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Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the fecal metagenome of weaned pigs

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ABSTRACT

Use of in-feed antibiotics such as chlortetracycline (CTC) in food animals is fiercely debated as a cause of antimicrobial resistance in human pathogens; as a result, alternatives to antibiotics such as heavy metals have been proposed. We used a total community DNA approach to experimentally investigate the effects of CTC and copper supplementation on the presence and quantity of antimicrobial resistance elements in the gut microbial ecology of pigs. Total community DNA was extracted from 569 fecal samples collected weekly over a 6-week period from groups of 5 pigs housed in 32 pens that were randomized to receive either control, CTC, copper, or copper plus CTC regimens. Qualitative and quantitative PCR were used to detect the presence of 14 tetracycline resistance (*tet*) genes and to quantify gene copies of *tet*A, *tet*B, bla_{CMY-2} (a 3rd generation cephalosporin resistance gene), and *pco*D (a copper resistance gene), respectively. The detection of *tet*A and *tet*B decreased over the subsequent sampling periods, whereas the prevalence of *tet*C and *tet*P increased. CTC and copper plus CTC supplementation increased both the prevalence and gene copy numbers of *tet*A, while decreasing both the prevalence and gene copies of *tet*B. In summary, *tet* gene presence was initially very diverse in the gut bacterial community of weaned pigs; thereafter, copper and CTC supplementation differentially impacted the prevalence and quantity of the various tetracycline, ceftiofur and copper resistance genes resulting in a less diverse gene population.

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1. Introduction

Antimicrobial resistance (AMR) is one of the major public health challenges of the 21st century (WHO, 1997, 2001; G8, 2013), and international collaboration is needed to combat this complex and multi-factorial issue (TATFAR, 2011; Doyle et al., 2013). The main public health concern about AMR in agriculture is the use of in-feed antibiotics in livestock for growth promotion, prevention and control of infectious diseases (Marshall and Levy, 2011).

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Chlortetracycline (CTC) and oxytetracycline are among the most commonly used in-feed antibiotics in pig production in the United States (Dewey et al., 1999; Apley et al., 2012). Tetracycline resistance is acquired mainly through mobile genetic elements (plasmids and transposons) (Roberts, 2011) and represents the most commonly observed resistance among a wide range of bacteria (Guillaume et al., 2000; Giguère, 2006). Forty-six tetracycline resistance (*tet* and *otr*) genes are currently known which confer bacterial resistance to tetracycline through efflux pumps, ribosomal protection, enzymatic inactivation, or as yet unknown mechanisms (Roberts, 2011).

Alternatives to antibiotics in agriculture are being sought because of the public health risk of AMR associated with the use of in-feed antibiotics in animals; one option is to use metals such as copper and zinc (Verstegen and Williams, 2002). Copper is an essential trace element that is sometimes used as a growth promoter at a dose elevated beyond that which is required for basic metabolic needs (Hasman et al., 2006). Intuitively, bacteria exposed to metals have developed the ability to resist high concentrations of metals, including copper (Brown et al., 1992). Among *E. coli*, in addition to chromosomally mediated copper homeostasis, a plasmid-borne copper resistance determinant (*pco*) has been described that confers resistance to copper (Rensing and Grass, 2003; Zimmermann et al., 2012). The *pco* determinant consists of seven genes arranged in two operons, *pco*ABCD and *pco*RS, with *pco*E as a separate gene (Rensing and Grass, 2003; Zimmermann et al., 2012). The *pco*ABCD genes are normally expressed as a polycistronic message from the same promoter under a two-component *pco*RS regulatory system, with stoichiometric production of the four gene products (Rouch and Brown, 1997); importantly, it is argued that expression of all four genes is required for full resistance (Zimmermann et al., 2012). On the other hand, the *pco*E gene is not strictly required for full expression of copper resistance; however, it acts as a sponge to sequester toxic copper and can thus provide additional time needed for the expression of the other *pco* genes (Zimmermann et al., 2012).

Antimicrobial resistance has been largely studied using culture-based methods; these typically involve bacterial isolation followed by sensitivity testing, or sometimes the testing of bacterial DNA isolated from bacterial culture for the presence of AMR genes (Patterson and Singer, 2006; Agga et al., 2014). These isolate-based methods likely underestimate the true magnitude and also the underlying dynamics of AMR since the majority of bacteria are non-cultivatable on existing culture media (Kim et al., 2011). Quantitative methods, based on the determination of gene copies from total community DNA, can give more accurate information on the impact of antimicrobial use on AMR element load in any given bacterial ecology (Patterson and Singer, 2006), such as the pig gut. Therefore, we used a culture-independent total community DNA approach to investigate the impact of in-feed therapeutic doses of CTC and elevated levels of copper supplementation on the presence, and quantity, of antimicrobial and copper resistance genes among the gut bacteria of pigs. Our hypotheses were that the in-feed use of tetracycline (CTC) expands certain tetracycline resistance genes and that copper supplementation in pigs differentially co-selects for certain tetracycline and third generation cephalosporin resistance genes along with copper resistance genes.

2. Materials and methods

2.1. Experimental design and DNA extraction

A complete description of the experimental design and the study animals was previously reported elsewhere (Agga et al., 2014). Briefly, a cluster randomized trial was employed to investigate the impact of in-feed supplementation of chlortetracycline and elevated levels of copper on antimicrobial resistance elements in the gut microbiota of nursery pigs. First, 160 weaned pigs (PIC 1050 barrows; PIC North America, Hendersonville, TN) 3 weeks of age were obtained from a single commercial pig farm and then randomized to one of 32 pens (at five pigs per pen), blocked by arrival body weight. Four treatment groups (described below) were randomly allocated to the 32 pens with eight pens per treatment group, further blocked by barn ($n = 2$), and with geographical distribution of pen treatments within each barn designed to minimize and balance any fecal cross-contamination between pens. After 2 weeks of farm adaptation, during which all pigs were provided only the basal diet supplemented with the metabolically recommended 16.5 ppm of copper sulfate ($CuSO_4$), the same diet (control) or else experimental doses of CTC at 550 mg/kg of feed (CTC group), elevated levels of $CuSO_4$ (125 mg/kg of feed) (copper group) or a combination of CTC and copper at the above doses (Copper + CTC group) were then fed continuously for 3 weeks. Fecal samples were collected from three pigs per pen (out of five pigs in total) on days 0, 7, 14, 21, 28 and 35. Only 569 (out of 576) fecal samples from the four experimental groups, consisting of control ($n = 143$), CTC ($n = 142$), copper ($n = 141$) and copper plus CTC ($n = 143$), were available for analysis. Seven fecal samples (6 from day 0 and 1 from day 7) were not sufficient for total DNA extraction and were excluded from analysis.

