Science of the Total Environment 889 (2023) 164183

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Limited impacts of high doses of dietary copper on the gut bacterial metal resistome explain negligible co-selection of antibiotic resistance

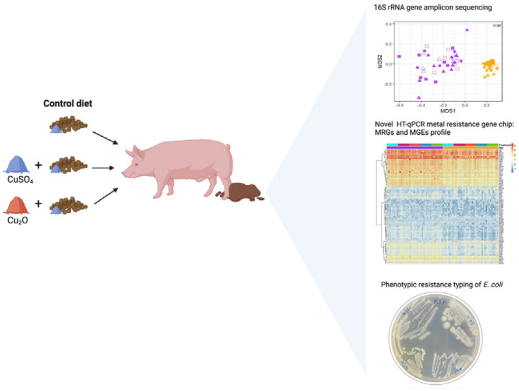
Asal Forouzandeh [a,b], Simon Bo Lassen [a], Julius Emil Brinck [a,i], Yan-Yan Zhou [c], Jiaojiao Zhu [d,e], David Solà-Oriol [b], Alessandra Monteiro [f], Xiuli Hao [d,e], Jian-Qiang Su [d], Hans H. Stein [g], J. Francisco Pérez [b], Kristian K. Brandt [a,h,*]

[a] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[b] Animal Nutrition and Welfare Service (SNiBA), Department of Animal and Food Sciences, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain
[c] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[d] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China
[e] Hubei Key Laboratory of Soil Environment and Pollution Remediation, College of Resources and Environment, Huazhong Agricultural University, Wuhan, China
[f] Animine, Annecy 74960, France
[g] Department of Animal Sciences, University of Illinois, Urbana 61801, USA
[h] Sino-Danish Center (SDC), University of Chinese Academy of Sciences, 380 Huaibeizhuang, Beijing, China
[i] National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

HIGHLIGHTS

- Pig gut microbiomes from a controlled high-dose Cu feeding trial were studied.
- The pig gut bacterial metal resistome was studied using a novel HT-qPCR chip.
- Only minor Cu impacts on gut bacterial community composition and metal resistome
- Cu selected for Cu resistance in *E. coli*, but not for Cu resistance genes (HT-qPCR)
- Our results explain why dietary Cu did not co-select for antibiotic resistance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Fang Wang

Keywords:
Antibiotic resistance
Bacterial community assembly
Co-selection
Escherichia coli
Gut microbiome
Metal resistance

ABSTRACT

High dietary intake of Cu has previously been linked to the selection of Cu resistance and co-selection of antibiotic resistance in specific gut bacteria. Based on a novel HT-qPCR metal resistance gene chip as combined with 16S rRNA gene amplicon sequencing and phenotypic resistance typing of *Escherichia coli* isolates, we here report the impacts of two contrasting Cu-based feed additives on the swine gut bacterial metal resistome and community assembly. DNA was extracted from fecal samples (n = 80) collected at day 26 and 116 of the experiment from 200 pigs allotted to five dietary treatments: negative control (NC) diet with 20 μg $CuSO_4$ g^{-1} and four diets added 125 or 250 μg $CuSO_4$ g^{-1} feed or 125 or 250 μg Cu_2O g^{-1} feed to the NC diet. Dietary Cu supplementation reduced the relative abundance of *Lactobacillus*, but it had negligible impacts on bacterial community composition relative to the gut microbiome maturation effect (time). The relative importance of different bacterial community assembly processes was not markedly affected by the dietary Cu treatments, and differences in swine gut metal resistome composition could be explained primarily by differences in bacterial community composition rather than by dietary Cu treatments. High dietary Cu

* Corresponding author at: Section for Microbial Ecology and Biotechnology, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark.
E-mail address: kkb@plen.ku.dk (K.K. Brandt).

http://dx.doi.org/10.1016/j.scitotenv.2023.164183
Received 20 February 2023; Received in revised form 10 May 2023; Accepted 11 May 2023
Available online 16 May 2023

intake (250 μg Cu g^{-1}) selected for phenotypic Cu resistance in *E. coli* isolates, but surprisingly it did not result in increased prevalence of the Cu resistance genes targeted by the HT-qPCR chip. In conclusion, the lacking impacts of dietary Cu on the gut bacterial metal resistome explain results from a previous study showing that even high therapeutic doses of dietary Cu did not cause co-selection of antibiotic resistance genes and mobile genetic elements known to harbor these genes.

1. Introduction

Antibiotic growth promoters have been widely used in swine production for many years (Dibner and Richards, 2005), but this agricultural practice has been banned, or at least tightly regulated, in many countries during recent years due to risks for the development and transfer of antibiotic resistance between animals and humans (You and Silbergeld, 2014; Zhao et al., 2021). Metals such as zinc (Zn) and copper (Cu) have received considerable attention as alternative growth promoters in pigs due to their antimicrobial activities (Højberg et al., 2005). Zn is primarily supplemented for weaners, whereas Cu is widely used in fattener diets.

The physiological Cu requirement for swine is 5 to 6 μg Cu g^{-1} diet (NRC, 2012), and the maximum dosage authorized in the EU is 25 μg Cu g^{-1}, with an exception of 150 μg Cu g^{-1} in case of suckling and weaning pigs up to 4 weeks after weaning (European Commission, 2018). However, in many non-EU countries, the commercial practice generally uses therapeutic doses of Cu (150 to 250 μg Cu g^{-1}) in swine diets as a growth promoter. Numerous studies have confirmed the growth-promoting effect of Cu in swine production (Espinosa et al., 2017; Espinosa and Stein, 2021; Pérez et al., 2011). The underlying animal growth-promoting mechanisms of dietary Cu are not yet fully understood, but several modes of action have been proposed (Espinosa and Stein, 2021). Cu speciation influences the biological activity of Cu with Cu^{+} being more toxic than Cu^{2+} (Rensing et al., 2018; Popov et al., 2020). Although Cu speciation will be affected during animal gut passage, recent animal feeding trials have demonstrated that monovalent copper oxide (Cu_2O) showed superior effects on animal performance and gut microbiome composition as compared to copper (II) sulfate ($CuSO_4$) (Blavi et al., 2021; Forouzandeh et al., 2021; Hamdi et al., 2018).

Animal gut bacteria can easily develop resistance to Cu and other metallic growth promoters (e.g. zinc) via adaptive mutations or acquisition of metal resistance genes (MRGs) by horizontal gene transfer (Aarestrup and Hasman, 2004; Hasman et al., 2005, 2006). Metal resistance genes are commonly located on plasmids or other mobile genetic elements (MGEs) and the use of elevated dietary Cu diets in pigs can thus be associated with a risk for co-selection (co-resistance) of antibiotic resistance genes (ARGs) (Hasman et al., 2005; Yazdankhah et al., 2014). Co-selection of MRGs and ARGs may also happen by cross-resistance or co-regulation (Baker-Austin et al., 2006). We recently reported the first controlled study investigating the impact of two contrasting Cu-based feed additives (growth promoters) on the overall swine gut microbiome and antibiotic resistome and found no evidence for the co-selection of ARGs (Brinck et al., 2023). However, we did not infer if the high-Cu diets (250 μg Cu g^{-1} feed) were indeed high enough to directly select for Cu resistance in the gut bacteria or whether co-selection of Cu and antibiotic resistance could still have happened in specific culturable bacterial species within the microbiome. Understanding these issues is important for a more complete characterization of the risks for Cu-induced co-selection of ARGs in pigs.

Very recently, a novel HT-qPCR metal resistance gene chip (MRG chip) has been made available to the scientific community (Zhu et al., 2022). The MRG chip allows for comprehensive quantification of known prokaryotic Cu resistance genes (CRGs) and other MRGs, and we, therefore, decided to make a follow-up study using frozen fecal matter samples from our previous studies (Blavi et al., 2021; Brinck et al., 2023). Compared to our previous antibiotic resistome study targeting ARGs (Brinck et al., 2023), we this time used the MRG chip to study the ability of dietary Cu to select for CRGs and other MRGs thereby shaping the gut bacterial metal resistome. In addition, we looked into the impacts of dietary Cu on the bacterial community assembly processes shaping the pig gut microbiome using iCAMP (Ning et al., 2020) and we used a cultivation-dependent approach targeting *Escherichia coli* to complement the HT-qPCR ARG data from our first study. Specifically, we generated the following hypotheses for our study: high-Cu diets (125 or 250 μg Cu g^{-1} feed) will affect bacterial community composition, diversity and underlying community assembly processes as revealed by 16S rRNA gene amplicon sequencing; high-Cu diets will increase the abundance of known genes that have been associated with Cu resistance in gut bacteria; high-Cu diets will co-select for genes conferring resistance to other metals; high-Cu diets will select for phenotypic Cu resistance in *E. coli* isolates; Cu resistant *E. coli* isolates are more resistant to contrasting classes of antibiotics than corresponding Cu susceptible isolates are (i.e. co-selection of Cu and antibiotic resistance).

