-negative or -positive *Enterococcus* spp. isolates from feces of piglets fed diets with or without added Cu and/or CTC[1]

	tcrB-negative enterococci		*tcrB*-positive enterococci	
Treatment groups[2]	No. of isolates	MIC, μg/mL	No. of isolates	MIC, μg/mL
Control	8	3.1	8	20.5
Cu	7	3.7	7	21.4
CTC	9	3.4	9	20.7
Cu + CTC	15	3.5	15	20.1

[1]A total of 320 barrows (DNA 200 × 400, DNA Genetics, Columbus, NE) weaned at approximately 21 d of age with 7.4 kg BW were used in a 28-d nursery study. Piglets were fed a common diet during an acclimation period from days –7 to –1 and treatment diets from days 0 to 28. Treatments consisted of control, with no addition of Cu or CTC in the diet; Cu, with 200 mg/kg added Cu from copper sulfate in the diet; CTC, with 440 mg/kg CTC in the diet; and Cu + CTC, with 200 mg/kg added Cu from copper sulfate and 440 mg/kg added CTC in the diet.
[2]Dietary treatments were arranged in a 2 × 2 factorial design with main effects of added Cu (0 vs. 200 mg/kg from copper sulfate) and chlortetracycline (0 vs. 440 mg/kg CTC). Dietary treatments had no effect on copper susceptibilities among enterococcal isolates ($P > 0.05$).

Table 6. Comparison of antibiotic susceptibilities between transferable copper resistance gene (*tcrB*)-positive and *tcrB*-negative *Enterococcus* spp. isolates from feces of piglets fed diets with or without added Cu and/or CTC[1]

	Mean minimum inhibitory concentration (MIC), μg/mL[2]				
Antimicrobial compounds	*tcrB*-negative	*tcrB*-positive	Susceptible MIC, μg/mL	Intermediate MIC, μg/mL	Resistant MIC, μg/mL
Chloramphenicol	32	16	≤8	16	≥32
Ciprofloxacin	1.1	7.7	≤1	2	≥4
Daptomycin	5.2	3.3	≤4	N/A	N/A[3]
Erythromycin	42	33.8	≤0.5	1–4	≥8
Gentamicin	16.8	31.6	≤500	N/A	>500
Kanamycin	102.4	115.2	≤512	N/A	≥1,024
Lincomycin	128	37.6	≤2	4	≥8
Linezolid	16	52.8	≤2	4	≥8
Nitrofurantoin	25.6	11.2	≤32	64	≥128
Penicillin	1.3	2.9	≤8	N/A	≥16
Streptomycin	102.4	78.4	≤1,000	N/A	>1,000
Synercid	5.4	5.6	≤1	2	≥4
Tetracycline	89.6	76.8	≤4	8	≥16
Tigecycline	1.95	1.2	≤0.25	N/A	N/A[4]
Tylosin tartrate	102.8	45.4	≤8	16	≥32
Vancomycin	2.9	2.7	≤4	8–16	≥32

[1]A total of 320 barrows (DNA 200 × 400, DNA Genetics, Columbus, NE) weaned at approximately 21 d of age with 7.4 kg BW were used in a 28-d nursery study. Piglets were fed a common diet during an acclimation period from days –7 to –1 and treatment diets from days 0 to 28. Treatments consisted of control, with no addition of Cu or CTC in the diet; Cu, with 200 mg/kg added Cu from copper sulfate in the diet; CTC, with 440 mg/kg CTC in the diet; and Cu + CTC, with 200 mg/kg added Cu from copper sulfate and 440 mg/kg added CTC in the diet. Dietary treatments had no effect on antimicrobial susceptibilities among enterococcal isolates ($P > 0.05$).
[2]For the purpose of averaging the transferable copper resistance gene (*tcrB*)-negative and *tcrB*-positive MIC, values that were ≥128 μg/mL were considered as 128 μg/mL.
[3]According to the Clinical and Laboratory Standards Institute, only a susceptible breakpoint has been established for daptomycin (≤ 4 μg/mL).
[4]According to the Clinical and Laboratory Standards Institute, only a susceptible breakpoint has been established for tigecycline (≤ 0.25 μg/mL).