Total community DNA was extracted from 200 mg of each fecal sample using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The DNA concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and stored at −20 °C until used. Total community DNA was used for the detection of 14 *tet* genes and for the quantification of two *tet* (*tet*A and *tet*B), bla_{CMY-2} and *pco*D gene copies per gram of feces. Positive control *E. coli* strains for *tet*, bla_{CMY-2} and *pco*D were obtained from Dr. Marilyn Roberts (Washington State University), University of Illinois (Odeh et al., 2002) and Dr. Henrik Hasman (the National Food Institute, Technical University of Denmark), respectively. DNA used as positive controls was extracted from these *E. coli* strains with the PureYield Plasmid Miniprep System (Promega Corporation, Madison, WI) following overnight culture in Luria-Bertani broth (Difco, BD, Sparks, MD) incubated at 37 °C.

2.2. PCR detection of tetracycline resistance genes

Previously developed multiplex PCR protocols and primers (Ng et al., 2001) with a pre-optimized multiplex

Table 1
Primers used in multiplex PCR assays for the detection of *tet* genes from fecal samples ($n = 569$) of pigs supplemented with chlortetracycline, copper, both or neither.

Multiplex group	Resistance gene*	Resistance mechanism	Primer	Primer sequence (5′-3′)	Amplicon size (bp)	Genebank accession No.
I	*tet*B	Efflux pump	*tet*B-F	TTG GTT AGG GGC AAG TTT TG	659	X61367
			*tet*B-R	GTA ATG GGC CAA TAA CAC CG		
	*tet*C	Efflux pump	*tet*C-F	CTT GAG AGC CTT CAA CCC AG	418	J01830
			*tet*C-R	ATG GTC GTC ATC TAC CTG CC		
	*tet*D	Efflux pump	*tet*D-F	AAA CCA TTA CGG CAT TCT GC	787	J01749
			*tet*D-R	GAC CGG ATA CAC CAT CCA TC		
II	*tet*A	Efflux pump	*tet*A-F	GCT ACA TCC TGC TTG CCT TC	210	L06798
			*tet*A-R	CAT AGA TCG CCG TGA AGA GG		
	*tet*E	Efflux pump	*tet*E-F	AAA CCA CAT CCT CCA TAC GC	278	L06940
			*tet*E-R	AAA TAG GCC ACA ACC GTC AG		
	*tet*G	Efflux pump	*tet*G-F	GCT CGG TGG TAT CTC TGC TC	844	S52437
			*tet*G-R	AGC AAC AGA ATC GGG AAC AC		
	*tet*K	Efflux pump	*tet*K-F	TCG ATA GGA ACA GCA GTA	169	S67449
			*tet*K-R	CAG CAG ATC CTA CTC CTT		
III	*tet*L	Efflux pump	tetL-F	TCG TTA GCG TGC TGT CAT TC	267	U17153
			tetL-R	GTA TCC CAC CAA TGT AGC CG		
	*tet*M	Ribosomal protection	tetM-F	CTG TTG AAC CGA GTA AAC CT	406	X90939
			tetM-R	GCA CTA ATC ACT TCC ATT TG		
	*tet*O	Ribosomal protection	*tet*O-F	AAC TTA GGC ATT CTG GCT CAC	515	Y07780
			*tet*O-R	TCC CAC TGT TCC ATA TCG TCA		
	*tet*S	Ribosomal protection	*tet*S-F	CAT AGA CAA GCC GTT GAC C	667	X92946
			*tet*S-R	ATG TTT TTG GAA CGC CAG AG		
IV	*tet*P	Ribosomal protection	*tet*P-F	CTT GGA TTG CGG AAG AAG AG	676	L20800
			*tet*P-R	ATA TGC CCA TTT AAC CAC GC		
	*tet*Q	Ribosomal protection	*tet*Q-F	TTA TAC TTC CTC CGG CAT CG	904	X58717
			*tet*Q-R	ATC GGT TCG AGA ATG TCC AC		
	*tet*X	Enzymatic inactivation	*tet*X-F	CAA TAA TTG GTG GTG GAC CC	468	M37699
			*tet*X-R	TTC TTA CCT TGG ACA TCC CG		

* *E. coli* strains with *tet*-positive plasmids were obtained from Dr. Marilyn Roberts (Washington State University).

PCR master mix kit (QIAGEN, Valencia, CA) were used to detect 14 different *tet* genes, grouped into 4 multiplex PCR assays, from each fecal sample (Table 1). A multiplex PCR reaction mixture of 50 μl consisted of 17 μl of nuclease-free water, 25 μl of master mix, 5 μl of the primer mix, and 3 μl of DNA template. A positive control mixture for each of the multiplex assays and a no-template control were included on each plate. Thermal cycling conditions were initial activation at 95 °C for 15 min followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s with a final extension at 72 °C for 10 min. PCR products were analyzed using capillary gel electrophoresis in the QIAxcel system (QIAGEN, Valencia, CA).

2.3. Quantification of resistance genes from fecal community DNA

We used Brilliant II SYBR green master mix (Agilent technologies, La Jolla, CA) based quantitative PCR (qPCR) for quantification of *tet*A, *tet*B, bla_{CMY-2}, and *pco*D genes from total fecal community DNA. All qPCR assays were performed in duplicate reactions in Mx3005P thermocycler (Stratagene Corporation, La Jolla, CA), and amplification data were analyzed with MXPro 4.1 (Stratagene Corporation, La Jolla, CA) software. The qPCR standard curves, defined as gene copy numbers versus cycle threshold (Ct) values, were generated using purified PCR products obtained from positive controls for each of the four genes. Ten-fold serial dilutions were made from known concentrations of purified PCR products and 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 gene copies per reaction were used to create a standard curve for *tet*A and *pco*D genes. For *tet*B and bla_{CMY-2} genes, 10^4, 10^3, 10^2, 10^1, and 10^0 dilutions were used to generate the standard curves. Standard curves were run in triplicate on each plate for all unknown samples. Non-template control (NTC), ATCC 25922 *E. coli* strain (negative control strain), along with template DNA from the positive control strains (positive control) were included in all qPCR reaction plates. The primers used for the quantification of the resistance genes are given in Table 2.