2. Materials and methods

2.1. Animal management and samples collection

Comprehensive information about the pig feeding trial with 200 growing pigs has been described by Blavi et al. (2021). In brief, the study involved 6-weeks old growing pigs with an average initial body weight of 11.5 ± 0.98 kg that were assigned to a randomized complete block design. Pig fecal matter was obtained on day 26 and 116 of the experiment (direct sampling from the rectum; 8 fecal samples per treatment group) as described previously (Brinck et al., 2023). Sampling time points were selected to get data on the effects of dietary Cu both during the initial animal growth stage (day 26) and shortly before animals would normally be sent for slaughter (day 116), when microbiomes were suspected to be most selected by the dietary Cu treatments. Fecal samples were immediately placed into liquid nitrogen and stored at −80° C. The dietary treatments included a negative control (NC) diet with 20 μg Cu g^{-1} ($CuSO_4$), as well as four diets added 125 μg Cu g^{-1} feed ($CuSO_4$ or Cu_2O) or 250 μg Cu g^{-1} feed ($CuSO_4$ or Cu_2O) on top of the NC diet. For short, the dietary Cu treatments were named 125 $CuSO_4$, 250 $CuSO_4$, 125 Cu_2O, and 250 Cu_2O, respectively. Concentrations of total Cu in the fecal samples were measured after 116 days in all treatments as described previously (Blavi et al., 2021) and amounted to (means ± standard deviations) 295 ± 74, 1531 ± 254, 2900 ± 438, 1404 ± 158, and 2356 ± 357 μg g^{-1} dry wt for the NC, 125 $CuSO_4$, 250 $CuSO_4$, 125 Cu_2O, and 250 Cu_2O treatments, respectively. Bioavailable Cu was determined after 116 days by a whole-cell bacterial bioreporter assay with values being reported previously (Brinck et al., 2023); i.e. below quantification limit of 7×10^{-4}, 0.47 ± 0.25, and 0.70 ± 0.50 μg g^{-1} for the NC, 250 $CuSO_4$, and 250 Cu_2O treatments, respectively.

2.2. Fecal DNA extraction

DNA was extracted from 250 mg thawed fecal samples ($n = 80$) using the DNeasy PowerSoil Pro DNA Isolation Kit (Qiagen, Germany) following the manufacturer's instructions. Nanodrop-1000 spectrophotometer (ThermoFisher Scientific, USA) and Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) were used to assess the quality and quantity of the isolated DNA. DNA concentrations varied between 50 and 280 ng μL^{-1} for the fecal samples. All DNA extracts had A260/A280 ratios >1.8. Extracted DNA was stored at -80 °C until used for 16S rRNA gene amplicon sequencing and HT-qPCR array.

2.3. 16S rRNA gene amplicon sequencing and data processing

The primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V4 region of the bacterial 16S rRNA gene by PCR (Walters et al., 2016). At Novogene Bioinformatics Technology Co. Ltd. (UK), a small-fragment library was created, followed by paired-end sequencing with the Novaseq PE250. The DADA2 pipeline (Callahan et al., 2016) in R version 4.0.5 was used to process the 16S rRNA gene amplicon sequences. Filtering and trimming of the reads were done using the default settings of DADA2. The paired-end reads were subsequently combined, and an amplicon sequence variant (ASV) database was built using the core sample inference algorithm and trained error models of DADA2 followed by the removal of chimeras. The taxonomy of the ASVs was assigned using the Silva 138.1 prokaryotic SSU taxonomic training data (Quast et al., 2013) formatted for DADA2. A phylogenetic tree was constructed based on the ASVs by using MAFFT (Katoh et al., 2002) and FastTree (Price et al., 2010). For downstream analysis, ASVs identified as mitochondrial DNA or chloroplast DNA were removed, and all samples were rarefied to an even sampling depth of 62,000 which was the minimum number of reads in all samples resulting in a total of 4164 ASVs.

2.4. High-throughput qPCR and data processing

High-throughput qPCR reactions were performed by the Wafergen SmartChip Real-time PCR system (ThermoFisher, USA), using a total of 86 validated primer sets targeting MRGs associated with Ag, Cu, Hg, Ni, Zn, Zn/Cd/Co/Pb, and MDR as well as MGEs (Table S1) according to Zhu et al. (2022). Out of the 86 gene targets assayed, 84 were detected targeting 45 MRGs and 8 MGEs. The detected MRGs were mostly associated with Zn/Cd/Co/Pb (17 primers targeting 9 genes), Cu (14 primers targeting 11 genes), and Zn (14 primers targeting 8 genes; Fig. S1A). Efflux pump, gene regulation, and uptake prevention were the most prevalent resistance mechanisms among all MRGs (91 %; Fig. S1B) and the detected MGEs only encoded integrases (4 primers) and transposases (9 primers; Fig. S1C).

All qPCR reactions were carried out in technical triplicates, with a non-template control included in each run. A threshold cycle (C_T) of 31 was used as the detection limit for the individual PCR reactions. C_T values higher than 31 were set to 0 and only genes detected in all triplicates were regarded as positive. Relative gene copy numbers were calculated with the formula: relative gene copy numbers $= 10^{(31\text{-}Ct)/(10/3)}$ as described previously (Looft et al., 2012), where Ct refers to HT-qPCR results. HT-qPCR data were normalized among samples by dividing the relative gene copy numbers by the corresponding 16S rRNA gene copy numbers.

2.5. Isolation and characterization of bacteria

In order to compare our data to our previous HT-qPCR ARG study (Brinck et al., 2023), bacterial isolates were exclusively obtained from fecal samples ($n = 24$) collected from the NC, 250 $CuSO_4$, and 250 Cu_2O treatments at the end of the experiment (d 116). Fecal samples were extracted by shaking 1 g of feces with 10 mL of 0.9 % sterile NaCl for 1 h on a rotary shaker (150 rev per min, 25 °C). Relevant dilutions of the liquid phase were prepared and spread in triplicates on Reasoner's 2A (R2A) agar plates (Reasoner and Geldreich, 1985). R2A medium was used, as the isolation campaign was part of a larger study also involving cultivation of bacteria from different aquatic and terrestrial environments. R2A plates were incubated for 3 d at 25 °C. After incubation, isolates ($n = 462$) were randomly picked from the plates and purified twice by re-streaking to obtain pure colonies. The isolates were stored at −80 °C in R2A containing 30 % (v/v) glycerol.

DNA was first extracted from 95 random isolates (treatments: NC, 250 $CuSO_4$, and 250 Cu_2O) grown on R2A agar plates using the Q-Extract DNA Extraction Solution (AMPLIQON, Odense, Denmark) according to the instruction manual. Isolates were subsequently identified by Sanger sequencing, which was conducted at Eurofins Genomics (Germany) using the 27F 16S rRNA gene primer. Most isolates were identified as *E. coli* (Table S2), and this species was therefore chosen as an indicator organism for our study. To identify *E. coli* isolates among the remaining isolates ($n = 462$), all were cultivated on *E. coli*-specific m-TEC ChromoSelect agar plates (Sigma-Aldrich, Germany) and incubated at 37 °C for 22–24 h. To verify the specificity of the m-TEC ChromoSelect agar, ten of the isolates capable of growing on the medium were randomly selected for Sanger sequencing and they were all identified as *E. coli*. Sixty-one percent of the 462 isolates were in this way identified as *E. coli* ($n = 284$; Fig. S2A). Out of the identified *E. coli* isolates, 17.6 % were from the NC group, whereas 36.3 % and 46.1 % belonged to the 250 $CuSO_4$ and 250 Cu_2O treatments, respectively (Fig. S2B).

2.6. Phenotypic determination of Cu and antibiotic resistance

All *E. coli* isolates ($n = 284$) were typed for Cu and antibiotic resistance as described previously (Berg et al., 2010). In brief, all isolates were cultivated on R2A agar plates containing either $CuSO_4$ (1 mM), ampicillin (32 mg/L), chloramphenicol (32 mg/L), colistin (64 mg/L), nalidixic acid (32 mg/L), streptomycin (32 mg/L), and tetracycline (16 mg/L). Resistance to the compounds was scored by visual growth inspection. The antibiotics were selected to represent contrasting classes of antibiotics with different modes of action. Antibiotics were obtained from VWR chemicals (Pennsylvania, USA). Isolates were defined as multidrug-resistant (MDR) when they were resistant to at least two of the screened antibiotics.

2.7. Statistical analyses

R version 4.0.5 (2021 − 03 − 31) was used for data exploration, graphics, and all statistical analyses. Bacterial community composition was examined with "microeco" version 0.10.1 and "ggplot2" version 3.3.3 R packages (Liu et al., 2021; Wickham, 2016). Alpha diversity of samples was displayed with Chao1 and Shannon measures, and statistical differences between treatment groups and time points were assessed by the Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) combined with Dunn's test for Multiple comparisons. Beta diversity of samples was displayed using Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity metrics. Differences in bacterial community composition among treatment groups and time points were investigated by perMANOVA. Differential abundance of ASVs was assessed among the different treatment groups applying analysis of compositions of microbiomes with bias correction with default settings (ANCOM-BC; Lin and Peddada, 2020).

The relative importance of different bacterial community assembly processes was investigated by iCAMP (Infer Community Assembly Mechanisms by Phylogenetic-bin-based null model analysis) as described previously (Ning et al., 2020). ASVs were initially divided based on their relative abundance. ASVs constituting <0.1 % in all samples were considered "rare", whereas ASVs with a relative abundance >1 % in one or more samples were considered "abundant"; remaining ASVs were classified as "intermediate" (Zhang et al., 2020). Dispersal limitation, homogenizing dispersal, and drift are primarily regarded as stochastic processes according to iCAMP null model theory (Zhou and Ning, 2017).

To examine the potential effects of Cu on the bacterial community structure and MRG/MGE composition, data from pigs with the same treatment and sampling time point were aggregated. MRG/MGE profiles were analyzed using the "vegan" R package version 2.6–2 (Oksanen et al., 2022). The function *adonis* was used to analyze variations in MRG/MGE compositions across treatment groups and time points using permutation multivariate analysis of variance (perMANOVA; Anderson, 2001). NMDS ordination using Bray-Curtis dissimilarity index was used to display dissimilarities of MRG/MGE compositions across treatments and time points. The "pheatmap" R package version 1.0.12 was used to create a heatmap of relative gene abundances. Significant differences in the relative abundance of MRG or MGE classes and also in individual genes were assessed by a one-way Analysis of Variance (ANOVA) test using the "dplyr" R package version

1.0.8. The *protest* function was used to complete the Procrustes test for correlation analysis between bacterial communities and MRG/MGE composition based on NMDS ordinations.

