Science of the Total Environment 857 (2023) 159609

Contents lists available at ScienceDirect

Science of the Total Environment

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

Impacts of dietary copper on the swine gut microbiome and antibiotic resistome

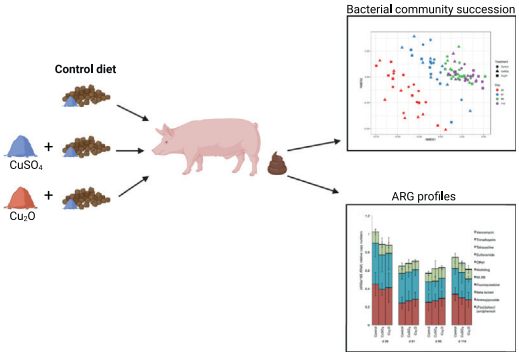
Julius Emil Brinck [a,g], Simon Bo Lassen [a,b], Asal Forouzandeh [a,c], Ting Pan [d], Yan-Zi Wang [d], Alessandra Monteiro [e], Laia Blavi [c], David Solà-Oriol [c], Hans H. Stein [f], Jian-Qiang Su [d], Kristian K. Brandt [a,b,*]

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

HIGHLIGHTS

- Impacts of two Cu-based feed additives on the swine gut microbiome were studied.
- Cu treatments increased bioavailable Cu in feces by at least 3 orders of magnitude.
- Antibiotic resistance genes were highly abundant and diverse in fecal samples.
- Microbiomes differed between pig growth stages, but only minor impacts of Cu.
- High levels of dietary Cu (250 ppm) did not co-select antibiotic resistance genes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Fang Wang

Keywords:
Antimicrobial resistance
Antibiotic resistance genes
Bacterial community composition
Co-selection
HT-qPCR
Pig microbiome

ABSTRACT

Restrictions on antibiotic growth promoters have prompted livestock producers to use alternative growth promoters, and dietary copper (Cu) supplementation is currently being widely used in pig production. However, elevated doses of dietary Cu constitute a risk for co-selection of antibiotic resistance and the risk may depend on the type of Cu-based feed additives being used. We here report the first controlled experiment investigating the impact of two contrasting Cu-based feed additives on the overall swine gut microbiome and antibiotic resistome. DNA was extracted from fecal samples ($n = 96$) collected at four time points during 116 days from 120 pigs allotted to three dietary treatments: control, divalent copper sulfate ($CuSO_4$; 250 μg Cu g^{-1} feed), and monovalent copper oxide (Cu_2O; 250 μg Cu g^{-1} feed). Bacterial community composition, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) were assessed, and bioavailable Cu ($[Cu]_{bio}$) was determined using whole-cell bacterial bioreporters. Cu supplementation to feed increased total Cu concentrations ($[Cu]_{total}$) and $[Cu]_{bio}$ in feces 8–10 fold and at least 670–1000 fold, respectively, but with no significant differences between the two Cu sources. The swine gut microbiome harbored highly abundant and diverse ARGs and MGEs irrespective of the treatments throughout the experiment. Microbiomes differed significantly between pig growth stages and tended to converge over time, but only minor changes in the bacterial community composition and resistome could be linked to Cu supplementation. A significant correlation between bacterial community composition (i.e., bacterial taxa present) and ARG prevalence patterns were observed by Procrustes

* 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.2022.159609
Received 23 July 2022; Received in revised form 17 October 2022; Accepted 17 October 2022
Available online 21 October 2022

analysis. Overall, results of the experiment did not provide evidence for Cu-induced co-selection of ARGs or MGEs even at a Cu concentration level exceeding the maximal permitted level for pig diets in the EU (25 to 150 μg Cu g^{-1} feed depending on pig age).

1. Introduction

Antibiotic resistance is recognized as one of the major threats to human health (WHO, 2014). Swine farms contribute to antibiotic resistance development and dissemination, and swine microbiomes comprise important reservoirs of antibiotic resistance genes (ARGs) due to the use of antimicrobials in swine production (Argudín et al., 2017). Due to the emergence of antibiotic resistance in pathogenic bacteria in animal production, the EU prohibited the use of antibiotic growth promoters (AGPs) in 2006 (EU, 2005). In the United States and China, AGPs administered in animal production became restricted in 2017 and 2020, respectively (U.S. Food and Drug Administration, 2018; Hu and Cowling, 2020). Due to the reduced use of AGPs, the livestock industry currently relies on alternative growth promoters such as in-feed supplementation with elevated concentrations of copper (Cu) or zinc (Zn) (Heo et al., 2013; Liu et al., 2018; Zhao et al., 2021).

However, dietary supplementation with Cu and Zn in swine production may also increase the risk of dissemination of antibiotic resistance via co-selection and mobilization of ARGs and their subsequent transfer to humans (Ashbolt et al., 2013; Yazdankhah et al., 2014; Zhao et al., 2018; Muurinen et al., 2021). Co-selection may occur if ARGs and metal resistance genes (MRGs) are genetically linked (co-resistance), if the same resistance mechanism confers resistance to both metals and antibiotics (cross-resistance), or if a common regulator controls the expression of resistance systems to both metals and antibiotics (co-regulation; Baker-Austin et al., 2006; Poole, 2017). Indeed, elevated doses of dietary Cu and Zn used for swine growth promotion have been shown to select for Cu or Zn resistance and to co-select for resistance to certain antibiotics in specific groups of swine gut bacteria harboring pathogenic strains (Hasman et al., 2006; Medardus et al., 2014; Yazdankhah et al., 2014). Very recently, orally administered veterinary medicinal products containing Zn have been prohibited in the EU (EU, 2017). This is likely to prompt European pig farmers to use the maximal allowed levels of Cu corresponding to 150 ppm Cu from lactation until 4 weeks after weaning, 100 ppm between the 5th and 8th weeks after weaning, and 25 ppm after the 8th week (regulation EU 2018/1039).

The animal growth-promoting effects of Cu and the underlying mechanisms have recently been reviewed (Espinosa and Stein, 2021). Copper ions exist in two states: oxidized cupric (Cu^{2+}) and reduced cuprous (Cu^{+}; Linder and Hazegh-Azam, 1996). Divalent copper (II) sulfate ($CuSO_4$) is a common growth promoter in pig diets (Park and Kim, 2016), but monovalent copper oxide (Cu_2O) also improve the growth performance of poultry (Hamdi et al., 2018; Forouzandeh et al., 2021) and swine (Blavi et al., 2021; Forouzandeh et al., 2022). Importantly, the two latter studies showed that $CuSO_4$ and Cu_2O exerted differential effects on several animal performance indicators, but it is presently not known if the ability of Cu to co-select ARGs depends on the metal sources being used. To shed light on this issue, we analyzed fecal samples collected from a previously reported experiment comparing the effects of $CuSO_4$ and Cu_2O on swine growth promotion during a 116-day feeding trial (Blavi et al., 2021; Forouzandeh et al., 2022). Our primary objective was to determine the effects of the two sources of dietary Cu on the swine gut microbiome and antibiotic resistome as assessed by 16S rRNA gene amplicon sequencing and high-throughput qPCR (HT-qPCR), respectively. In order to assess the selective pressures imposed by the two forms of dietary Cu, we further measured Cu bioavailability using a whole-cell bacterial bioreporter assay. In addition to being the first study comparing effects of two contrasting sources of Cu in high-Cu diets on antibiotic resistance in pigs, our study is also the first to specifically examine impacts of high-Cu diets on the swine gut antibiotic resistome using a bacterial community-wide, cultivation-independent approach.