For the quantification of *tet*A, the total qPCR reaction mixture of 20 μl consisted of 6.25 μl water, 10 μl master mix (Agilent Technologies, La Jolla, CA), 0.75 μl reference dye (30 nm), 0.5 μl each of the primers, and 2 μl template DNA. The thermal cycling program was 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 28 s. For *tet*B quantification, a total volume of 20 μl reaction consisted of 6.825 μl of water, 10 μl of master mix, 0.375 μl of reference dye, 0.4 μl of each of the primers, and 2 μl of template DNA. Thermal reaction conditions were 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Gene copies of the bla_{CMY-2} gene were quantified in a 20-μl mixture consisting of 5.25 μl of water, 10 μl of master mix, 0.75 μl of reference dye, 1 μl each of the primers, and 2 μl of template DNA. Thermal profiles consisted of 40 cycles of denaturation at 95 °C for 10 s, annealing at 52 °C for 30 s,

Table 2
Primers used for the quantification of resistance genes from fecal samples of pigs supplemented with chlortetracycline, copper, both or neither.

Genes	Primer	Primer sequence (5′-3′)	Amplicon size (bp)	Reference
*tet*A	*tet*A-F	GCT ACA TCC TGC TTG CCT TC	210	(Ng et al., 2001)
	*tet*A-R	CAT AGA TCG CCG TGA AGA GG		
*tet*B	*tet*B-F1	CAG CAA GTG CGC TTT GGA TGC TG	101	(Borjesson et al., 2009)
	*tet*B-R1	TGA GGT GGT ATC GGC AAT GA		
	585F	CAG ACG CGT CCT GCA ACC ATT AAA		
bla_{CMY-2}[a]	1038R	TAC GTA GCT GCC AAA TCC ACC AGT	454	(Alali et al., 2009)
*pco*D[b]	*pco*D-F	ATC AGCA GGC AGG ACA ATA C	103	This study
	*pco*D-R	CTG ATG TGG GTA TTA GCT GGA TT		

[a] Positive E. *coli* strain for bla_{CMY-2} was obtained from University of Illinois (Odeh et al., 2002).
[b] Positive E. *coli* with pRJ1004 for *pco*D was obtained from Dr. Henrik Hasman (the National Food Institute, Technical University of Denmark).

and extension at 79 °C for 17 s. For *pco*D gene quantification, 20-μl reaction volume comprised of 6.45 μl of water, 10 μl of master mix, 0.75 μl of reference dye, and 0.4 μl of the primers and 2 μl of DNA template. The qPCR thermal conditions were 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. In all qPCR assays, melting curve analysis was performed with a final dissociation step at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. All qPCR reactions were initiated by a denaturation step of 95 °C for 10 min to activate the HotStarTaq DNA polymerase enzyme.

2.4. Statistical analysis

A full factorial analysis in STATA 12.1 (STATA Corporation, College Station, TX) was used to analyze the effects of treatments over the study period. In all statistical analyses a P-value < 0.05 was considered significant for identifying differences across treatment groups or sampling periods with Bonferroni-adjusted significance tests for multiple comparisons. Model selection was based on manual elimination of non-significant terms starting with a full model (a model that included all 3- and 2-way interactions and the main effects of treatment and sampling time), and thereafter first removing the non-significant highest-order interaction, then 2-way interactions, and finally main effects terms, where applicable. The fit of all models was assessed by likelihood ratio (LR) χ^2 test comparing the final model with the null model; further, the final model itself was built by adding model terms and then comparing the full to nested model at each step using the LR test.

2.4.1. Prevalence of tetracycline resistance genes

For analysis of the detection prevalence of *tet* genes, the weekly sampling dates were categorized into pre-treatment (day 0), during treatment (days 7, 14, 21), and post-treatment periods (days 28 and 35). Binary outcomes (presence/absence) of the *tet* genes were initially presented as frequencies along with the 95% exact binomial confidence intervals. The median (with 95% CI) was used to compare the number of *tet* genes detected per fecal sample by treatment groups using the Kruskal–Wallis test. Because all other genes had sparse cells, indicating either low prevalence or full sample gene saturation, further multivariable statistical analyses were performed only for *tet*A, *tet*B, and *tet*P genes. Random effects logistic regression with pen as a random effect was used to analyze the fixed effects of treatment, sampling period and their interactions. Bivariate probit regression with a robust clustered standard error accounting for pen effect was used to model the binary outcomes of *tet*A and *tet*B simultaneously to adjust for their biological dependencies. Ordinal logistic regression with a robust standard error to adjust for clustering by pen was fit to evaluate the effect of treatment and sampling period on the number of different *tet* genes detected per fecal sample. Our unit of analysis was the binary outcome for each fecal sample (presence or absence of the *tet* genes) and our experimental unit was pen.

2.4.2. Quantification of resistance genes

Multilevel mixed-effects linear regression models were used to assess the effects of the two treatments and sampling day on three outcomes: (1) absolute (non-standardized) gene copy numbers per gram of feces; (2) standardized quantities of the genes normalized to the total DNA concentration in the original fecal samples; and (3) relative quantities of *tet*A to *tet*B per gram of feces. The models were three-level hierarchical models in which individual gene copy readings (our unit of analysis) were nested within fecal sample (duplicate reactions per sample), which in turn were nested within pen (our experimental unit). Each reaction reading from qPCR was used as the unit of analysis. On each sampling day, fecal samples were collected from three pigs among the five pen mates to ensure balanced data for each pen by accounting for any unforeseen within trial mortality; therefore, pig was not included as a random effect in the models. The models included the random effects of duplicate reactions per sample and pen, and fixed effects of treatments, sampling day, and their interactions.