Logistic regression with binomial distribution using the generalized linear model (GLM) function *glm* was used to do an ANOVA-type analysis of Cu and antibiotics resistance in isolates using treatment as a distinguishing factor. The model was subsequently compared against a model not distinguished by treatment and the significance of the models was tested using the Chi-square test.

3. Results

3.1. Impacts of high Cu diets on gut bacterial community composition and assembly

After quality filtering, the 80 fecal samples yielded a total of 6,166,891 paired-end 250-bp sequences, ranging between 62,280 and 96,631 sequences per sample. After rarefication, the 4164 ASVs that were formed from the sequences were allocated to 25 distinct phyla and 463 genera. *Firmicutes* comprised the dominant phylum among all treatments and time points presented at a mean relative abundance of 91.5 % followed by *Bacteroidota,* and *Euryarchaeota* both with a mean relative abundance of 2.5 % (Fig. S3). *Clostridium* sensu stricto *1* was the most abundant genus representing up to 21.5 % of all ASVs at the first time point and 47.10 % at the second time point (Fig. S4).

Overall, bacterial community composition and diversity were affected primarily by time (gut microbiome maturation effect), whereas only minor impacts of the dietary Cu treatments were observed. Hence, none of the dietary Cu treatments influenced species richness (Chao1) or Shannon diversity index suggesting that ASV-level alpha diversity of the gut bacterial community was not affected by the dietary Cu treatments (Fig. S5AB). By contrast, the richness and diversity of the gut bacterial communities decreased as pigs aged (Kruskal-Wallis and Dunn's test, $P < 0.01$; Fig. S5C). Likewise, the bacterial community composition was highly affected by time (*perMANOVA*, $R^2 = 0.52$, $P < 0.001$; Fig. 1) with only minor impacts of dietary Cu treatments. No clear overall impact of dietary treatments on the bacterial community composition was observed at the first time point (d 26; perMANOVA, $R^2 = 0.12$, $P = 0.224$; Fig. S6), but minor impacts were observed at the second time point as visualized in the NMDS ordination plot (d 116; *perMANOVA*, $R^2 = 0.19$, $P < 0.01$; Fig. S6).

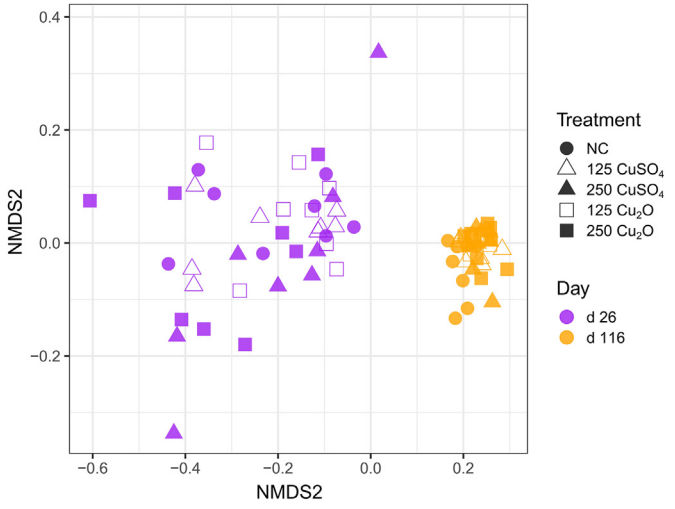


Fig. 1. Differences in bacterial community composition as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity index across all fecal samples ($n = 80$) based on the relative abundance of amplicon sequence variants (ASVs). Communities grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}), and by time (day): 26 (purple dots), and 116 (orange dots).

Dietary Cu treatments altered the relative abundance of several genera (e.g. *Ligilactobacillus*, *Oscillospira*, *Prevotella*, *Roseburia*, *Schwartzia*, and *Streptococcus*) compared to the NC after 26 days (ANCOM-BC, $P < 0.05$; Fig. S7). However, only the relative abundance of *Dialister* and *Lactobacillus* was affected by the dietary treatments after 116 days (Fig. S7). The relative abundance of *Dialister* was higher in the 125 Cu_2O group compared with the NC, whereas the relative abundance of *Lactobacillus* was higher in the NC group compared with 125 Cu_2O and 250 $CuSO_4$ or Cu_2O groups (d 116; ANCOM-BC, $P < 0.05$).

We further explored the relative importance of different bacterial community assembly processes across different time points and treatments using iCAMP (Fig. 2). Overall, drift was the most important community assembly mechanism across all time points and dietary Cu treatments. Moreover, dispersal limitation and homogenous selection also constituted major community assembly processes after 26 days, whereas homogenizing dispersal became important for shaping the bacterial community composition after 116 days. The importance of drift was highest within the abundant fraction of the community, whereas it was less important among taxa belonging to the intermediate and rare fractions of the community. Drift, homogenous selection, and dispersal limitation were the most influential factors driving the changes in community assembly at the first time point (d 26) whereas at the second time point (d 116) drift was the most important process in community assembly of abundant taxa. Importantly, the different dietary Cu treatments had no clear impact on the relative importance of the different community assembly processes responsible for shaping the bacterial community.

3.2. Impacts of high Cu diets on MRGs/MGEs of gut microbiota

The composition of the metal resistome was primarily affected by time (gut microbiome maturation effect; *perMANOVA*, $P < 0.001$; Fig. 3), whereas it was not significantly impacted by dietary Cu at any of the time points (*perMANOVA*, $P > 0.1$; Fig. S8). Likewise, dietary Cu treatments did not markedly affect the number of detected MRGs (ANOVA, $P > 0.05$; Fig. S9A) or the relative abundance pattern of individual MRGs (Fig. 4). The cumulative relative abundance of all quantified MRGs was also similar across the dietary Cu treatments after both 26 and 116 days (ANOVA, $P > 0.1$; Fig. S9B). The relative abundance of MRGs (normalized to the corresponding 16S rRNA gene copy number) averaged 0.20 across all samples at the first time point (range: 0.04 - 0.7), while it averaged 0.07 at the second time point (range: 0.01 - 0.18).

By contrast, the dietary Cu treatments significantly affected the MGE profile (*perMANOVA*, $P < 0.05$; Fig. S10), although overall dietary Cu impacts were rather modest as indicated from the relative abundance pattern of individual MGEs (Fig. 4). The dietary Cu treatments affected the cumulative relative abundance of transposases after 26 days (ANOVA, $P = 0.0002$; Fig. S9D), but not after 116 days (ANOVA, $P > 0.01$). Overall, the cumulative relative abundance (day 26 and 116; normalized to the corresponding 16S rRNA gene copy number) of MGEs ranged from 0.02 to 0.3 with an average of 0.1. A more in-depth examination of individual MGEs revealed that dietary Cu treatments significantly impacted only the *IS1216* transposase gene after 26 days (ANOVA, $P = 0.016$; Fig. S11). Hence, the relative abundance of this gene was higher with Cu_2O (125 and 250 μg g^{-1}) supplementation as compared with 250 $CuSO_4$. Dietary Cu treatments did also not markedly affect the number of detected MGEs (ANOVA, $P > 0.05$; Fig. S9C).

The lacking or modest impacts of dietary Cu treatments on MRGs and MGEs were not surprising given that only a small subset of the gene targets have previously been indicated to confer resistance to Cu in bacteria. Hence, we specifically evaluated the impacts of dietary Cu on the relative abundance of 11 genes (*pcoC*, *copA*, *cusB*, *cutC*, *tcrA*, *cueO*, *cueR*, *cusR*, *cusS*, *tcrY*, and *pcoD*; targeted by 14 primers) previously shown to confer resistance to Cu (Fig. 5). The number of detected CRGs was similar across all treatments (ANOVA, $P > 0.1$; Fig. 5A). Remarkably, dietary Cu treatments did not affect the cumulative relative abundance of CRGs (ANOVA,