2. Materials and methods

2.1. Description of Cu supplementation swine feeding trial

Pig fecal samples were derived from a feeding trial carried out at the University of Illinois in Urbana-Champaign, Illinois, USA. The feeding trial protocol was reviewed and approved by the University of Illinois' Institutional Animal Care and Use Committee. Offsprings of L 359 boars mated to Camborough females (60 barrows, and 60 gilts; Pig Improvement Company, Hendersonville, TN) were used in the feeding trial, which has previously been described in detail (Blavi et al., 2021; Forouzandeh et al., 2022). Importantly, no antibiotics were administered during the study. In brief, a total of 120 pigs with an average initial body weight of 11.5 ± 0.98 kg were used in a 116 d trial. Pigs were randomly allotted to a randomized complete block design with two blocks of 60 pigs, three treatments, five pigs per pen, and four replicate pens per treatment in each block. Treatments were arranged as follows: control diet with 20 μg $CuSO_4$ g^{-1} feed (control group), which is the minimal recommended dose of Cu for growing/finishing pigs, control diet supplemented with 250 μg $CuSO_4$ g^{-1} feed ($CuSO_4$ group), or control diet supplemented with 250 μg Cu_2O g^{-1} feed (Cu_2O group). A total of eight fecal samples per treatment group were collected directly from the rectum of the pigs at four different time points (26, 61, 96, and 116 d after the start of the experiment). Fecal samples were subsequently placed into liquid nitrogen and stored at -80 °C until samples were shipped on dry ice to the University of Copenhagen.

2.2. Fecal DNA extraction

DNA was extracted from thawed fecal samples (0.25 g, $n = 96$) using the DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen) according to the instruction manual. The quality and quantity of the extracted DNA were assessed using nanodrop and Qubit. DNA concentrations varied between 80 and 220 ng μl^{-1} for the fecal samples. The A260/A280 ratios were >1.8 for all DNA extracts. Extracted DNA was freeze-dried and used for 16S rRNA gene amplicon sequencing (Section 2.3) and high-throughput qPCR (Section 2.4).

2.3. 16S rRNA gene amplicon sequencing and data processing

The V4 region of the bacterial 16S rRNA gene was amplified by PCR using the modified forward primer pair 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) (Walters et al., 2016). A small-fragment library was constructed, followed by paired-end sequencing using the Novaseq PE250 at Novogene Bioinformatics Technology Co. Ltd. (UK). The 16S rRNA gene amplicon sequences were processed using the DADA2 pipeline (Callahan et al., 2016) in R version 3.6.3. Default settings were used for filtering and trimming. Identical sequencing reads were combined using the dereplication function. Paired-end reads were merged, chimeras were removed, an amplicon sequence variant (ASV) table was constructed, and taxonomy was assigned using the RDP trainset 16/release 11.5 (Callahan et al., 2017). A phylogenetic tree was constructed using the "DECIPHER" (Wright, 2015) and the "phangorn" R packages. DADA2 data outputs were combined into a phyloseq object using the "phyloseq" package for further analysis.

2.4. High-throughput qPCR and data processing

HT-qPCR reactions were performed by the Wafergen SmartChip Real-time PCR system, using a total of 384 validated primer sets targeting 319

ARGs and 57 MGEs, as previously described (Stedtfeld et al., 2018). Out of the 96 fecal samples analyzed, a total of 20 samples could not be included in the HT-qPCR analysis due to a technical error during HT-qPCR analysis and insufficient amounts of DNA to repeat the analysis. As a consequence, between five and eight replicates per treatment group were available at each time point.

All qPCR reactions were performed in technical duplicates and for each run, a non-template control was included. 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 both duplicates 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 and 31 refers to the detection limit. HT-qPCR data were normalized among samples by dividing the relative gene copy numbers by the corresponding 16S rRNA gene copy numbers. Cell-specific ARG copy numbers were calculated assuming an average of four 16S rRNA genes per bacterium (Klappenbach et al., 2001).

2.5. Total and bioavailable Cu in fecal samples

Total Cu ($[Cu]_{total}$) in fecal samples was analyzed using inductively coupled plasma-optical emission spectrometry as described previously (Blavi et al., 2021).

Bioavailable Cu ($[Cu]_{bio}$) was determined in fecal samples ($n = 18$) collected at the end of the experiment (d 116) using a whole-cell bacterial bioreporter assay based on two isogenic, bioluminescent strains of *Pseudomonas fluorescens* (Tom-Petersen et al., 2001; Nybroe et al., 2008). In brief, 0.2 g feces samples were mixed with 1 ml Milli-Q water in a 5 ml Falcon tube and incubated for 2 h on a horizontal shaker (200 rpm, 22 °C). Subsequently, the samples were centrifuged (10.000 *g*, 22 °C, 10 min) and supernatants were collected and stored at −20 °C for later bioreporter analysis. $[Cu]_{bio}$ was operationally defined as Cu species that were able to induce expression of Cu-regulated *luxAB* genes in *P. fluorescens* DF57-Cu15 bioreporter cell suspensions within a 1.5 h incubation period (Brandt et al., 2008). *P. fluorescens* DF57-40E7 bioreporter cells with constitutive expression of *luxAB* genes were used as a reference bioreporter strain allowing for corrections of sample matrix effects (e.g. masking of emitted light) as described in detail previously (Brandt et al., 2008).

2.6. Statistical analyses

All statistical analysis and data exploration were completed in R version 3.6.3. Data from pigs of the same treatment and sampling time point were grouped to investigate potential Cu effects on the bacterial community structure and ARG/MGE composition. Bacterial community composition was examined with "phyloseq" and "ggplot2" R packages (Callahan et al., 2016; Wickham, 2016). Alpha diversity of samples was displayed using the *plot_richness* function with Chao1 and Shannon measures, and statistical differences between treatment groups and time points were assessed by Wilcoxon rank-sum test. Beta diversity of samples was displayed using non-metric multidimensional scalings (NMDS) based on Bray-Curtis dissimilarity metrics using the function *plot_ordination*. Indicator genera (*Streptococcus*, *Lactobacillus*, and *Bifidobacterium*) affected by dietary Cu treatments were selected based on previous studies (Højberg et al., 2005; Muurinen et al., 2021; Zhang et al., 2019), and the relative abundance was assessed between treatment groups. Differential abundance of ASVs was evaluated among the different treatment groups using analysis of compositions of microbiomes with bias correction (ANCOM-BC) as described by Lin and Peddada (2020). Permutation multivariate analysis of variance (PERMANOVA) was used to investigate differences in bacterial community composition or ARG/MGE compositions among treatment groups and time points with the function *adonis* (Anderson, 2001). ARG/MGE profiles were analyzed using "vegan" R package (Oksanen et al., 2019). Principal coordinates analysis (PCoA) was performed using Bray-Curtis dissimilarity metrics to display dissimilarities of ARG/MGE compositions across treatments and time points with the function *betadisper*. Procrustes test for correlation analysis between bacterial communities and ARG/MGE composition based on NMDS ordinations was completed with the *protest* function. Mantel's test using Spearman's rank correlation was used to further analyze the links between bacterial community structure and ARG/MGE profiles using the *mantel* function (Legendre and Legendre, 1998). Heatmap was produced with the "pheatmap" R package. Significant differences in the relative abundance of ARG or MGE classes (grouped according to classes of antibiotics that they confer resistance to or the type of MGE that they encoded) were assessed by one-way Analysis of Variance (ANOVA) combined with Dunnett's test for pairwise comparisons (Dunnett, 1955). The ANOVA test was performed using the *anova* function and the *glht* function of in "multicomp" package in R. To investigate significant differences in relative abundance of individual genes across treatments groups, Kruskal-Wallis rank sum test was conducted using the *kruskal.test* function of the "stats" R package (Kruskal and Wallis, 1952). Significant Spearman's rank correlations between the relative abundance (genes/16S rRNA gene) of individual ARGs/MGEs and the relative abundance (%) of bacterial taxa at the Genus level were determined using the *associate* function of the Microbiome package. *P*-values were adjusted using the Benjamin-Hochberg procedure, and only correlations with a *p*-value below 0.01 and a correlation coefficient above 0.5 or below −0.5 were considered statistically robust. Network analysis was carried out using Cytoscape version 3.8.2 with CoNet plugin as previously described (Hu et al., 2017). ANOVA was used to examine differences in fecal Cu content ($[Cu]_{total}$) and the fraction of $[Cu]_{bio}$ among samples from the different treatments.