To investigate the relative abundance of *tet*A and *tet*B in the fecal samples, the ratio of *tetA* to *tet*B was derived by dividing *tet*A gene copies by *tet*B gene copies present in 1 g of feces. We have previously reported that the prevalence of *tet*A was significantly higher than that of *tet*B from *E. coli* isolates characterized from the fecal samples (Agga et al., 2014). Gene copies were normalized by dividing gene copies per μl to the initial DNA concentration (expressed as ng/μl) and then were subsequently $\log_{10}$ transformed. Three reactions for *tet*A and 11 reactions for bla_{CMY-2} had no CT values. In each case, only one of the duplicate readings had no CT value, thus, it did not yield a negative

result at the sample level. To deal with missing observations, these observations were initially assigned a value of zero, while for later analyses we added half the value of the lowest nonzero value observed in the samples to all observations (following Boyer et al., 2013). The estimated gene copy numbers in each reaction were back-calculated to gene copies per gram of wet feces for each sample, which was then transformed to $\log_{10}$ to achieve normality. Multivariate multiple linear regression models were used to simultaneously model the four resistance genes (*tet*A, *tet*B, bla_{CMY-2} and *pco*D) together to account for their biological dependencies; that is, the genes were quantified from the same fecal sample arising from pigs under the same treatment and period effects. Pairwise Pearson correlations between resistance genes with Bonferroni-adjusted significance tests to adjust for multiple comparisons were calculated, and principal component analysis (PCA) plots were generated to display these relationships.

3. Results

3.1. Prevalence of tetracycline resistance genes

Three genes, *tet*D, *tet*K, and *tet*S, were not detected in any of the fecal samples, while *tet*E (from two control pens during the pre-treatment period) and *tet*G (one sample from a control and a copper pen during the treatment period) were each detected in only two fecal samples. In contrast, *tet*O, *tet*Q, and *tet*X genes were detected in 100% of the fecal samples, regardless of the treatment group or period. The prevalence and distribution of the remaining six genes (*tet*A, *tet*B, *tet*C, *tet*L, *tet*M and *tet*P), cross tabulated by treatment group and sampling period, are shown in Table 3. Since regression models for most of the individual *tet* genes failed to converge, as a result of either gene scarcity or saturation, only *tet*A, *tet*B and *tet*P were modeled beyond these descriptive analyses.

3.2. Multilevel mixed-effects logistic regression analysis for the prevalence of tetA, tetB and tetP

Results of individual multilevel mixed-effects logistic regression analysis for *tet*A, *tet*B, and *tet*P analyses are shown in Fig. 1. The single three way (CTC × copper × sampling period) and all two way interactions (CTC × copper, CTC × sampling day and copper × sampling day) were non-significant ($P > 0.05$). Overall, CTC supplementation was significantly associated with increased detection of *tet*A (OR = 1.7 [95% CI = 1.1–2.8]). The detection of both *tet*A and *tet*B decreased significantly ($P < 0.001$) across the sampling periods. In contrast, the detection probability for *tet*P demonstrated a significant ($P < 0.05$) increasing trend across sampling periods.

Across all treatment groups and sampling days, unadjusted prevalence of *tet*A (77% [95% CI = 74–81%])

Table 3
Prevalence (95% CI) of the various *tet* genes detected by multiplex PCR from total community DNA obtained from fecal samples of pigs fed diets supplemented with chlortetracycline (CTC), copper, both or neither.

tet genes	Treatment group	Treatment period		
		Before ($n = 24$)[a]	During ($n = 72$)	After ($n = 48$)
*tet*A	Control	91.3 (72.0–98.9)	68.1 (56.0–78.6)	68.8 (53.7–81.3)
	CTC	95.5 (77.2–99.9)	73.6 (61.9–83.3)	77.1 (62.7–88.0)
	Copper	95.2 (76.2–99.9)	73.6 (61.9–83.3)	62.5 (47.4–76.0)
	Copper + CTC	100 (85.8–100)	83.1 (72.3–91.0)	81.3 (67.4–91.1)
*tet*B<	Control	87.0 (66.4–97.2)	63.9 (51.7–74.9)	35.4 (22.2–50.5)
	CTC	100 (84.6–100)	51.4 (39.3–63.3)	41.7 (27.6–56.8)
	Copper	90.5 (69.6–98.8)	66.7 (54.6–77.3)	35.4 (22.2–50.5)
	Copper + CTC	95.8 (78.9–99.9)	46.5 (34.5–58.7)	45.8 (31.4–60.8)
*tet*C	Control	95.7 (78.1–99.9)	100 (95.0–100)	100 (92.6–100)
	CTC	100 (84.6–100)	95.8 (88.3–99.1)	100 (92.6–100)
	Copper	85.7 (63.7–97.0)	100 (95.0–100)	100 (92.6–100)
	Copper + CTC	95.8 (78.9–99.9)	95.8 (88.1–99.1)	100 (92.6–100)
*tet*L	Control	95.7 (78.1–99.9)	100 (95.0–100)	100 (92.6–100)
	CTC	100 (84.6–100)	98.6 (92.5–100)	100 (92.6–100)
	Copper	100 (83.9–100)	100 (95.0–100)	100 (92.6–100)
	Copper + CTC	100 (85.8–100)	100 (94.9–100)	100 (92.6–100)
*tet*M	Control	100 (85.2–100)	100 (95.0–100)	97.9 (88.9–99.9)
	CTC	100 (84.6–100)	100 (95.0–100)	97.9 (88.9–99.9)
	Copper	100 (83.9–100)	97.2 (90.3–99.7)	100 (92.6–100)
	Copper + CTC	100 (85.8–100)	95.8 (88.1–99.1)	97.9 (88.9–99.9)
*tet*P	Control	39.1 (19.7–61.5)	59.7 (47.5–71.1)	95.8 (85.7–99.5)
	CTC	31.8 (13.9–54.9)	66.7 (54.6–77.3)	79.2 (65.0–89.5)
	Copper	14.3 (3.0–36.3)	73.6 (61.9–83.3)	89.6 (77.3–96.5)
	Copper + CTC	37.5 (18.8–59.4)	85.9 (75.6–93.0)	81.3 (67.4–91.1)

[a] Number of fecal samples tested per treatment group. Ranged from 22 to 24 (pretreatment period), 71 to 72 (treatment period), and was constant at 48 post-treatment period.
95% confidence interval (CI) is an exact confidence interval based on binomial distribution.