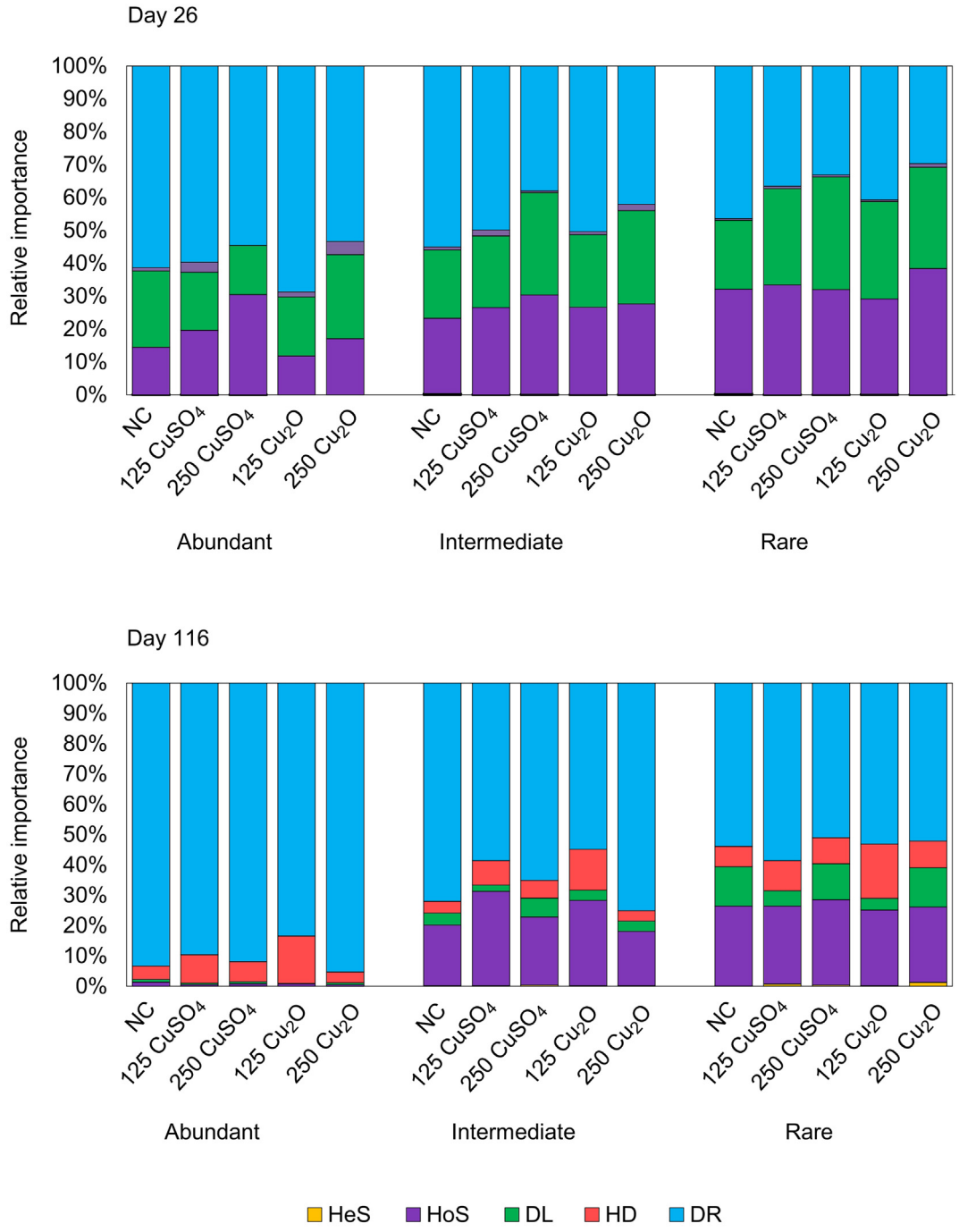


Fig. 2. Relative importance of bacterial community assembly processes based on the principle of the null models employed by iCAMP in response to dietary Cu treatments: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}). The relative importance of heterogenous selection (HeS), homogenous selection (HoS), dispersal limitation (DL), homogenizing dispersal (HD), and drift (DR) was determined separately for abundant, intermediate, and rare bacterial taxa.

$P > 0.1$; Fig. 5B), but the relative abundance of the *cueO* gene was decreased by dietary Cu treatments (only significant for 125 Cu_2O, 250 $CuSO_4$ and 250 Cu_2O treatments) relative to NC (ANOVA, $P < 0.001$; Fig. 6). Moreover, the cumulative relative abundance of CRGs was significantly lower after 116 days as compared to 26 days (ANOVA, $P < 0.05$).

3.3. Linkage between bacterial community composition and MRGs/MGE profiles

Procrustes analysis was performed to investigate whether MRG or MGE profiles could be linked to bacterial community composition (i.e. ASVs present and their relative abundance). MRG profiles showed a significant correlation with bacterial community composition (Procrustes sum of squares $M^2 = 0.54$, $R^2 = 0.68$, $P = 0.0001$, 9999 free permutations; Fig. S12A), whereas there was no significant correlation between MGE profiles and the bacterial community composition (Procrustes sum of squares $M^2 = 0.98$, $R^2 = 0.14$, $P = 0.33$, 9999 free permutations; Fig. S12B). However, Spearman's rank correlation analysis revealed correlations ($P < 0.05$; Fig. S13) among some bacterial genera and MRGs/MGEs within the swine gut microbiome. The most striking example was the co-occurrence of *Streptococcus* and the *IS1216* transposase gene with most entities being highly abundant in the two Cu_2O treatments after 26 days ($R = 0.69$, $P < 0.0001$; Fig. 7). The abundance of *IS1216* transposase gene was also

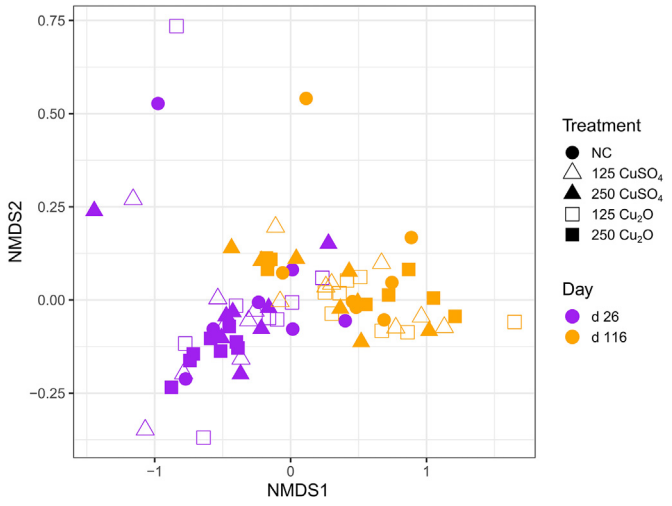


Fig. 3. Differences in the metal resistance genes (MRGs) composition of the gut microbiome as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity index across all fecal samples ($n = 80$) grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}), and by time (day): 26 (purple dots), and 116 (orange dots).

tightly correlated with *Streptococcus* after 116 days ($R = 0.67$, $P < 0.0001$), however, there was no significant difference between the treatments. The data from the second time point (day 116) revealed a remarkably tight correlation between the *cueO* gene and the genus *Lactobacillus* ($R = 0.83$, $P < 0.0001$; Fig. 7). The correlation was also evident from the first time point (day 26) with no variation among the treatments ($R = 0.92$, $P < 0.0001$).

3.4. Phenotypic resistance to Cu and antibiotics in E. coli isolates

The negligible impacts of dietary Cu on metal resistance gene profiles (HT-qPCR) prompted us to further investigate phenotypic Cu resistance in gut bacterial isolates. *E. coli* was chosen as our indicator species as most of the strains enriched and isolated on the used non-selective medium belonged to this species. Supplementation with high levels of dietary Cu selected for Cu resistance in fecal *E. coli* isolates compared to the NC treatment (GLM, 250 $CuSO_4$, $P < 0.001$; 250 Cu_2O, $P < 0.05$; Fig. 8A). Among the NC treatment ($n = 50$), only 24 % were Cu resistant, whereas corresponding numbers for the 250 $CuSO_4$ ($n = 103$) and 250 Cu_2O ($n = 131$) treatments were 72 % and 41 %, respectively. The fraction of Cu-resistant *E. coli* isolates was significantly higher for the 250 $CuSO_4$ group as compared to the 250 Cu_2O group (GLM, $P < 0.05$).

Contrary to our hypothesis, Cu resistant *E. coli* isolates were not more resistant to antibiotics as compared to Cu susceptible isolates (Fig. 8B). By contrast, Cu sensitive isolates were more resistant to ampicillin and streptomycin than corresponding Cu resistant isolates were (GLM, $P < 0.001$). A closer look at the results revealed that this observation could primarily be linked to isolates derived from the two dietary Cu treatments (Fig. S14). In particular, Cu sensitive isolates derived from the 250 Cu_2O group exhibited a high level of ampicillin and streptomycin resistance. Overall, supplementation of 250 Cu_2O, irrespective of Cu sensitivity, increased phenotypic resistance of *E. coli* isolates to ampicillin, and streptomycin compared with the NC and 250 $CuSO_4$ (GLM, $P < 0.05$; Fig. S15).

4. Discussion

4.1. Gut microbiome composition and bacterial community assembly processes

The gut microbiome was dominated by ASVs belonging to the phylum *Firmicutes* across all treatments and time points, which is in line with previous studies of the swine gut (Forouzandeh et al., 2022; Lamendella et al., 2011; Niu et al., 2015). However, the relative abundance of *Firmicutes* was 25 to 50 % higher than in our previous study of the same feeding trial even though some of the samples used for DNA extraction were indeed taken from the same animals. This can be explained by the different DNA extraction kits used in the two studies following the commercial discontinuation of the kit used in the previous study (Brinck et al., 2023; Sperling et al., 2018). Nevertheless, the main conclusions regarding the impacts of dietary Cu on the gut bacterial resistome were similar in the two studies with a significant gut microbiome maturation effect (time) and rather minor impacts being observed for the dietary Cu treatments.

Dietary Cu treatments altered the relative abundance of a few genera. The relative abundance of *Lactobacillus* was reduced after 116 days in response to both dietary Cu treatments in line with our previous study (Brinck et al., 2023). The lacking effect of Cu supplementation on bacterial diversity and composition and the analogous effect of Cu supplements on swine gut microbiome in our experiment was in accordance with former studies where they compared the effect of 150 and 250 μg g^{-1} of Cu_2O and $CuSO_4$ on the composition of the microbial community in broilers ileum (Forouzandeh et al., 2021) or swine colon (Forouzandeh et al., 2022).

We investigated the relative importance of contrasting bacterial community assembly processes using iCAMP. No clear impacts of dietary Cu treatments on the relative importance of the community assembly processes responsible for shaping the bacterial community were observed. Drift was the most influential community assembly mechanism in the outcome of this experiment regardless of whether bacterial communities were stressed by high Cu concentration or not. Counterintuitively, drift became more important with pig age, even though it is generally thought that stochastic community assembly processes are most important during early phases of gut colonization (Seki et al., 2022). Our interpretation of the iCAMP output is that drift became more important over time for explaining animal-to-animal gut microbiome differences due to the observed convergence of bacterial community composition over time (this study, Brinck et al., 2023). Hence, the very small differences observed after 116 days were almost entirely driven by stochastic events.