3. Results

3.1. Total and bioavailable Cu in feces

The fecal Cu content ($[Cu]_{total}$) in samples obtained from both Cu treatment groups was 8–10 times higher than in the corresponding control samples ($P < 0.001$), confirming the differences in Cu concentration in dietary treatments (Table 1). In addition, $[Cu]_{total}$ levels were slightly higher for the $CuSO_4$ group compared with the Cu_2O group ($P < 0.05$; Table 1), but we also notice considerable replicate-to-replicate variability for these data indicating some heterogeneity. The level of bioavailable Cu ($[Cu]_{bio}$) was at least 670–1000 fold higher in fecal samples obtained from the two Cu treatments compared with the corresponding control ($P < 0.05$), but $[Cu]_{bio}$ levels were not significantly different between the two Cu treatments (ANOVA and Tukey's test, $P = 0.453$; Table 1). In control samples, $[Cu]_{bio}$ was below the detection limit of the assay (<0.0007 μg g^{-1}). The relative Cu bioavailability (i.e. $[Cu]_{bio}/[Cu]_{total}$) was not different between the two Cu treatments (ANOVA and Tukey's test, $P = 0.114$).

3.2. Impacts of dietary Cu and pig growth stage on bacterial community composition

After quality filtering, a total of 10,899,893 paired-end 250-bp sequences were acquired from all fecal samples ($n = 96$), ranging between 47,132 to 137,805 sequences per sample. Sequences were clustered into 9682 ASVs assigned to 48 different phyla and 704 genera. Phylogenetic

Table 1
Bioavailable Cu ($[Cu]_{bio}$) determined with *Pseudomonas fluorescens* bioreporter assay in fecal samples ($n = 18$) from pigs allocated to different dietary Cu treatments: control (no Cu supplementation), $CuSO_4$ (copper sulfate, 250 μg Cu g^{-1} feed), or Cu_2O (monovalent copper oxide, 250 μg Cu g^{-1} feed). The total fecal Cu content ($[Cu]_{total}$) determined using inductively coupled plasma-optical emission spectrometry is shown. Means ± standard deviations are shown.

Treatment	$[Cu]_{bio}$ (μg g^{-1})	$[Cu]_{total}$ (μg g^{-1})	$[Cu]_{bio}/[Cu]_{total}$ (%)
Control	$<7 \times 10^{-4}$ [a]	295 ± 74	$<2.49 \times 10^{-4}$
$CuSO_4$	0.47 ± 0.25	2900 ± 438	0.016 ± 0.007
Cu_2O	0.70 ± 0.50	2356 ± 357	0.029 ± 0.019

[a] $[Cu]_{bio}$ of control samples was below the detection limit ($<7 \times 10^{-4}$ μg g^{-1}).

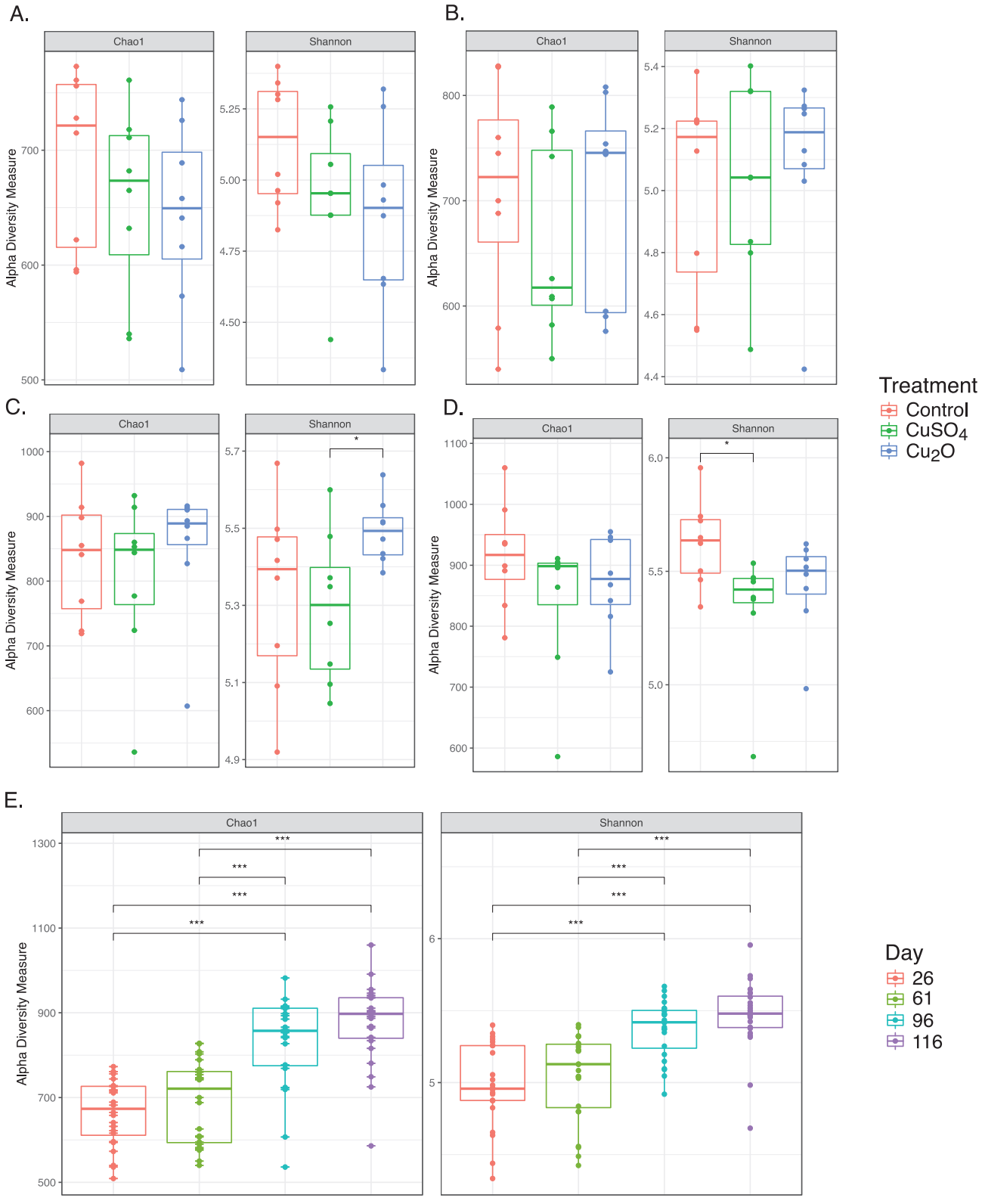


Fig. 1. Boxplots showing alpha diversity (Chao1 and Shannon Index) of the bacterial community across all fecal samples (n = 96). Communities grouped by treatment (dietary Cu): Control, $CuSO_4$ (copper sulfate, 250 μg g^{-1}), and Cu_2O (monovalent copper oxide, 250 μg g^{-1}) during gut microbiome maturation (days post-weaning): A. 26, B. 61, C. 96, and D. 116. E. Communities grouped by degree of gut microbiome maturation (days post-weaning): 26, 61, 96, and 116. Boxes represent the interquartile range (IQR) 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. Every sample is represented by a colored dot. Dots beyond the end of the whiskers are outliers. Solid lines and asterisks indicate a significant difference between groups. The level of significance is indicated as follows: Wilcoxon rank-sum test; *** $P < 0.001$, * $P < 0.05$.

analysis of 16S rRNA gene amplicon sequences showed that the swine gut microbiome was dominated by *Firmicutes* and *Bacteroidetes* phyla among all treatments and time points (Fig. S1). At a higher phylogenetic resolution, *Prevotella* spp. was consistently one of the most abundant genera representing up to 30 % of all classifiable bacteria (Fig. S2).

Species richness estimator (Chao1) or Shannon diversity indices indicated that alpha diversity of the gut bacterial community was not affected by the two Cu treatments at any time point. Shannon diversity index revealed significant differences between the two Cu treatments 96 days after the start of the experiment and between the $CuSO_4$ group and the control group 116 days after the start of the experiment, but the differences were not consistent. By contrast, the richness and diversity of the gut bacterial communities increased as pigs became older (Wilcoxon rank-sum test, $P < 0.001$) (Fig. 1).