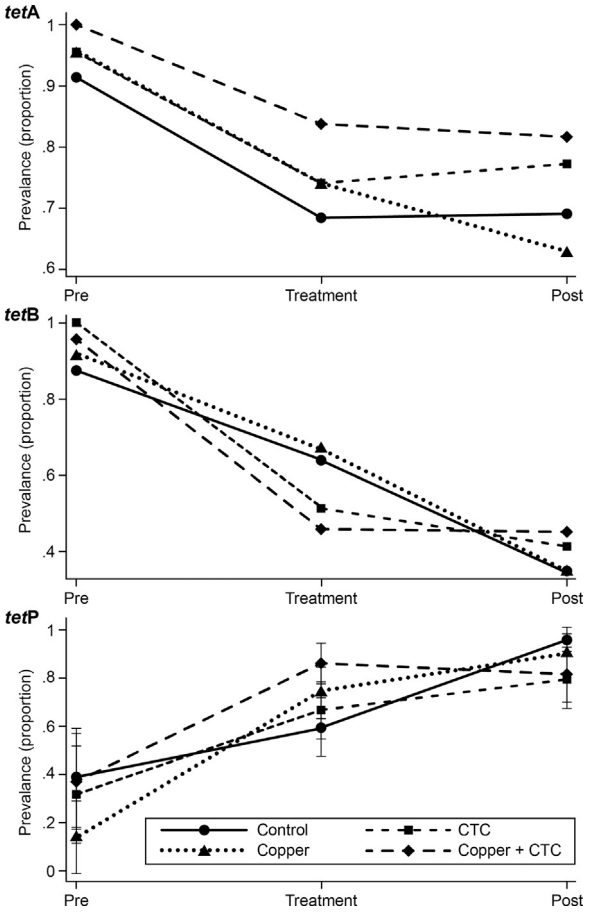

Fig. 1. Model adjusted prevalence for the detection of *tet*A, *tet*B, and *tet*P from fecal samples of pigs. Results are shown by treatment groups (chlortetracycline (CTC), copper, both, or neither) and by treatment periods (pre-treatment, during treatment and post-treatment). The model was unable to return the 95% confidence interval (CI) for *tet*A and *tet*B due to their 100% prevalence in the CTC + copper, and CTC group respectively in the pre-treatment period. *tet*P graph is shown with 95% CI.

was significantly higher than that of *tet*B (57% [95% CI = 53–61%]). The prevalence of *tet*A was significantly ($P < 0.05$) higher than that of *tet*B in the post-treatment samples, except for within the CTC-supplemented group. The prevalence of *tet*A (83% [95% CI = 72–91%]) was significantly higher than that of *tet*B (46.5% [95% CI = 35–59%]) in the copper plus CTC-supplemented groups during the treatment period alone. Bivariate probit analysis of *tet*A and *tet*B showed that probability of detecting both genes together dropped significantly from pretreatment levels through the treatment phase. Adjusted for biological interdependences (and pen effect), a significant ($P < 0.001$) 3-way interaction was observed between sampling period, copper, and CTC supplementation for both genes. The combined supplementation of copper and CTC tended only to increase the probability of *tet*A detection, whereas it did not affect *tet*B when compared to the control group.

3.3. Analysis of multiple co-detected tet genes using ordinal logistic regression

The median number of *tet* genes detected per fecal sample was eight across all samples. The median number of *tet* genes detected per sample did not differ significantly ($P > 0.05$) among the treatment groups across all treatment periods. The number of genes detected per fecal sample ranged from 4 to 10 (control), 5 to 9 (copper), 6 to 9 (CTC), and 5 to 9 (copper plus CTC). The distributions of the number of *tet* genes detected per fecal sample is illustrated in Fig. 2. The frequency and distribution of the different *tet* gene combinations is listed in Table 4. Across all samples, 26 distinct *tet* gene combinations were observed. Control, CTC, and copper plus CTC groups each contained 13 unique genotypic profiles, whereas the copper group had 14 unique profiles. The top three most common genotypic profiles observed were of 8- and 9-gene combinations. Fecal samples obtained during the treatment period (proportional OR = 0.5 [95% CI = 0.3–0.7]) and post-treatment period (prop. OR = 0.5 [95% CI = 0.3–0.9]) tended

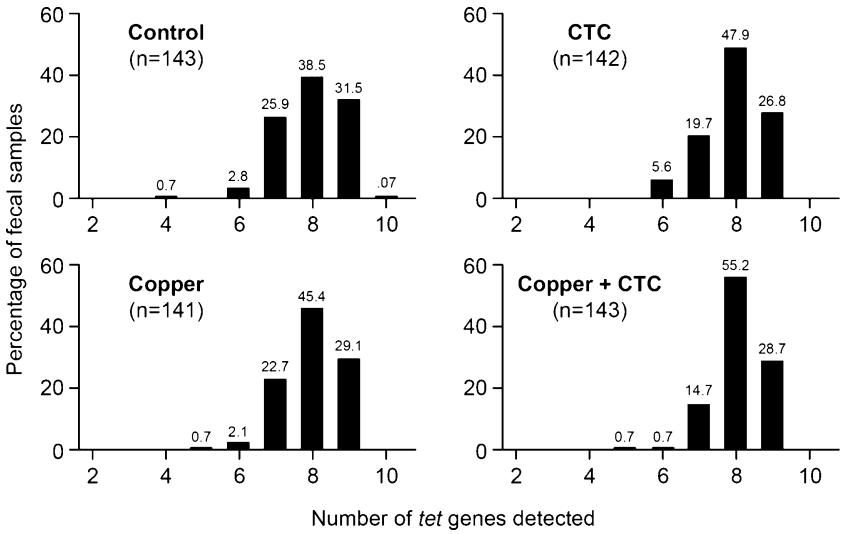

Fig. 2. Percentage of tetracycline resistance genes detected from a single fecal sample from pigs supplemented with chlortetracycline (CTC), copper, both, or neither.

Table 4
Frequency distribution of *tet* genes detected from fecal samples of pigs supplemented with chlortetracycline, copper, both or neither.