4.2. Selection and co-selection of bacterial resistance to Cu and other metals

Cu supplementation had a negligible effect on the swine gut bacterial metal resistome, which was primarily affected by gut microbiome maturation (i.e. time). The absence of dietary Cu treatment effects on CRG and MRG profiles contrasts with our phenotypic Cu resistance data demonstrating a significantly higher frequency of Cu-resistant *E. coli* isolates from the two high-Cu dietary treatments than from the corresponding control treatment. The higher frequency of Cu-resistant isolates among the dietary Cu treatment groups indicates that elevated doses of dietary Cu actually had the ability to select for Cu resistant bacterial strains via toxicant-induced selection. This is in line with previous cultivation-dependent studies demonstrating that high doses of dietary Cu (125 to 208 μg Cu^{2+} g^{-1}) could be linked to acquired Cu resistance in *Enterococcus faecium* and *E. faecalis* via the plasmid-borne Cu resistance gene *tcrB* (Amachawadi et al., 2010, 2011; Hasman et al., 2006). Interestingly, more recent studies did not obtain evidence for an effect of high Cu supplementation on Cu resistance in *E. faecalis* or *E. coli* isolates collected from broilers feces (Forouzandeh et al., 2021) or in *E. faecalis* isolates collected from swine feces (Capps et al., 2020; Villagómez-Estrada et al., 2020).

Under the influence of dietary Cu treatments, only the relative abundance of the *CueO* gene was affected, but the Cu supplemented diet actually decreased *CueO* abundance. *CueO* encodes for an enzyme with a high cuprous oxidase activity, which protects *E. coli* against Cu toxicity, but the CueO enzyme may also have other cell functions unrelated to Cu (Singh et al., 2004). Altogether, the HT-qPCR MRG chip data did thus not provide any evidence for selection of CRGs even though dietary Cu selected for phenotypic Cu resistance in *E. coli* isolates. This finding demonstrates that the relative abundances of CRGs targeted by the MRG chip may constitute a poor proxy for phenotypic Cu resistance in specific groups of bacteria and

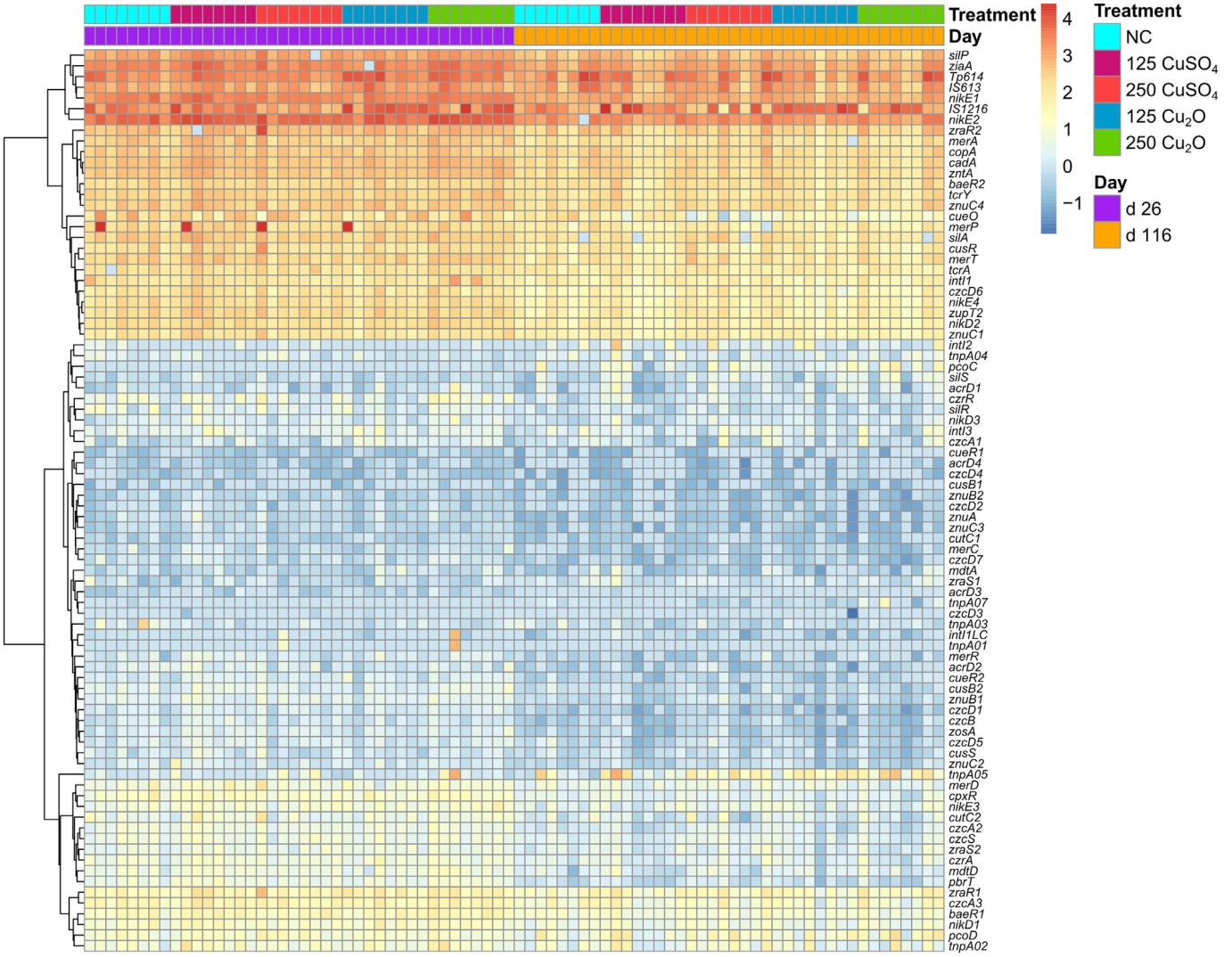


Fig. 4. Heatmap showing the impacts of dietary Cu treatments and time on 86 gene targets related to metal resistance, mobile genetic elements, and multidrug resistance. The color gradient represents the log-transformed relative gene abundance (normalized to the corresponding 16S rRNA gene copy numbers). Rows represent the results of each primer set (assay) shown on the y-axis. Columns represent fecal samples ($n = 80$) grouped by dietary Cu treatments: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}) and by time (day): 26, and 116. Rows were clustered based on Euclidean distances.

it is thus advised to complement the MRG chip with phenotypic resistance data for specific species of bacteria to infer whether high levels of dietary Cu can select for Cu resistance via toxicant-induced succession. In addition, it should also be mentioned that some CRGs (e.g. *pcoA*, *cusA*, and *tcrB*) were not covered by the used MRG chip.

Rather than indicating an important role of dietary Cu in shaping the MRG profiles, our results showed a significant correlation between MRG profiles and bacterial community composition. This is consistent with our previous study showing a similar correlation between ARG profiles and bacterial community composition (Brinck et al., 2023). Although we did not observe a major treatment effect on the overall gut microbiome, the relative abundance of *Lactobacillus* declined in response to Cu treatments after 116 days. Previous studies have demonstrated a similar decrease in *Lactobacillus* abundance following Cu or Zn supplementation in pigs and poultry (Forouzandeh et al., 2021; Højberg et al., 2005; Mei et al., 2009), and it is therefore highly likely that *Lactobacillus* species are directly impacted by metal toxicity in animals fed with supplements of Cu or Zn. Likewise, the relative abundance of *cueO* decreased under the influence of elevated Cu. These findings exemplify how dietary Cu treatments may affect the composition of the swine gut bacterial metal resistome via changes in bacterial community composition.

4.3. Cu-induced co-selection of MGEs

Unlike MRGs, the MGE profiles were in general not well correlated to changes in the bacterial community composition, but the dietary Cu treatments did affect the MGE profile to some extent. Specifically, the *IS1216* transposase gene was significantly more abundant in dietary treatments with Cu_2O (125 and 250 μg g^{-1}) suggesting that this Cu source could possibly imply an increased risk for horizontal transfer of antibiotic resistance determinants among gut bacteria. The abundance of the *IS1216* transposase gene was highly correlated with *Streptococcus* after both 26 and 116 days, but *IS1216* may also play a prominent role for gene transfer in *Enterococcus* species of animal origin (Liu et al., 2012; Xu et al., 2010).

4.4. Cu-induced co-selection of antibiotic resistance in pigs

Pig production is generally considered a hotspot for the development and transfer of antibiotic resistance and since metals such as Cu and Zn are frequently used in parallel with antibiotics it is often also considered a hotspot specifically for metal-induced co-selection of antibiotic resistance (Zhao et al., 2021). Indeed, it has been shown that multi-resistance plasmids conferring resistance to both metals and antibiotics are present in

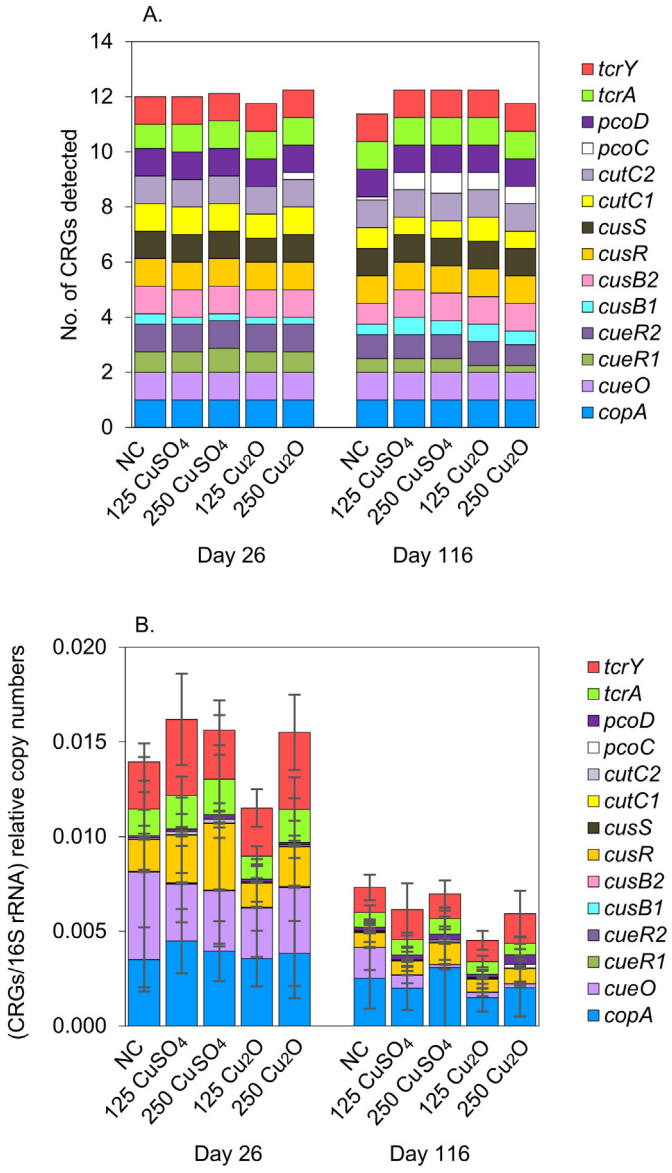


Fig. 5. Impacts of dietary Cu treatments and time on the prevalence of Cu resistance genes (CRGs) in pig fecal samples (n = 80) grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}) and by time (day): 26, and 116. A. Average number of detected CRGs; B. Average cumulative relative abundance of CRGs. The cumulative relative abundance refers to the sum of relative abundance for all targeted CRGs. Error bars show the standard deviation.