A gut microbiome maturation effect was also indicated by an altered configuration of bacterial communities as pigs became older (Fig. 2). Hence, NMDS ordinations indicated that microbiomes clustered according to the time of sampling and that community composition tended to converge (less variability between samples) after 96 d consistent with a mature gut microbiome developing over time. Specifically, approx. 31 % of the variance was explained by the age of the pigs (PERMANOVA, $R^2 = 0.307$, $P < 0.001$) (Fig. 2).

Cu treatments did not affect bacterial community composition at the phylum level. Hence, NMDS ordinations displayed no clustering of replicate samples from each Cu treatment, demonstrating no marked overall effects of Cu supplementation on gut bacterial community composition at any time point of the experiment (PERMANOVA, $R^2 = 0.019$, $P = 0.48$) (Fig. 2; Fig. S3). Indicator genera (*Streptococcus*, *Lactobacillus*, and *Bifidobacterium*) were selected based on previous studies (see Section 2.6). The relative abundance of the indicator genera was reduced at most time points in both Cu treatment groups when compared with the control (ANOVA, $P < 0.05$) (Fig. 3). This pattern was confirmed using the ANCOM-BC approach, which showed differentially abundant ASVs among different treatment groups at each time point (Figs. S4–S6). In general, both Cu treatments tended to decrease the relative abundances of a wide range of ASVs during the experiment, whereas only minor changes were observed when comparing the two Cu treatments. Correspondingly, the relative abundances of *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* ASVs were consistently reduced in response to both Cu treatments compared with the control (ANCOM-BC, $P < 0.05$). In contrast, the relative abundances of different ASVs including *Alloprevotella*, *Blautia*, and *Eubacterium hallii* were increased in response to the Cu treatments at certain time points.

3.3. Impacts of dietary Cu and pig growth stage on ARGs and MGEs

In total, 193 unique ARGs and 39 MGEs were detected by HT-qPCR chip analysis. The detected ARGs conferred resistance to all major classes of antibiotics targeted, of which most conferred resistance to aminoglycosides (42), multidrug determinants (33), Macrolide-Lincosamide-Streptogramins B (MLSBs) (30), and β-lactams (27) (Fig. S7A). The most common resistance mechanism was antibiotic deactivation, contributing to nearly half of the detected ARGs (Fig. S7B). Among detected MGEs, most encoded insertional sequences (18), plasmids (10), and transposases (8) (Fig. S7C).

No differences among treatment groups for ARG and MGE profiles were observed when microbiomes from all time points were compared (Fig. 4). Likewise, the diversity of detected ARGs and MGEs were not different between the two Cu treatment groups, but increased between the first time point (d 26) and later time points (d 61, 96, and 116) ($P < 0.001$) (Fig. 5A, B).

Overall, the gut antibiotic resistome appeared unaffected by dietary Cu treatments (Fig. 5). The relative abundance of ARGs (normalized to the corresponding 16S rRNA gene copy number) ranged from 0.31 to 1.46 with an average of 0.71, whereas the relative abundance of MGEs fluctuated from 0.21 to 2.70 with an average of 0.97. The most abundant ARGs conferred resistance to aminoglycosides (primarily *aph3-III*, *aphA3*, *sat4*, *ant6-Ia*, and *ant6-Ib*), MLSBs (primarily *lnuC*, *ermB*, *ermF*, and *ermQ*), and tetracyclines (primarily *tet44* and *tet32*). The relative abundance of ARGs was greater in samples collected at the first time point (d 26) compared with the other time points ($P < 0.001$). The relative abundance of MGEs was highly dominated by transposase encoding genes (primarily *Tp614* and *IS613*), constituting 99.7 % of all MGEs detected. To further explore the less abundant MGE groups, transposases were removed from the dataset.

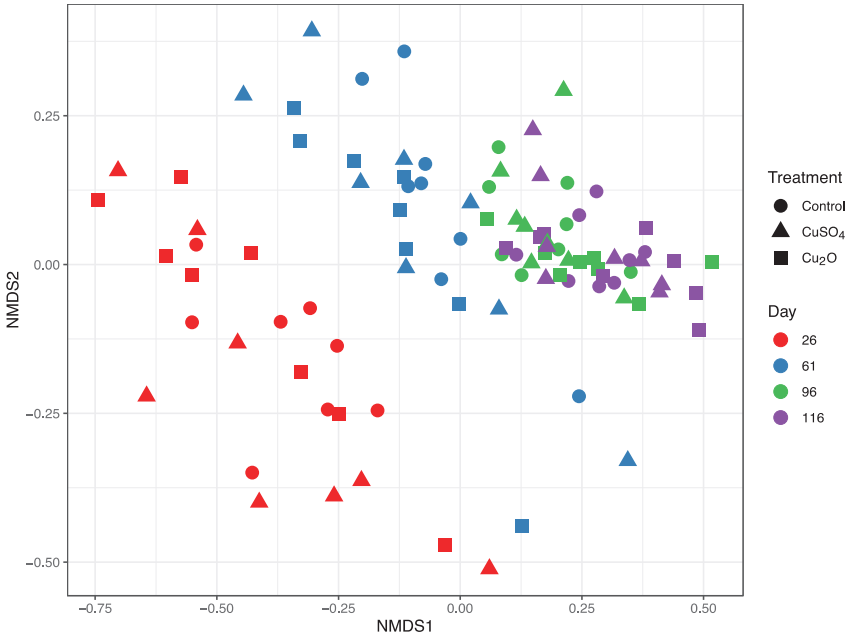


Fig. 2. Differences in bacterial community composition as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity index across all fecal samples ($n = 96$) based on the relative abundance of amplicon sequence variants (ASVs). Communities grouped by treatment (dietary Cu): Control (circles), $CuSO_4$ (copper sulfate, 250 μg g^{-1}) (triangles), and Cu_2O (monovalent copper oxide, 250 μg g^{-1}) (squares), and by the degree of gut microbiome maturation (days post-weaning): 26 (red dots), 61 (blue dots), 96 (green dots), and 116 (purple dots).

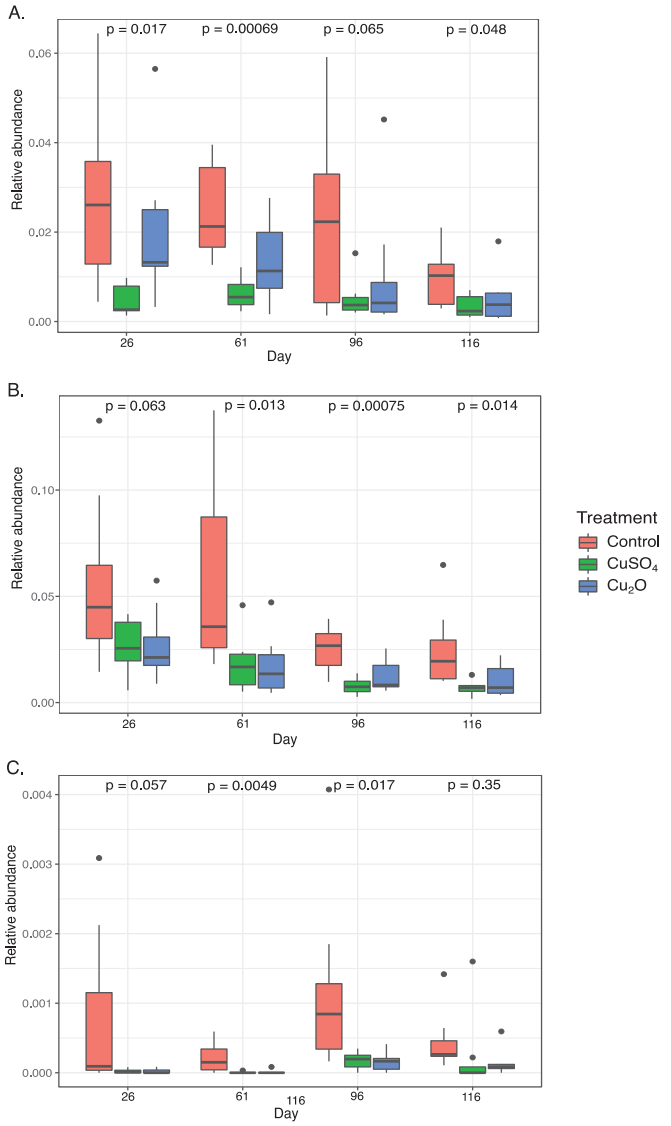


Fig. 3. Boxplots showing the relative abundance of indicator genera affected by dietary Cu treatments (Control; $CuSO_4$, 250 μg g^{-1}; Cu_2O, 250 μg g^{-1}) during gut microbiome maturation (26, 61, 96, and 116 d post-weaning). A. *Streptococcus*, B. *Lactobacillus*, or C. *Bifidobacterium*. Boxes 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. One-way ANOVA was used to evaluate differences in relative abundance between samples of different treatment groups at each time point (*p*-values are shown).