Genotypic profiles[b]	Treatment groups				Total (n = 569)
	Control[a] (n = 143)	CTC (n = 142)	Copper (n = 141)	Copper + CT (n = 143)	
a_b_c_e_l_m_o_p_q_x	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_b_c_e_l_m_o_q_x	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_b_c_g_l_m_o_q_x	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
a_b_c_l_m_o_p_q_x	43 (30.1)	38 (26.8)	40 (28.4)	41 (28.7)	162 (28.5)
a_b_c_l_m_o_q_x	24 (16.8)	27 (19)	26 (18.4)	27 (18.9)	104 (18.3)
a_b_c_l_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
a_b_c_l_o_q_x_	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.2)
a_b_c_m_o_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
a_b_l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	2 (1.4)	3 (0.5)
a_b_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
a_c_g_l_m_o_p_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_c_l_m_o_p_q_x_	24 (16.8)	32 (22.5)	26 (18.4)	44 (30.8)	126 (22.1)
a_c_l_m_o_q_x_	9 (6.3)	10 (7)	7 (5)	4 (2.8)	30 (5.3)
a_c_l_o_p_q_x_	0 (0)	0 (0)	1 (0.7)	2 (1.4)	3 (0.5)
a_l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	1 (0.7)	2 (0.4)
a_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
b_c_l_m_o_p_q_x_	7 (4.9)	7 (4.9)	12 (8.5)	6 (4.2)	32 (5.6)
b_c_l_m_o_q_x_	7 (4.9)	4 (2.8)	3 (2.1)	1 (0.7)	15 (2.6)
b_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
c_l_m_o_p_q_x_	21 (14.7)	12 (8.5)	20 (14.2)	12 (8.4)	65 (11.4)
c_l_m_o_q_x_	3 (2.1)	7 (4.9)	1 (0.7)	1 (0.7)	12 (2.1)
c_l_o_p_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
c_l_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
l_o_p_q_x_	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.2)
m_o_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)

[a] The distribution of the genes is given as the frequency (percentage calculated from the total number of fecal samples tested per treatment group).
[b] Lowercase letters represent the different *tet* genes (i.e., *tet*A, B, C, E, G, L, M, O, P, Q, X).

to have fewer numbers of *tet* genes, suggesting a decreasing trend in the diversity of *tet* genes detected in the pig fecal flora over time.

3.4. Quantification of resistance genes from fecal samples

Model-adjusted mean $\log_{10}$ copies per gram of feces for each of the four genes are depicted in Fig. 3. Mean $\log_{10}$ copies of *tet*A, *tet*B, and $bla_{\text{CMY-2}}$ decreased significantly ($P < 0.05$) over time; however, mean $\log_{10}$ copies of *pco*D increased significantly ($P < 0.001$) on days 7 and 14, returning to baseline following day 21. Mean *pco*D gene quantities were significantly higher in the control group than in the other treatment groups on day 14; however, *pco*D gene copies in the copper plus CTC group were significantly higher than the control samples on days 21 and 35 (Fig. 3).

Multivariate analysis (analyzing *tet*A, *tet*B, $bla_{\text{CMY-2}}$ and *pco*D genes together) yielded the same conclusions as the individual gene results, with small differences seen in the coefficients and the *P*-values. The independent variables (copper, CTC, day, and all possible interactions) were significant as a group ($F_{23,\,1112} = 9.89$, $P < 0.0001$). Following the multivariate regression, all pairwise correlations between residual values of the genes were positive except for *tet*A and *pco*D. The Breusch–Pagan test of independence was significant ($\chi^2_6 = 590.544$, $P < 0.0001$), indicating, as expected, that the residuals of the four genes were not independent from each other. Fig. 4 shows principal component analysis plot for the relationship between $\log_{10}$ gene copies of the resistance genes. The highest pairwise correlation was observed between *tet*A and $bla_{\text{CMY-2}}$ ($\rho = 0.66$) followed by pairwise correlation between *tet*B and $bla_{\text{CMY-2}}$ ($\rho = 0.57$).

Results of resistance gene copy analyses, with response variables adjusted to the initial DNA concentration of the total community DNA in the fecal sample, gave essentially the same results as the analysis based on non-standardized copies of genes, with some minor differences in the coefficients and *P*-values (data not shown).

Considering the relative ratios of *tet*A to *tet*B, the mean copies of *tet*A generally were higher than *tet*B copies. The *tet*A to *tet*B ratio was significantly different from unity on days 7 ($P = 0.015$) and 14 ($P = 0.006$) across all samples and treatment groups when compared with the baseline. Copper plus CTC supplementation drove *tet*A copies even higher on days 14 and 21 when compared to those of control and copper-only supplemented samples on the same days, respectively (Fig. 5).

4. Discussion

Antimicrobial resistance studies in pigs have heretofore focused largely on specific cultivatable bacterial species rather than the entire gut microbiota and have typically relied upon culture-based approaches (Mathew et al., 1998; Alali et al., 2008; Abley et al., 2012). However, a few culture independent approaches based on total community DNA also have been used to study the ecology of antimicrobial resistance genes in pigs (Aminov et al., 2001; Pakpour

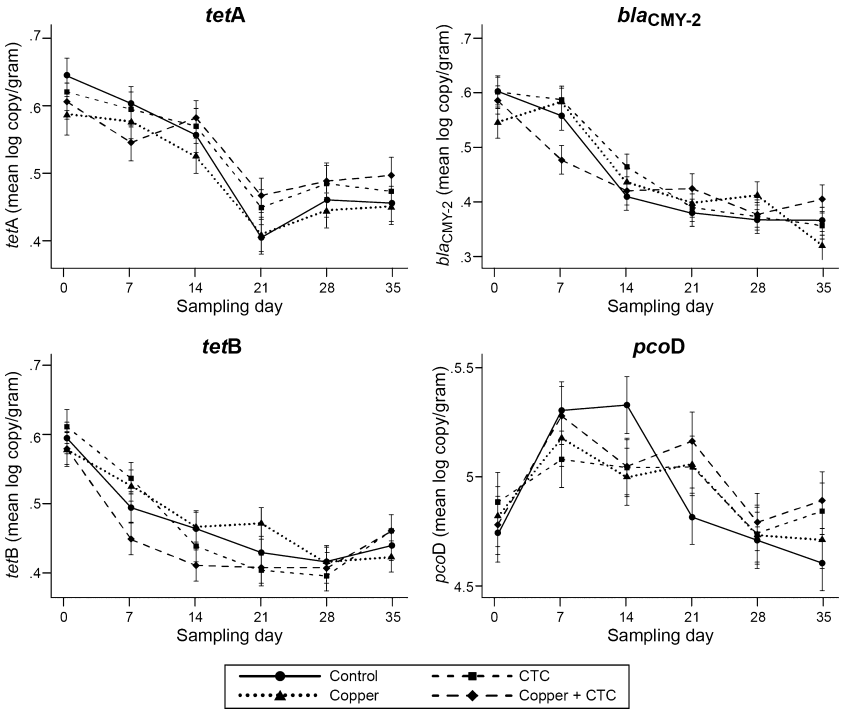

Fig. 3. Non-standardized mean $\log_{10}$ copies of resistance genes per gram of fecal sample from pigs.