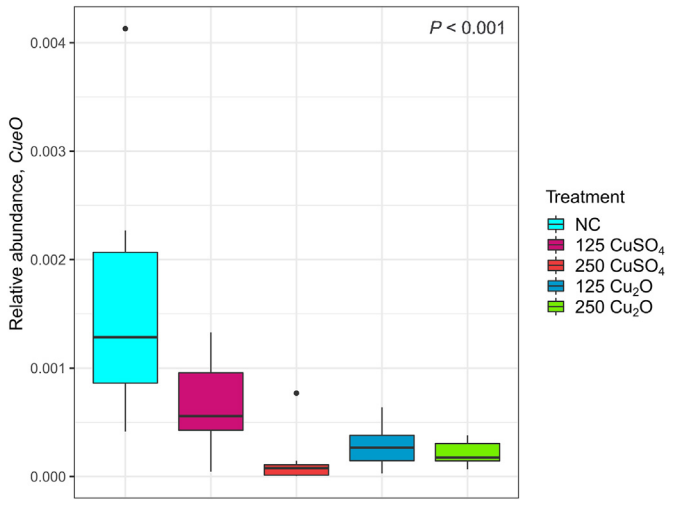


Fig. 6. Boxplot showing the impacts of dietary Cu treatments on the average relative abundance of the *cueO* gene after 116 days of experiment. Relative gene abundance grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}). Boxes represent the interquartile range between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the boxes defines the median. Whiskers show the lowest and highest values within 1.5 times the IQR from the first and third quartiles. Outliers are represented by black dots. A one-way ANOVA test was used to evaluate differences in relative abundance between samples of different treatment groups (*P*-value is shown).

gut bacteria and that they are probably enriched in bacteria of livestock fecal origin compared with other environments (Fang et al., 2016; Pál et al., 2015). It has also been shown that exposure to Cu and Zn can co-select for increased levels of resistance against β-lactams in *E. coli* isolates from pigs in Germany (Hölzel et al., 2012) and against chloramphenicol and ciprofloxacin in *E. coli* isolates from pigs in China (Zhang et al., 2019). Based on our phenotypic resistance data from *E. coli* isolates from pigs in Illinois, we could reject the hypothesis that Cu resistant *E. coli* isolates were more resistant to contrasting classes of antibiotics than corresponding Cu susceptible isolates were. Hence, our data did not indicate co-selection of Cu resistance and antibiotic resistance in *E.coli* from pigs supplemented with dietary Cu supplements in accordance with our previous microbiome-wide study showing that the studied dietary Cu treatments did not co-select for ARGs (Brinck et al., 2023). Interestingly, our data are consistent with recent studies showing no co-occurrence of ARGs and MRGs in assembled metagenomes derived from dairy slurry containing elevated levels of both Cu and Zn (Baker et al., 2022).

In conclusion, we propose that the lacking evidence for Cu-induced co-selection of antibiotic resistance in our pig feeding trial (Brinck et al., 2023; this study) can be explained at least in part by the negligible impacts of the imposed dietary Cu treatments on the gut bacterial metal resistome as observed in this study. Hence, dietary Cu did not select for known CRGs or multidrug resistance genes targeted by the used HT-qPCR MRG Chip indicating a low risk for co-selection via co-resistance or cross-resistance mechanisms, respectively (Baker-Austin et al., 2006). We hypothesize that the negligible impacts of dietary Cu on the gut microbiome reflect the fact that high levels of dietary Cu have been in use for many pig generations and associated legacy effects of this agricultural practice. Hence, CRGs may already have become stably integrated into the fecal microbiome consistent with the weaker impacts of dietary Cu on Enterococci in recent studies (Capps et al., 2020; Villagómez-Estrada et al., 2020) as compared to older studies (Amachawadi et al., 2010, 2011; Hasman et al., 2006). Similar legacy effects have previously been proposed for feeding trials with elevated doses of antibiotics in pigs showing no development of antibiotic resistance (Pollock et al., 2020) and may have important consequences such as enhanced persistence of resistance to antimicrobial agents even at low concentrations (Salyers and Amábile-Cuevas, 1997; Andersson and Hughes, 2011).

Despite the growing knowledge on the antimicrobial activity of Cu (Espinosa and Stein, 2021), much remains to be learned about the impacts of dietary Cu supplements on the swine gut bacterial resistome and its implications for transmission and dissemination of antibiotic resistance to humans. Cu-based feed additives can reduce the amount of total microbial protein in the gut and have significant growth-promoting effects in pigs (Espinosa et al., 2019), but clearly benefits must be carefully balanced against the potential risks for co-selection and persistence of antibiotic resistance in pig gut bacteria and the subsequent transfer of antibiotic resistance to humans via various pathways (Zhao et al., 2021).

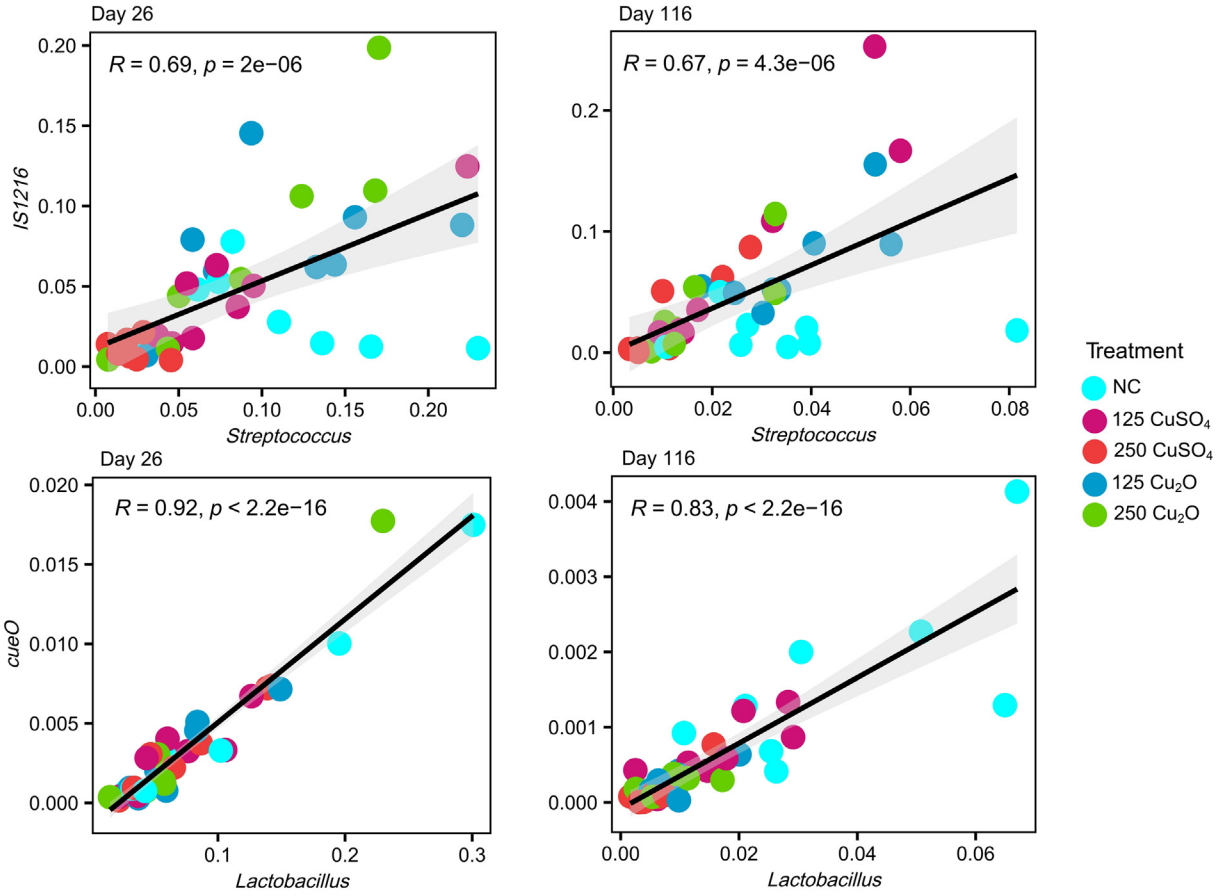


Fig. 7. Spearman's rank correlations between the relative abundance of *IS1216* and *cueO* genes (genes/16S rRNA gene) and the relative abundance (%) of bacterial genera on day 26 or 116 of the experiment.