The relative abundance of genes encoding insertional sequences (primarily *IS91*, *IS26*, and *IS1247*), plasmids (primarily *trb-C*), and integrases (primarily *intl2*) varied between approx. 6×10^{-4} and 9×10^{-3} throughout the trial, but no Cu treatment effects were observed (Fig. S8). When normalized against the number of bacterial cells, the relative ARG copy number ranged between 1.24 and 5.84, and averaged 2.84 ARGs per cell. For MGEs (primarily transposases), the relative copy number ranged between 0.84 and 10.8 with an average of almost 4 MGEs per cell.

The composition of the antibiotic resistome changed ($P < 0.05$) during gut microbiome maturation (Fig. 6). Hence, irrespective of dietary Cu treatment, PCoA ordination demonstrated that resistomes from different time points clustered together (PCoA ordination), indicating that the ARG composition changed during gut microbiome maturation (Fig. 6A). The age of pigs explained approximately 32 % of the variance in the ARG composition (PERMANOVA, $R^2 = 0.323$, $P < 0.001$). By contrast, the dietary Cu treatments could not be distinguished using PCoA, indicating that they shared similar ARG profiles (Fig. 6B). Similar patterns were observed for MGE profiles, which changed composition over time (PERMANOVA, $R^2 = 0.252$, $P < 0.01$) without being affected by dietary Cu treatments (Fig. S9). However, when comparing the relative abundance of each individual gene, a few genes were impacted ($P < 0.05$) by Cu treatments at the different time points (Fig. S10-S13). For instance, the genes *arr3*, *ermX*, *aac(6)-ir*, *pAKD1-incP1*, and *lnuB* were reduced by the Cu treatments in samples collected 26 d after the start of the experiment compared with the control (Kruskal-Wallis, $P < 0.05$). In general, different genes were impacted at different time points, and only a few genes appeared to be consistently impacted throughout the trial. These genes included *tetM*, *lnuB*, and *lnuA*, which all were reduced by dietary Cu during multiple time points. Moreover, a gene associated with enterococci (i.e., a group of bacteria containing *Enterococcus* and *Streptococcus*) was reduced by the Cu treatments throughout multiple time points, which corresponds to the data obtained for the *Streptococcus* genus by the 16S rRNA gene amplicon sequencing.

We further performed network analysis to visualize co-occurrence patterns between individual ARGs and MGEs (Fig. S14). Networks of the different treatment groups were quite similar with only few strong ($r > 0.8$) and statistically significant ($P < 0.05$) correlations being observed. However, the networks of the control group appeared to be slightly simpler (i.e., less edges) compared to both Cu treatments. The transposase encoding gene, *tnpA-5*, was correlated with the beta-lactamase marker, *ampC*. In addition, the IS element, *IS630*, correlated with the tetracycline resistance gene, *tetD*. These correlations between antibiotic resistance determinants indicate the potential for horizontal transfer of ARGs in the swine gut.

3.4. Linkage between bacterial community composition and ARGs/MGEs

Procrustes analysis was used to examine the correlation between ARG and MGE profiles generated from HT-qPCR data and bacterial community composition as revealed by 16S rRNA gene amplicon sequencing analysis. ARG profiles were correlated with bacterial community composition (Procrustes sum of squares $M^2 = 0.82$, $R^2 = 0.42$, $P = 0.0001$, 9999 free permutations) (Fig. S15A). MGE profiles were also correlated with bacterial community composition (Procrustes sum of squares $M^2 = 0.95$, $R^2 = 0.24$, $P = 0.03$, 9999 free permutations), but not as clear as the ARG profiles (Fig. S15B). A Mantel's test using Spearman's rank correlation was also performed and confirmed a correlation between the ARG profile and the bacterial community composition based on Bray-Curtis metrics ($R^2 = 0.14$, $P = 0.01$, 999 free permutations). For the MGE profile, Mantel's test did not confirm a correlation with bacterial community structure ($R^2 = 0.01$, $P = 0.42$, 999 free permutations).

Spearman's rank correlation analysis revealed positive and negative correlations ($P < 0.05$) among bacterial genera and ARGs/MGEs in the swine gut (Fig. S16–17). A positive correlation ($P < 0.05$) in occurrence of *Bifidobacterium* and the tetracycline resistance gene, *tetM*, was observed. This corresponds well with the other data, indicating that Cu treatments reduce the abundance of *Bifidobacterium*, and thereby, also the *tetM* gene. In addition, *Streptococcus* and *Lactobacillus* were positively correlated ($P < 0.05$) with the lincomycin resistance gene, *lnuA*. In addition, *Escherichia* and *Shigella* genera were highly correlated ($P < 0.05$) with numerous multidrug resistance genes (*acrB*, *acrF tolC*, *IS26*, *mdtA*, *mdtE*, *mdtH*, *sugE*).

4. Discussion

4.1. Dietary Cu supplements had a marginal impact on the gut microbiome

To the best of our knowledge, we here report the first controlled longitudinal study examining the impacts of two contrasting Cu sources on the swine gut bacterial community and resistome. The microbiomes were dominated by ASVs belonging to the phyla *Firmicutes* and *Bacteroidetes* and the genus *Prevotella* spp., in accordance with previous studies on the swine gut (Kim et al., 2011; Lamendella et al., 2011; Niu et al., 2015). The