use of antibiotics for growth promotion, implementation and development of antibiotic alternatives are crucial to maintain food animal production and health. One alternative is feeding Cu at levels above NRC requirements. However, due to the potential link with AMR, the practice raises concerns similar to those that drove the ban on dietary use of antibiotics for growth promotion in food animals. To understand the effects of using antibiotic alternatives, it is important to understand the complexity of AMR, including selection pressures that facilitate the acquisition of resistance genes. In this study, we evaluated the effects of feeding normal and elevated levels of Cu, alone or in combination with CTC, on growth performance, prevalence and quantification of *tcrB*, prevalence of *tet*(M) and *erm*(B), and Cu and antimicrobial susceptibility determinations of *Enterococcus* spp. isolates from feces of weaned piglets.

The growth benefits of feeding levels of Cu above requirements to weaned pigs are well established (Cromwell et al., 1998; Hill et al., 2000; Bikker et al., 2016; Carpenter et al., 2018) and seem to be largely driven by increased feed intake, although the efficiency of gain is also improved (Stahly et al., 1980; Hill et al., 2001; Bikker et al., 2016). In the present study, the addition of 200 mg/kg Cu as Cu sulfate improved rate of gain of nursery pigs by 8% during the initial period up to day 14, mostly driven by increased feed intake and resulted in heavier piglets at the end of the 28 d. This is in agreement with a recent study by Carpenter et al. (2018). Interestingly, the improvement in growth

rate was mostly observed in the first 14-d period (7.5 to 11.5 kg BW) with no effect in the next 14-d period (11.5 to 19.5 kg BW) or overall (7.5 to 19.5 kg BW).

Antibiotics has also been shown to be efficacious at improving rate of gain and feed intake in weaned pigs (Stahly et al., 1980; Dritz et al., 2002; Puls et al., 2019). In the present study, the addition of 440 mg/kg CTC improved growth rate, feed intake, and "G:F" in the overall 28-d feeding period, resulting in heavier piglets at the end of the treatment period. The improvement in the efficiency of gain has not been consistently associated with the addition of in-feed antibiotics in nursery diets. Previous studies have reported improvements in feed efficiency similar to the present study (Stahly et al., 1980; Gottlob et al., 2004), whereas others have not (Dritz et al., 2002; Puls et al., 2019). The lack of interactive effects between Cu and CTC suggests that the responses of Cu and CTC on the growth performance of weaned pigs are as efficacious when added alone or in combination in nursery diets (Hill et al., 2001).

In this study, the *tcrB* gene was detected in four *Enterococcus* species: *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*. In previous studies, the *tcrB* gene was detected in only two species, *E. faecalis* and *E. faecium* (Amachawadi et al., 2011). Species distributions may equate differently based upon the number of samples collected, the number of isolates selected for speciation, and the number of screened species in each study. The species detected in this study were similar to Danish (Hasman and Aarestrup, 2002; Hasman et al., 2006) and Australian (Mazaheri et al., 2010) studies. The overall species distribution in this study was unlike other study reports, as most species reported in the United States other than *E. faecalis* and *E. faecium* are *E. durans* and *E. malodoratus* (Devriese et al., 1994; Thal et al., 1995). The variation of *Enterococcus* species across studies could be due to many factors such as age at sample time, which could represent a given animal that has had either more or less time to develop a diverse microflora. Additionally, selecting just a few isolates of many colonies from a fecal sample could, by chance, be a limiting factor in unveiling the true variance of *Enterococcus* species in each sample.

There was only a small proportion of *tcrB*-positive isolates (39/384, 10.2%) in this study. The low prevalence of *tcrB* in enterococci observed in this study was comparable to a previous study (Amachawadi et al., 2011) and suggests that this resistance gene is not being transferred at a high rate in enterococci, specifically. Regardless, enterococci remain an important gut bacteria to monitor as they are increasingly recognized as pathogens that can cause illnesses like bacteremia and infective endocarditis in humans (Arias and Murray, 2008). Studies in Denmark that used high levels of Cu found a high prevalence of *tcrB* in *Enterococcus* spp. isolates from swine collected at slaughter which could be associated with the age of the animal (development of resistant microflora), the duration (birth to slaughter), and concentration (175 to 200 mg/kg) of Cu exposure (Hasman and Aarestrup, 2002; Hasman et al., 2006). The presence of *tcrB* in the control group (8/39, 20.5%) suggests that there are naturally resistant isolates present in piglets. The prevalence of *tcrB*-positive isolates did not have a significant treatment effect despite being numerically higher in the Cu with CTC treatment group compared with other treatment groups. Most *tcrB*-positive *Enterococcus* spp. isolates were from sample day 14 (56.4%) compared with days 0 (15.4%) and 28 (28.2%).