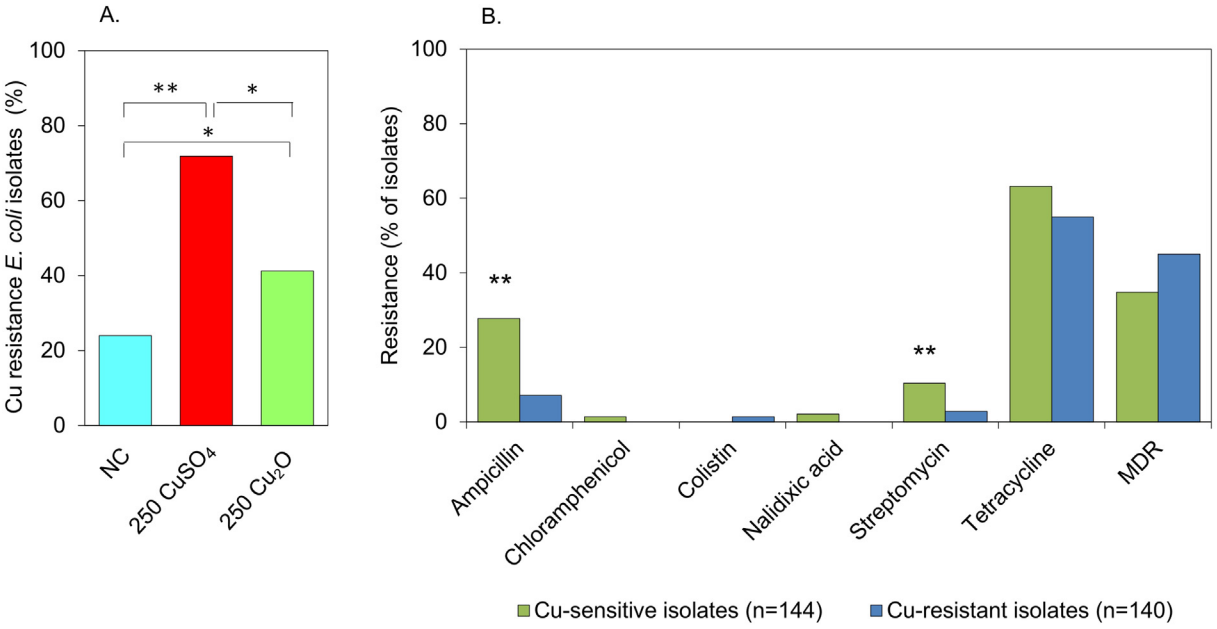


Fig. 8. Phenotypic resistance to Cu and 6 antibiotics in fecal *Escherichia coli* isolates. A. Impacts of dietary Cu treatments on the frequency of Cu resistance among *E. coli* isolates ($n = 284$): NC (negative control; 50 isolates), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}; 103 isolates), and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}; 131 isolates); B. Frequency of antibiotic resistance and multidrug resistance (MDR) among Cu-sensitive ($n = 144$) and Cu-resistant ($n = 140$) *E. coli* isolates pooled together from the three dietary Cu treatments. The level of significance is indicated as follows: Generalized linear model (GLM); ** $P < 0.001$, * $P < 0.05$.

Funding

Asal Forouzandeh was supported by Agéncia de Gestió d'Ajusts Universitaris i de Recerca de la Generalitat de Catalunya (Ph.D. grant 2019FI_B 00282). The support of the UAB-Banco de Santander Talent Program is also thanked for the provision of a research scholarship for David Solà-Oriol. Analytical costs were covered by University of Copenhagen and the Sino-Danish Center (SDC).

Data availability

Data will be made available on request.

Declaration of competing interest

Alessandra Monteiro is an employee at Animine. The European Code of Conduct for Research Integrity is followed by Animine (Drenth, 2010). There are no conflicts of interest among the other authors.

Acknowledgments

Susanne Iversen is acknowledged for technical assistance. Daliang Ning is acknowledged for helpful comments on the iCAMP algorithm (Ning et al., 2020).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164183.

References

Aarestrup, F.M., Hasman, H., 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet. Microbiol. 100, 83–89. https://doi.org/10.1016/J.VETMIC.2004.01.013.

Amachawadi, R.G., Shelton, N.W., Jacob, M.E., Shi, X., Narayanan, S.K., Zurek, L., Dritz, S.S., Nelssen, J.L., Tokach, M.D., Nagaraja, T.G., 2010. Occurrence of tcrB, a transferable copper resistance gene, in fecal enterococci of swine. Foodborne Pathog. Dis. 7, 1089–1097. https://doi.org/10.1089/fpd.2010.0540.

Amachawadi, R.G., Shelton, N.W., Shi, X., Vinasco, J., Dritz, S.S., Tokach, M.D., Nelssen, J.L., Scott, H.M., Nagaraja, T.G., 2011. Selection of fecal enterococci exhibiting tcrB-mediated copper resistance in pigs fed diets supplemented with copper. Appl. Environ. Microbiol. 77, 5597–5603. https://doi.org/10.1128/AEM.00364-11.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46. https://doi.org/10.1111/J.1442-9993.2001.01070.PP.X.

Andersson, D.I., Hughes, D., 2011. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol. Rev. 35, 901–911. https://doi.org/10.1111/j.1574-6976.2011.00289.x.

Baker, M., Williams, A.D., Hooton, S.P.T., Helliwell, R., King, E., Dodsworth, T., María Baena-Nogueras, R., Warry, A., Ortori, C.A., Todman, H., Gray-Hammerton, C.J., Pritchard, C.W., Iles, E., Cook, R., Emes, R.D., Jones, M.A., Kypraios, T., West, H., Barrett, D.A., Ramsden, S.J., Gomes, R.L., Hudson, C., Millard, A.D., Raman, S., Morris, C., Dodd, C., Kreft, J.-U., Hobman, J.L., Stekel, D.J., 2022. Antimicrobial resistance in dairy slurry tanks: a critical point for measurement and control. Environ. Int. 169, 107516. https://doi.org/10.1016/j.envint.2022.107516.

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–182. https://doi.org/10.1016/j.tim.2006.02.006.

Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O., Brandt, K.K., 2010. Cu exposure under field conditions Coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. Environ. Sci. Technol. 44, 8724–8728. https://doi.org/10.1021/es101798r.

Blavi, L., Solà, D., Monteiro, A., Pérez, J.F., Stein, H.H., 2021. Inclusion of dicopper oxide instead of copper sulfate in diets for growing–finishing pigs results in greater final body weight and bone mineralization, but reduced accumulation of copper in the liver. J. Anim. Sci. 99, 1–10. https://doi.org/10.1093/jas/skab127.

Brinck, J.E., Lassen, S.B., Forouzandeh, A., Pan, T., Wang, Y.-Z., Monteiro, A., Blavi, L., Solà-Oriol, D., Stein, H.H., Su, J.-Q., Brandt, K.K., 2023. Impacts of dietary copper on the swine gut microbiome and antibiotic resistome. Sci. Total Environ. 857, 159609. https://doi.org/10.1016/j.scitotenv.2022.159609.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Capps, K.M., Amachawadi, R.G., Menegat, M.B., Woodworth, J.C., Perryman, K., Tokach, M.D., Dritz, S.S., DeRouchey, J.M., Goodband, R.D., Bai, J., Apley, M.D., Lubbers, B.V., Nagaraja, T.G., 2020. Impact of added copper, alone or in combination with chlortetracycline, on growth performance and antimicrobial resistance of fecal enterococci of weaned piglets. J. Anim. Sci. 98, 1–11. https://doi.org/10.1093/JAS/SKAA003.

Dibner, J.J., Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84, 634–643. https://doi.org/10.1093/ps/84.4.634.

Drenth, P.J.D., 2010. A European code of conduct for research integrity. All European Academies 14. https://allea.org/wp-content/uploads/2015/09/A-European-Code-of-Conduct-for-Research-Integrity_final.10.10.pdf (Accessed 14 November 2022).

Espinosa, C.D., Stein, H.H., 2021. Digestibility and metabolism of copper in diets for pigs and influence of dietary copper on growth performance, intestinal health, and overall immune status: a review. J. Anim. Sci. Biotechnol. 12, 13. https://doi.org/10.1186/s40104-020-00533-3.

Espinosa, C.D., Fry, R.S., Usry, J.L., Stein, H.H., 2017. Copper hydroxychloride improves growth performance and reduces diarrhea frequency of weanling pigs fed a corn–soybean meal diet but does not change apparent total tract digestibility of energy and acid hydrolyzed ether extract. J. Anim. Sci. 95, 5447. https://doi.org/10.2527/jas2017.1702.

Espinosa, C.D., Fry, R.S., Kocher, M.E., Stein, H.H., 2019. Effects of copper hydroxychloride and distillers dried grains with solubles on intestinal microbial concentration and apparent ileal and total tract digestibility of energy and nutrients by growing pigs. J. Anim. Sci. 97, 4904–4911. https://doi.org/10.1093/jas/skz340.

European Commission, 2018. Commission implementing regulation (EU) 2018/1039 of 23 July 2018. EFSA J. 2018.

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J., Liu, Y., 2016. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from E. coli isolates of food-producing animals. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep25312.

Forouzandeh, A., Blavi, L., Abdelli, N., Melo-Duran, D., Vidal, A., Rodríguez, M., Monteiro, A.N.T.R., Pérez, J.F., Darwich, L., Solà-Oriol, D., 2021. Effects of dicopper oxide and copper sulfate on growth performance and gut microbiota in broilers. Poult. Sci. 100, 101224. https://doi.org/10.1016/j.psj.2021.101224.

Forouzandeh, A., Blavi, L., Pérez, J.F., D'Angelo, M., González-Solé, F., Monteiro, A., Stein, H.H., Solà-Oriol, D., 2022. How copper can impact pig growth: comparing the effect of copper sulfate and monovalent copper oxide on oxidative status, inflammation, gene abundance, and microbial modulation as potential mechanisms of action. J. Anim. Sci. 100, 2015–2019. https://doi.org/10.1093/jas/skac224.