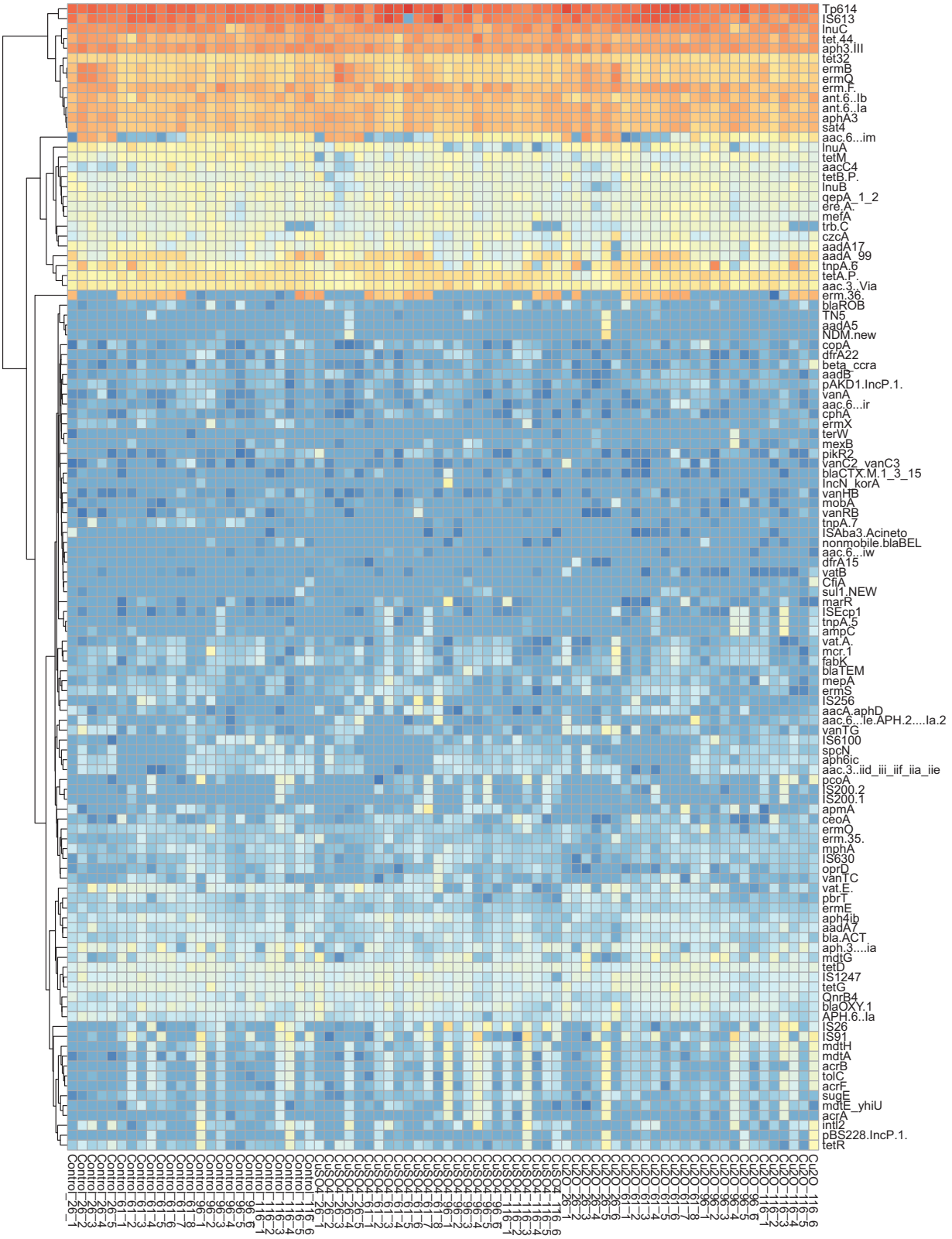


Fig. 4. Heatmap of most abundant genes ($n = 116$) related to antibiotic resistance and mobile genetic elements. The colour gradient represents the log-transformed relative gene abundance (normalized to the corresponding 16S rRNA gene copy numbers). Rows represent the results of each primer set (assay) shown on the y-axis. Columns represent fecal samples ($n = 76$) grouped by treatment (dietary Cu): Control, $CuSO_4$ (copper sulfate, 250 μg g^{-1}), and Cu_2O (monovalent copper oxide, 250 μg g^{-1}). The labels on the x-axis (i.e. Control_26_1) indicate the treatment, days post-weaning (26, 61, 96, or 116), and replicate number of the samples. Rows were clustered based on Euclidean distances.

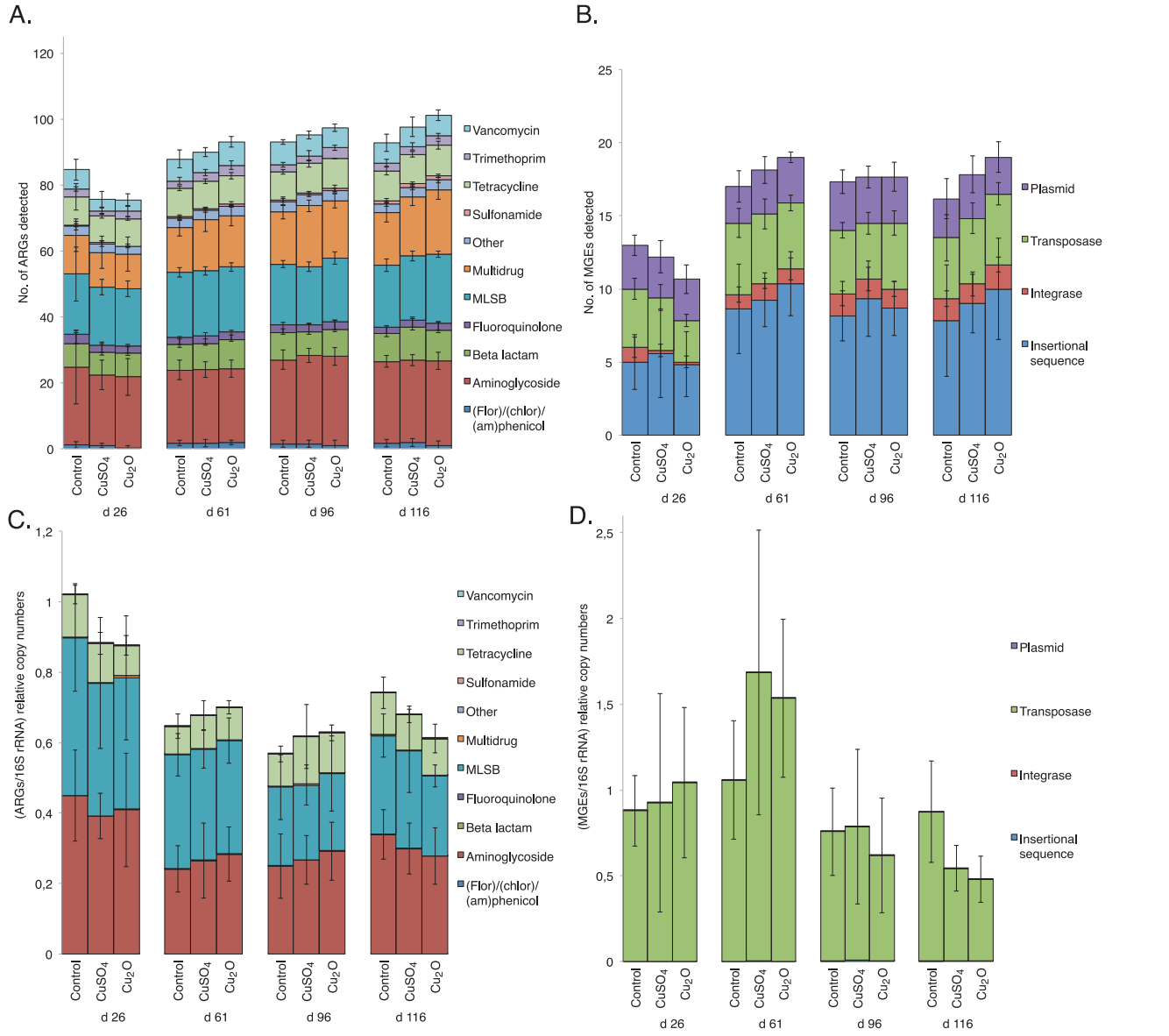


Fig. 5. Diversity and relative abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in pig fecal samples ($n = 76$) derived from three dietary Cu treatments (Control; $CuSO_4$, 250 μg g^{-1}; Cu_2O, 250 μg g^{-1}). Fecal samples were taken 26, 61, 96, and 116 days post-weaning. A. Average number of unique ARGs; B. Average number of unique MGEs C. Average relative abundance of ARGs; D. Average relative abundance of MGEs. The relative abundance data refer to the sum of relative abundance for all targeted ARGs or MGEs, respectively. MLSB, Macrolide-Lincosamide-Streptogramin B resistance. Error bars show the standard deviation.

bacterial community composition and antibiotic resistome (e.g., the relative abundance of ARGs and MGEs) were different among pig growth stages and microbiomes tended to converge over time, indicating gut microbiome maturation as reported previously (Kim et al., 2011; Lu et al., 2014; Niu et al., 2015). ARG profiles correlated with changes in bacterial community composition during gut microbiome maturation, indicating that bacterial community composition was a determining factor for the composition of the swine gut resistome. Previous swine gut microbiome studies have also demonstrated correlations between phylogeny and ARGs (Looft et al., 2012; Munk et al., 2018), whereas other studies have indicated a partial uncoupling between phylogenetic composition and antibiotic resistance profiles under selective pressures from antibiotics (Johnson et al., 2016) or non-antibiotic pig growth promoters (Muurinen et al., 2021).

Both Cu supplements consistently reduced the relative abundance of gut commensal bacteria belonging to the genera *Lactobacillus* and *Streptococcus*, which has also been reported in previous studies with $CuSO_4$ (Højberg et al., 2005; Zhang et al., 2019). In addition, the relative abundance of ASVs belonging to the genus *Bifidobacterium* also decreased in response to both Cu treatments consistent with findings from a recent in-feed metal (Zn + Cu) supplement trial in pigs (Muurinen et al., 2021). Correspondingly, the relative abundance of a few ARGs (*tetM*, *lnuB*, and *lnuA*) was consistently reduced in response to both Cu treatments throughout the experiment, and these genes all correlated with the relative abundance of the three genera (*Streptococcus*, *Lactobacillus*, and *Bifidobacterium*) that were affected by the dietary Cu treatments.