et al., 2012). In this study, we experimentally investigated the effects of supplementation of therapeutic doses of CTC and copper over a 3-week period on the presence and quantities of tetracycline, 3rd generation cephalosporins (*bla*$_{CMY-2}$), and copper resistance elements in post-weaning pig feces.

4.1. Prevalence of tetracycline resistance genes

We investigated the impact of in-feed CTC and copper supplementation on the prevalence of 14 *tet* genes (out of a potential of 46 known genes); these particular genes were chosen based on previous detection reports from pig feces (Pakpour et al., 2012). We identified a large diversity of *tet* genes in the pig gut, including among control pigs in pens that were not directly exposed to CTC or copper. This result is consistent with previous reports (Aminov et al., 2001; Kazimierczak et al., 2009; Barkovskii and Bridges, 2012; Looft et al., 2012; Holman and Chenier, 2013) indicating high background tetracycline resistance in pigs; as such, this can make it difficult to compare and contrast the effects of treatment interventions based solely on presence or absence of a gene. Most of the *tet* genes were present either in 100% of the samples or were present at very low prevalence leading to zero cell frequency; consequently we were unable to analyze the impact of treatment on 11 genes out of the 14 *tet* genes targeted.

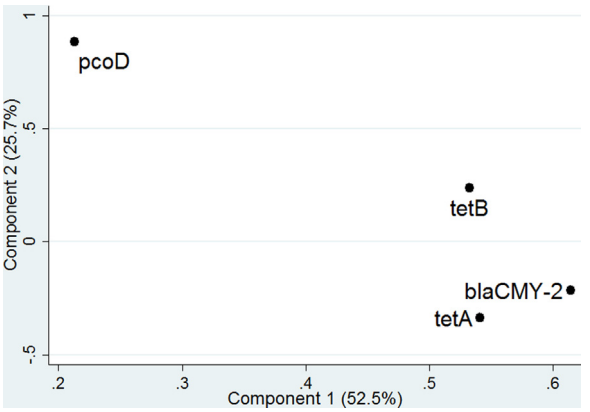

Fig. 4. Principal component analysis plot showing the relative association between *tet*A, *tet*B, *bla*$_{CMY-2}$ and *pco*D genes quantified from fecal samples of pigs supplemented with chlortetracycline or copper.

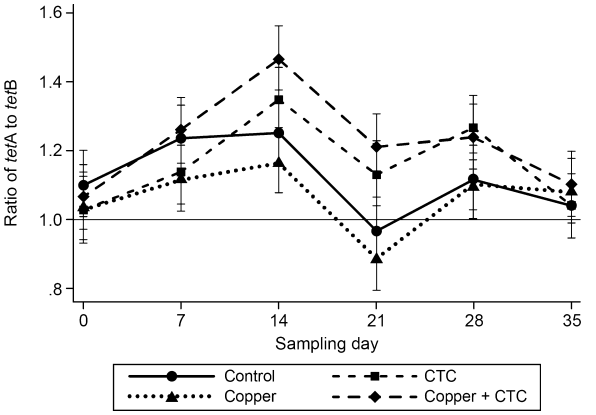

Fig. 5. Ratios of *tet*A to *tet*B genes quantified from the fecal samples of pigs supplemented with chlortetracycline and copper, graphed by treatment groups and sampling days. The horizontal line serves as reference at a ratio of 1.

The efflux genes *tet*A and *tet*B, generally reported only in Gram-negative enteric bacteria, are carried on large conjugative plasmids, which also can carry other antibiotic as well as heavy metal resistance genes. This obviously contributes to a widespread dissemination of multidrug-resistant enteric bacteria (Chopra and Roberts, 2001). The *tet*B gene has the widest host range in Gram-negative bacteria among the various *tet* genes. We observed a decrease in the prevalence of both *tet*A and *tet*B in fecal community DNA across subsequent sampling periods, which is likely to have been attributed to an aging effect of the pigs, regardless of prior or current treatments. On the other hand, we observed an upward trend in the detection probability for *tet*C and *tet*P across the same aging period of the pigs. This overall trend could be the result of a gradual shift in the underlying microbial population of the gut as pigs age and continue to consume more solid feedstuffs (Kim et al., 2011). This is supported by the increase, or more stable occurrence, of the predominantly Gram-positive *tet* genes (P, O, Q, L, and M) and a decrease in Gram-negative *tet* genes (A and B). Copper supplementation significantly increased the prevalence of *tet*P during the treatment period. *tet*P, a ribosomal protection gene, has previously been reported from *Clostridium* species, which tend to initially expand and then contract in numbers as pigs age (Norman et al., 2009; Kim et al., 2011).

Interestingly, in the bivariate analysis of *tet*A and *tet*B, the sole presence of *tet*A was significantly higher in the copper plus CTC-supplemented group during the treatment period compared with the control group. This result contrasted sharply with the prevalence of *tet*B-only detection which was significantly lowered compared to the control group. This result may suggest that CTC supplementation, either alone or in combination with copper, can favor *tet*A over *tet*B.