Hamdi, M., Solà, D., Franco, R., Durosoy, S., Roméo, A., Pérez, J.F., 2018. Including copper sulphate or dicopper oxide in the diet of broiler chickens affects performance and copper content in the liver. Anim. Feed Sci. Technol. 237, 89–97. https://doi.org/10.1016/j.anifeedsci.2018.01.014.

Hasman, H., Franke, S., Rensing, C., 2005. Resistance to metals used in agricultural production. In: Aarestrup, F.M., Schwarz, S., Shen, J., Cavaco, L. (Eds.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington, DC, USA, pp. 99–114 https://doi.org/10.1128/9781555817534.ch7.

Hasman, H., Kempf, I., Chidaine, B., Cariolet, R., Ersbøll, A.K., Houe, H., Hansen, H.C.B., Aarestrup, F.M., 2006. Copper resistance in Enterococcus faecium, mediated by the tcrB gene, is selected by supplementation of pig feed with copper sulfate. Appl. Environ. Microbiol. 72, 5784–5789. https://doi.org/10.1128/AEM.02979-05.

Højberg, O., Canibe, N., Poulsen, H.D., Hedemann, M.S., Jensen, B.B., 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Appl. Environ. Microbiol. 71, 2267–2277. https://doi.org/10.1128/AEM.71.5.2267-2277.2005.

Hölzel, C.S., Müller, C., Harms, K.S., Mikolajewski, S., Schäfer, S., Schwaiger, K., Bauer, J., 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environ. Res. 113, 21–27. https://doi.org/10.1016/J.ENVRES.2012.01.002.

Katoh, K., Misawa, K., Kuma, K.I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066. https://doi.org/10.1093/nar/gkf436.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47, 583–621. https://doi.org/10.1080/01621459.1952.10483441.

Lamendella, R., Santo Domingo, J.W., Ghosh, S., Martinson, J., Oerther, D.B., 2011. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol. https://doi.org/10.1186/1471-2180-11-103.

Lin, H., Peddada, S. Das, 2020. Analysis of compositions of microbiomes with bias correction. Nat. Commun. 11, 1–11. https://doi.org/10.1038/s41467-020-17041-7.

Liu, Y., Wang, Y., Wu, C., Shen, Z., Schwarz, S., Du, X.D., Dai, L., Zhang, W., Zhang, Q., Shen, J., 2012. First report of the multidrug resistance gene cfr in Enterococcus faecalis of animal origin. Antimicrob. Agents Chemother. 56, 1650–1654. https://doi.org/10.1128/AAC.06091-11.

Liu, C., Cui, Y., Li, X., Yao, M., 2021. Microeco: an R package for data mining in microbial community ecology. FEMS Microbiol. Ecol. 97, 255. https://doi.org/10.1093/FEMSEC/FIAA255.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., Stanton, T.B., 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. U. S. A. 109, 1691–1696. https://doi.org/10.1073/PNAS.1120238109.

Mei, S.F., Yu, B., Ju, C.F., Zhu, D., Chen, D.W., 2009. Effect of different levels of copper on growth performance and cecal ecosystem of newly weaned piglets. Ital. J. Anim. Sci. 9, 378–381. https://doi.org/10.4081/ijas.2010.e71.

Ning, D., Yuan, M., Wu, L., Zhang, Y., Guo, X., Zhou, X., Yang, Y., Arkin, A.P., Firestone, M.K., Zhou, J., 2020. A quantitative framework reveals ecological drivers of grassland microbial community assembly in response to warming. Nat. Commun. 11. https://doi.org/10.1038/s41467-020-18560-z.

Niu, Q., Li, P., Hao, S., Zhang, Y., Kim, S.W., Li, H., Ma, X., Gao, S., He, L., Wu, W., Huang, X., Hua, J., Zhou, B., Huang, R., 2015. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. Sci. Rep. 5, 1–7. https://doi.org/10.1038/srep09938.

NRC, 2012. Nutrient Requirements of Swine. 11th ed. Natl. Acad. Press, Washington, DC.

Oksanen, J., Simpson, G.L., Blanchet, F.G., 2022. Vegan: Community Ecology Package. R package version 2.6–2. [WWW Document]. http://CRAN.Rproject.org/package=vegan. URL https://github.com/vegandevs/vegan NeedsCompilation.

Pál, C., Papp, B., Lázár, V., 2015. Collateral sensitivity of antibiotic-resistant microbes. Trends Microbiol. https://doi.org/10.1016/j.tim.2015.02.009.

Pérez, V.G., Waguespack, A.M., Bidner, T.D., Southern, L.L., Fakler, T.M., Ward, T.L., Steidinger, M., Pettigrew, J.E., 2011. Additivity of effects from dietary copper and zinc on growth performance and fecal microbiota of pigs after weaning. J. Anim. Sci. 89, 414–425. https://doi.org/10.2527/jas.2010-2839.

Pollock, J., Muwonge, A., Hutchings, M.R., Mainda, G., Bronsvoort, B.M., Gally, D.L., Corbishley, A., 2020. Resistance to change: AMR gene dynamics on a commercial pig farm with high antimicrobial usage. Sci. Rep. 10, 1–10. https://doi.org/10.1038/s41598-020-58659-3.

Popov, S., Saphier, O., Popov, M., Shenker, M., Entus, S., Shotland, Y., Saphier, M., 2020. Factors enhancing the antibacterial effect of monovalent copper ions. Curr. Microbiol. 77, 361–368. https://doi.org/10.1007/s00284-019-01794-6.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One 5, e9490. https://doi.org/10.1371/journal.pone.0009490.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

Reasoner, D.J., Geldreich, E.E., 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7. https://doi.org/10.1128/AEM.49.1.1-7.1985.

Rensing, C., Moodley, A., Cavaco, L.M., McDevitt, S.F., 2018. Resistance to metals used in agricultural production. Microbiol. Spectr. 6, 1–24. https://doi.org/10.1128/microbiolspec.ARBA-0025-2017.

Salyers, A.A., Amábile-Cuevas, C.F., 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents Chemother. 41, 2321–2325.

Seki, D., Schauberger, C., Hausmann, B., Berger, A., Wisgrill, L., Berry, D., 2022. Individuality of the Extremely Premature Infant Gut Microbiota Is Driven by Ecological Drift. , p. mSystems 7 https://doi.org/10.1128/MSYSTEMS.00163-22.

Singh, S.K., Grass, G., Rensing, C., Montfort, W.R., 2004. Cuprous oxidase activity of CueO from Escherichia coli. J. Bacteriol. 186, 7815–7817. https://doi.org/10.1128/JB.186.22.7815-7817.2004.

Sperling, T., O'neil, D., Nieciecki, V., Smith, P., Schroeer, S., Scholle, N., Polidori, M., Sprenger-Haussels, M., 2018. Novel Procedure for Improved Results in Soil Microbiome Analysis Using the DNeasy ® PowerSoil ® pro Kit. QIAGEN - sample to insight https://qiagen.moscow/upload/pdf/AN_DNeasy_Powersoil_Pro_Kit.pdf.

Villagómez-Estrada, S., Pérez, J.F., Darwich, L., Vidal, A., van Kuijk, S., Melo-Durán, D., Solà-Oriol, D., 2020. Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota. J. Anim. Sci. 98, 1–15. https://doi.org/10.1093/jas/skaa117.

Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R., 2016. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 1. https://doi.org/10.1128/mSystems.00009-15.

Wickham, H., 2016. Data Analysis. Springer, Cham, pp. 189–201 https://doi.org/10.1007/978-3-319-24277-4_9.

Xu, X., Lin, D., Yan, G., Ye, X., Wu, S., Guo, Y., Zhu, D., Hu, F., Zhang, Y., Wang, F., Jacoby, G.A., Wang, M., 2010. vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium. Antimicrob. Agents Chemother. 54, 4643–4647. https://doi.org/10.1128/AAC.01710-09.

Yazdankhah, S., Rudi, K., Bernhoft, A., 2014. Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. Microb. Ecol. Health Dis. 25. https://doi.org/10.3402/mehd.v25.25862.

You, Y., Silbergeld, E.K., 2014. Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion. Front. Microbiol. 5, 284. https://doi.org/10.3389/FMICB.2014.00284/BIBTEX.

Zhang, Y., Zhou, J., Dong, Z., Li, G., Wang, J., Li, Y., Wan, D., Yang, H., Yin, Y., 2019. Effect of dietary copper on intestinal microbiota and antimicrobial resistance profiles of Escherichia coli in weaned piglets. Front. Microbiol. 10, 1–11. https://doi.org/10.3389/fmicb.2019.02808.

Zhang, H., Hou, F., Xie, W., Wang, K., Zhou, X., Zhang, D., Zhu, X., 2020. Interaction and assembly processes of abundant and rare microbial communities during a diatom bloom process. Environ. Microbiol. 22, 1707–1719. https://doi.org/10.1111/1462-2920.14820.

Zhao, Y., Yang, Q.E., Zhou, X., Wang, F.-H., Muurinen, J., Virta, M.P., Brandt, K.K., Zhu, Y.-G., 2021. Antibiotic resistome in the livestock and aquaculture industries: status and solutions. Crit. Rev. Environ. Sci. Technol. 51, 2159–2196. https://doi.org/10.1080/10643389.2020.1777815.

Zhou, J., Ning, D., 2017. Stochastic community assembly: does it matter in microbial ecology? Microbiol. Mol. Biol. Rev. 81. https://doi.org/10.1128/mmbr.00002-17.

Zhu, J., Huang, Qiong, Peng, X., Zhou, X., Gao, S., Li, Y., Luo, X., Zhao, Y., Rensing, C., Su, J., Cai, P., Liu, Y., Chen, W., Hao, X., Huang, Qiaoyun, 2022. MRG Chip: a high-throughput qPCR-based tool for assessment of the heavy metal(loid) Resistome. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.2c00488.