The concentration of fecal *tcrB* was not affected by the inclusion of Cu alone or in combination with CTC. Using the NCBI primer BLAST tool, we identified seven bacterial cocci, in addition to *Enterococcus*, that contained the *tcrB* sequence with 100% match. These cocci included three species of *Trichococcus* (*pasteurii*, *collinsii*, and *ilyis*), *Aerococcus viridans*, and two species of *Streptococcus* (*pseudoporcinus* and *agalactiae*). The presence of these cocci in the swine gut is known, but it is possible a few of them may be contributing to *tcrB* amplification in our qPCR assay. Enterococci that possess the *tcrB* gene, homologous to *copB*, are more capable of exporting Cu from the cell, thereby avoiding Cu's cytotoxic effect, than bacteria that lack the *tcrB* gene. This is likely the reason that we saw a near-7-fold increase in resistance to Cu in *tcrB*-positive enterococci as compared with *tcrB*-negative enterococci (20 vs. 3 mM), which is supported by previous studies (Hasman and Aarestrup, 2002; Amachawadi et al., 2010).

It is known that macrolide resistance gene *erm*(B) and vancomycin resistance gene *vanA* can reside on the same plasmid as *tcrB* (Hasman and Aarestrup, 2002) and that feeding dietary Cu to piglets can co-select for erythromycin and vancomycin resistance (Hasman et al., 2006). In our study, both *tcrB*-negative and *tcrB*-positive isolates were positive for the *erm*(B) gene, and were resistant to erythromycin, disabling us from evaluating the co-selection of macrolide resistance in conjunction with the selection of *tcrB*. Additionally, both *tcrB*-negative and *tcrB*-positive isolates carried the *tet*(M) gene, which is not surprising given the high transferability of this gene and others by mobile genetic elements (Hegstad et al., 2010). Large portions of day 0 and day 28 isolates were resistant to the macrolide antibiotics, erythromycin and tylosin, which is comparable to a former study (Amachawadi et al., 2010). The past use of tylosin as a growth promoter, and both past and present use to treat enteric infections, may be an unintended contributing factor in the selection of AMR. Most of the isolates were resistant to tetracycline as well, which is not surprising based on a previous study that demonstrated the co-selection of tetracycline resistance with macrolide resistance (Cauwerts et al., 2007). In this study, the phenotypic resistance of both macrolides and tetracycline is supported genetically as well.

Enterococci are commensal bacteria found in both animals and humans. Antimicrobial of enterococci is a primary concern in human medicine (Moellering, 1992) because enterococci are important nosocomial and opportunistic pathogens (Puchter et al., 2018). Animals, like humans, can serve as a reservoir of resistance genes capable of transferring to other subjects (Kojima et al., 2010). Enterococci tend to become resistant to medically important antibiotics by acquiring resistance genes via intra- (Amachawadi et al., 2010) and inter-species (Amachawadi et al., 2011) conjugal transfer. It has been shown that the transfer of genetic elements can occur between *Enterococcus* spp. and other gut bacteria (Bertram and Durre, 1991). Because of the propensity of *Enterococcus* spp. to house and share AMR genes, human–human, animal–animal, and human–animal exposures to resistant enterococci are concerning and warrant investigation as in this study.

The results of this study show that 200 mg/kg added Cu or 440 mg/kg CTC in nursery diets exerts growth promotional effects in weaned pigs and are as efficacious when added alone as in combination with nursery diets. Added Cu, with or without a selection pressure of CTC, did not increase Cu-resistant enterococci and did not co-select resistance to antibiotics.

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Conflict of interest statement

The authors report no conflict of interest.

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