4.2. Quantification of resistance genes

Quantitative measurements in longitudinal studies of AMR resistance genes based on the entire fecal microbiome are crucial to better monitor the dynamics of AMR (Lowrance et al., 2007; Alali et al., 2009). To our knowledge, this is the first longitudinal study to quantitatively measure and directly compare the abundance of two *tet* genes (*tet*A and *tet*B), a 3rd generation cephalosporin resistance gene (bla_{CMY-2}), and a Gram-negative copper resistance determinant (*pco*D) among fecal samples obtained from pigs treated (or untreated) with copper and CTC. Log_{10} mean copies of *tet*A, *tet*B, and bla_{CMY-2} genes all decreased over time, whether standardized to DNA concentration or not. In some measure of contrast, *pco*D gene quantities increased at first during treatment, followed by a gradual decrease through the remaining sampling days. All of these declines in resistance elements are likely to be due to the aging effect of the pigs, as previously reported elsewhere (Langlois et al., 1988; Berge et al., 2010). This declining trend in gene copy numbers also could be attributed to changes in the underlying bacterial population, with Gram-positive bacteria gradually replacing Gram-negative bacteria as pigs move from post-weaning to grower stages of production (Kim et al., 2011). The four resistance genes we targeted exclusively confer resistance to Gram-negative bacteria, whereas the underlying bacterial population (as represented by the total community DNA) largely expands as a result of an increase in Gram-positive bacteria.

Supplementation with copper alone had opposing effects on the abundance of the *tet* genes as it tended to decrease gene copies of *tet*A but increase the levels of *tet*B (Fig. 3). Interestingly, we observed that copper and CTC supplementation significantly reduced the quantities of bla_{CMY-2} on the 7th day after treatment; however, as with *tet*A, when CTC was supplemented alone bla_{CMY-2} gene copies were higher than in the control group during the treatment period (Fig. 3). Paradoxically, the log_{10} mean copies of *pco*D in the control group were significantly higher than the copper treatment groups on day 14 (Fig. 3). The *pco*D gene copies remained more or less constant through time in the entire treatment group. This finding remains unexplained; however, it may be that the advantages conferred by the copper resistance gene for Gram negative bacteria (*pco*) versus that for Gram positive bacteria (*tcr*B) are of a lesser magnitude, as has been shown in the minimum inhibitory concentration (MIC) distributions in work by others (Hasman and Aarestrup, 2002; Amachawadi et al., 2011, 2013). Rouch and Brown (Rouch and Brown, 1997) earlier pointed out that *pco*-mediated copper resistance is an auxiliary mechanism that cooperates with the host bacterial cell copper management systems; in essence, it can modestly extend the range of environmental copper concentrations over which the bacterial cell can survive but does not provide a large leap in MIC with its presence.

Generally, the gene copies of *tet*A were several orders of magnitude higher than those of *tet*B and bla_{CMY-2}, indicating that, overall, the microbial population possesses more *tet*A. This result was also reflected in the analysis of the ratio of *tet*A to *tet*B detection, in which *tet*A concentration was generally much higher than that of *tet*B. Greater abundance of *tet*A from pig manure using qPCR absolute quantification was also earlier reported compared to that of the *tet*B gene (Yu et al., 2005). The high correlation between *tet*A and bla_{CMY-2} we observed could indicate that these two genes are genetically linked, as reported elsewhere (Call et al., 2010), and that 3rd generation cephalosporin resistance can be maintained and enhanced through co-selection by CTC supplementation in pig feed; therefore, in the absence of direct selective pressure from ceftiofur or other cephalosporins (Barbosa and Levy, 2000). We also previously reported the presence of high association between the prevalences of *tet*A and bla_{CMY-2} gene carriage in *E. coli* isolates obtained from the same study pigs (Agga et al., 2014).

In addition to the absolute quantification method, we also used a relative quantification method by normalizing resistance gene copies to the total DNA concentration (instead of, for example, a variable referent gene such as 16s rRNA) in the fecal sample. This method was also previously used by Holman and Chenier (Holman and Chenier, 2013) to investigate the impact of subtherapeutic doses of tylosin and CTC on *tet* and *erm* gene copies from pig fecal samples. Furthermore Alali et al. (2009) arrived at same conclusions by comparing both absolute gene copies and gene copies

normalized to the 16S rRNA gene. In our analysis, both non-standardized and standardized methods produced the same conclusions. This suggests that the treatments did not perturb the fecal flora as a whole, yielding roughly the same denominator. Theoretically, a total fecal community DNA approach does not differentiate whether bacterial DNA is from live or dead bacteria at the time of analysis; although this is of little consequence for studying gut microflora, it could have an impact on studies that extend into the farm environment. The AMR gene pools can serve as a constant source of horizontal gene transfer among bacterial population sharing the same ecology (Marshall et al., 2009).

The major drawback of the total community approach is that it is impossible to attribute the resistance genes to particular bacteria. In essence, this makes such analysis of a classically ecological nature, albeit in an opposite (micro) direction than the usual analyses, which are typically macro-ecological and well-documented in the epidemiological and even in the sociological literature. Thus, the approach is subjected to a unique form of bias known as the "ecological fallacy," in which an inference made at the aggregated level (in this study of genes interpreted at pen or sample level) should not be assumed to apply at the disaggregated level (e.g., the bacteria level). Such an approach gives an overall picture of the resistance genes in the gut at a micro-ecological level, and this may be used for evaluating the impact of growth promoters in agriculture. It is not a stand-alone approach; rather, it should be relied upon to supplement other approaches such as isolate-based analysis to avoid making inappropriate policy decisions on the basis of a single outcome measure.

5. Conclusions

This is the first experimental study to investigate the individual and combined effects of CTC and copper supplementation on the presence of *tet* genes and on the quantities of tetracycline and cephalosporin resistance genes in the gut microbial ecology of pigs. In general, *tet* genes were diverse in the total gut bacterial community of the study pigs; importantly, copper and CTC supplementation differentially expanded several of the resistance genes studied. These results suggest that such supplementation may favor expansion of certain genes, perhaps at the expense of other gene targets, especially during the treatment period. Our study indicated that in-feed CTC supplementation is associated with increased quantities of *tet*A and $bla_{\text{CMY-2}}$ genes. The presence of strong correlation between quantities of *tet*A and $bla_{\text{CMY-2}}$ genes, coupled with common practice of tetracycline use in swine production, would indicate that 3rd generation cephalosporin resistance can be maintained in swine population through genetic linkage with *tet*A even in the absence of cephalosporin use. Copper supplementation in swine production, on the other hand, was associated with lowered $bla_{\text{CMY-2}}$ gene copies; and it was not associated with increased *pco*D gene quantity. Copper supplementation as an alternative to antibiotics in swine production and the actual role of *pco*D as copper resistant determinant in the gut microbial community of pigs should be further elucidated.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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