Although the two Cu supplements exerted similar and rather subtle impacts on the fecal microbiomes, some differences between the two Cu treatments were demonstrated in previous studies (Blavi et al., 2021; Forouzandeh et al., 2022). Hence, Cu_2O was a more efficient pig growth promoter than $CuSO_4$, but $CuSO_4$ resulted in greater Cu concentrations in the liver and spleen confirming different bioavailability of the two Cu sources when fed to pigs. Likewise, Cu_2O was more effective than $CuSO_4$ at reducing the relative abundance of *Streptococcaceae* and *Lactobacillaceae* when fed to broiler chickens, (Forouzandeh et al., 2021). Strongly contrasting effects of mono- and divalent Cu forms in bacteria are well-known from in vitro studies, and monovalent Cu inhibit growth and deplete populations

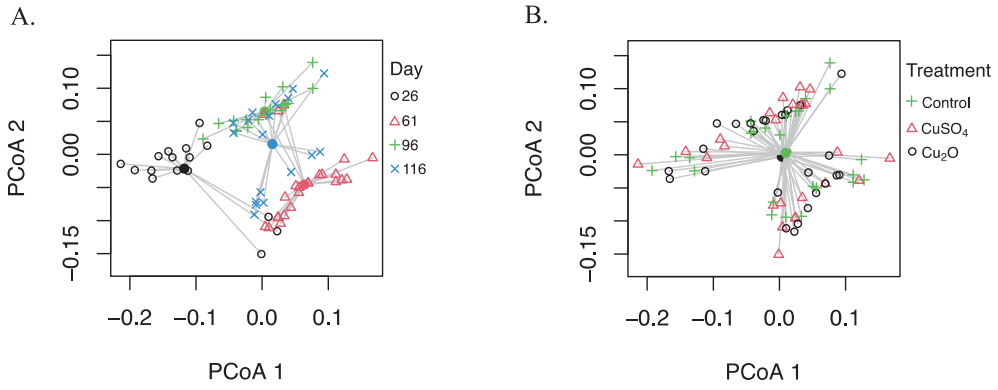


Fig. 6. Differences in gut bacterial resistome composition as revealed by principal coordinates analysis (PCoA) of the relative abundance (normalized to the corresponding 16S rRNA gene copy numbers) of antibiotic resistance genes (ARGs) using Bray-Curtis dissimilarity index. A. Resistomes grouped by degree of gut microbiome maturation (days post-weaning): 26 (black circles), 61 (red triangles), 96 (green plus signs), and 116 (blue crosses). B. Resistomes grouped by treatment (dietary Cu): Control (green crosses); $CuSO_4$, 250 μg g^{-1} (red triangles); Cu_2O, 250 μg g^{-1} (black circles).

of *E. coli* and *Staphylococcus aureus* much more effectively than divalent Cu (Saphier et al., 2018; Popov et al., 2020). However, the differential effects of mono- and divalent Cu sources was expected to be much weaker in the pig gut due to the convergence of chemical Cu speciation during gut passage. This hypothesis is in accordance with results from the present study demonstrating that bioavailability of fecal Cu to bacteria was not different between the two sources of Cu as assessed by our *P. fluorescens* bioreporter assay.

4.2. Does Cu-based feed supplements constitute a risk for co-selection of ARGs in livestock?

Results of the present experiment demonstrate that swine gut microbiomes, including ARGs and MGEs, were only modestly affected by dietary Cu treatments and that the observed changes in ARG profiles was linked to phylogeny rather than co-selection of antibiotic resistance. This main finding is remarkable, as the two dietary Cu sources resulted in at least 670- or 1000-fold increased levels of bioavailable Cu in feces. Bioavailable Cu to bacteria (i.e., *P. fluorescens* bioreporter) thus increased by at least two orders of magnitude more than total Cu did as a result of dietary Cu supplementation demonstrating a high bioavailability of Cu added to feed as $CuSO_4$ or Cu_2O. Such levels of Cu are highly likely to exert significant selection pressure for Cu resistance in gut bacteria in accordance with previous research (Hasman et al., 2006; Medardus et al., 2014; Yazdankhah et al., 2014). The current results thus indicate that gut bacterial communities may develop Cu resistance without affecting the overall bacterial community composition in a similar fashion as previously reported for bacterial communities in soil (Brandt et al., 2010).

The observed inability of Cu-based growth promoters to co-select ARGs (our study) is consistent with results from a related experiment investigating effects of non-antibiotic growth promoters including a feed supplement treatment with both ZnO (2–3 g kg^{-1}) and $CuSO_4$ (125 mg kg^{-1}) (Muurinen et al., 2021). However, results of these studies employing similar experimental designs (controlled field experiments during one pig growth cycle) and methodologies (HT-qPCR and 16S rRNA gene amplicon sequencing) contrast with other reports indicating risk for Cu- and/or Zn-induced co-selection of antibiotic resistance in the pig gut and other environments as reviewed previously (Yazdankhah et al., 2014; Wales and Davies, 2015; Poole, 2017). Several studies have demonstrated co-selection of resistance to Cu and antibiotics in specific groups of pig gut bacteria. It is also well-known that some plasmids contain genes conferring resistance to both Cu and antibiotics and such plasmids are enriched in bacteria of fecal origin relative to other environments (Pal et al., 2015; Fang et al., 2016). Moreover, a recent high-throughput metagenomic study in pigs indicated that metals contributed to the maintenance of antibiotic resistance in pig farming, of which pathogens belonging to *Enterobacteriaceae* tended to have more DNA-fragments with co-occurring ARGs, MRGs, and MGEs (Li et al., 2022). These results are consistent with results from the present experiment indicating that the relative abundance of *Escherichia/Shigella* was highly correlated with several multidrug resistance genes, of which *acrB/F* and *mdtA/E/H* can confer cross-resistance towards antibiotics and metals including Cu. Transcription of these genes encoding multidrug efflux pumps can be activated by the BaeRS two-component system, which is induced by Cu and Zn stress (Nishino et al., 2007; Nishino et al., 2005).

Despite a clear co-selection potential in swine gut microbiomes (as judged from the literature), we did not observe any evidence that high dietary levels of Cu did actually co-select ARGs. We propose two potential explanations for this result. The first explanation has been termed 'ecological fallacy' and relates to the used total microbiome approach based on analysis of metagenomic DNA (Agga et al., 2015). The strength of the used fecal microbiome approach is that it allows experimenters to study the total bacterial community with very high community coverage, but this at the same time offers some limitations, as it is not possible to link the presence of specific ARGs and MGEs to specific taxa with high confidence. Hence, it is indeed possible that Cu could have co-selected for Cu and antibiotic resistance in specific groups of gut bacteria in our study. On the other hand, it is also possible that selection for Cu resistance may lead to enhanced antibiotic susceptibility in other groups of gut bacteria by a phenomenon termed 'collateral sensitivity' (Pál et al., 2015) and such effects may also be overlooked by the used total microbiome approach. It is also a possibility that Cu-induced changes in the antibiotic resistome may simply be a result of a Cu-induced change in the bacterial community composition as different taxa tend to harbor different ARGs.

The second explanation for a lack of Cu-induced co-selection of antibiotic resistance is that the studied pig microbiomes carried high background levels of ARGs and MGEs combined with high persistence of ARGs (Kazimierczak et al., 2009; Looft et al., 2012; Pollock et al., 2020; Muurinen et al., 2021). Hence, the previous use of antibiotics may have supported the establishment of a high background level of antibiotic resistance in the swine gut microbiomes giving rise to legacy effects from previous generations of pigs. High background levels of antibiotic resistance in swine may also explain why only limited or transient effects of antibiotic treatment on the gut microbiome have been observed in some previous experiments (Holman and Chénier, 2014; Pollock et al., 2020). Antibiotic resistance is typically associated with a fitness cost and in the absence of selection pressure, this should in principle benefit the antibiotic-susceptible bacteria enabling them to outcompete the resistant bacteria over time (Andersson and Hughes, 2010). However, antibiotic resistance at the community level can be highly persistent for a number of reasons

such as fitness-compensatory evolution, cost-free adaptive mutations, and co-selection of ARGs (Andersson and Hughes, 2010). Even if antibiotic resistance comes with a fitness cost, antibiotic-susceptible bacteria may not always be able to replace resistant populations belonging to the same species, if the latter has been already firmly established in the gut due to priority effects resulting in colonization resistance (Segura Munoz et al., 2022).

5. Conclusions and perspectives

The swine gut microbiome is of significant concern for public health as the transmission of antibiotic resistant bacteria (including pathogens) to humans may occur via various environmental transmission pathways (Muloi et al., 2018; Zhao et al., 2021). The combined use of metallic growth promotors (mainly Cu and Zn) and antibiotics in pig production constitutes a risk for co-selection of metal and antibiotic resistance in bacteria. However, results from the present experiment did not provide evidence for the role of Cu in the co-selection of ARGs and MGEs even at high levels of dietary Cu exceeding the maximal permitted level for pig diets in the EU (25 to 150 μg Cu g^{-1} feed depending on pig age). Although reassuring, the obtained results do not rule out the possibility that Cu may co-select for antibiotic resistance in some specific groups of gut bacteria as reported in earlier studies. Cu feed supplements may also pose a risk for the long-term persistence of ARGs in pig microbiomes, and there is a need for controlled long-term studies over several pig generations to study this issue. Cu and other metals can modulate conjugal plasmid transfer within microbiomes (Klümper et al., 2016; Song et al., 2020). Hence, horizontal gene transfer of ARGs should also be considered in future studies aiming to investigate the impacts of metallic growth promoters on the pig gut microbiome.

CRediT authorship contribution statement

Julius Emil Brinck: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing – original draft. **Simon Bo Lassen:** data curation, formal analysis, methodology, software, validation, writing – review & editing, supervision. **Asal Forouzandeh:** writing – review & editing. **Ting Pan:** investigation, writing – review & editing. **Yan-Zi Wang:** investigation, writing – review & editing. **Alessandra Monteiro:** writing – review & editing. **Laia Blavi:** resources, writing – review & editing. **David Solà-Oriol:** writing – review & editing. **Hans H. Stein:** writing – review & editing. **Jian-Qiang Su:** writing – review & editing, supervision. **Kristian K. Brandt:** conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, writing – review & editing. All authors read and approved the final manuscript.

Funding

This work was supported by Animine, a company specializing in the precision mineral nutrition of food-producing animals, in Annecy, France.

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). Laia Blavi is now an AB Neo employee. There are no conflicts of interest among the other authors.

Acknowledgments

Susanne Iversen is acknowledged for technical assistance and support. The support of the UAB-Banco de Santander Talent Program is also thanked for the provision of research scholarship for David Solà-Oriol.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159609.

References

Agga, G.E., Scott, H.M., Vinasco, J., Nagaraja, T.G., Amachawadi, R.G., Bai, J., Norby, B., Renter, D.G., Dritz, S.S., Nelssen, J.L., Tokach, M.D., 2015. Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the fecal metagenome of weaned pigs. Prev. Vet. Med. 119, 179–189. https://doi.org/10.1016/J.PREVETMED.2015.02.008.

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., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. 84 (8), 260–271. https://doi.org/10.1038/nrmicro2319 2010.

Argudín, M.A., Deplano, A., Meghraoui, A., Dodémont, M., Heinrichs, A., Denis, O., Nonhoff, C., Roisin, S., 2017. Bacteria from Animals as a Pool of Antimicrobial Resistance Genes. Antibiot. 6 (12), 12–16. https://doi.org/10.3390/ANTIBIOTICS6020012 2017.

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G.J., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001. https://doi.org/10.1289/ehp.1206316.

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.

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. 1–10. https://doi.org/10.1093/jas/skab127.

Brandt, K.K., Holm, P.E., Nybroe, O., 2008. Evidence for bioavailable copper-dissolved organic matter complexes and transiently increased copper bioavailability in manure-amended soils as determined by bioluminescent bacterial biosensors. Environ. Sci. Technol. 42, 3102–3108. https://doi.org/10.1021/es071916+.

Brandt, K.K., Frandsen, R.J.N., Holm, P.E., Nybroe, O., 2010. Development of pollution-induced community tolerance is linked to structural and functional resilience of a soil bacterial community following a five-year field exposure to copper. Soil Biol. Biochem. 42, 748–757. https://doi.org/10.1016/j.soilbio.2010.01.008.

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.

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 11, 2639–2643. https://doi.org/10.1038/ismej.2017.119.

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 10 June 2021).

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1096–1121. https://doi.org/10.1080/01621459.1955.10501294.

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. Animal Sci. Biotechnol. 12. https://doi.org/10.1186/s40104-020-00533-3.

EU, 2005. Ban on antibiotics as growth promoters in animal feed enters into effect (1831/2003/EC). http://europa.eu/rapid/press-release_IP-05-1687_en.htm. (Accessed 20 May 2021).

EU, 2017. Commission Implementing Decision of 26.6.2017 concerning, in the framework of Article 35 of Directive 2001/82/EC of the European Parliament and of the Council, the marketing authorisations for veterinary medicinal products containing 'zinc oxide' to be administered orally to food producing species, COM(2017) 4529 final.

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., Pérez, J., 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, 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. 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., 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.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. (Berl) 97, 207–237. https://doi.org/10.1111/J.1439-0396.2012.01284.X.

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.

Holman, D.B., Chénier, M.R., 2014. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. FEMS Microbiol. Ecol. 90, 599–608. https://doi.org/10.1111/1574-6941.12419 Hu, H.-W., Wang, J.-T., Li, J., Shi, X.-Z., Ma, Y.-B., Chen, D., 2017. Long-term nickel contamination increases the occurrence of antibiotic resistance genes in agricultural soils. Environ. Sci. Technol. 51, 790-800.

Hu, Y.J., Cowling, B.J., 2020. Reducing antibiotic use in livestock. China. Bull. World Health Organ. 98, 360–361. https://doi.org/10.2471/BLT.19.243501.

Hu, H.-W., Wang, J.-T., Li, J., Shi, X.-Z., Ma, Y.-B., Chen, D., 2017. Long-term nickel contamination increases the occurrence of antibiotic resistance genes in agricultural soils. Environmental Science and Technology 51 (2), 790–800. https://doi.org/10.1021/acs.est.6b03383.

Johnson, T.A., Stedtfeld, R.D., Wang, Q., Cole, J.R., Hashsham, S.A., Looft, T., Zhu, Y.G., Tiedje, J.M., 2016. Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. MBio 7. https://doi.org/10.1128/mBio.02214-15.

Kazimierczak, K.A., Scott, K.P., Kelly, D., Aminov, R.I., 2009. Tetracycline resistome of the organic pig gut. Appl. Environ. Microbiol. 75, 1717–1722. https://doi.org/10.1128/AEM.02206-08.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J., Isaacson, R.E., 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Vet. Microbiol. 153, 124–133. https://doi.org/10.1016/j.vetmic.2011.05.021.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. Rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184. https://doi.org/10.1093/NAR/29.1.181.

Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., Smets, B.F., 2016. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 2017 111 (11), 152–165. https://doi.org/10.1038/ismej.2016.98.

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. 11, 1–17. https://doi.org/10.1186/1471-2180-11-103/FIGURES/6.

Legendre, P., Legendre, L., 1998. Numerical ecology. 2nd Englis. ed. Elsevier.

Li, X., Rensing, C., Vestergaard, G., Arumugam, M., Nesme, J., Gupta, S., Brejnrod, A.D., Sørensen, S.J., 2022. Metagenomic evidence for co-occurrence of antibiotic, biocide and metal resistance genes in pigs. Environ. Int. 158, 106899. https://doi.org/10.1016/J.ENVINT.2021.106899.

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.

Linder, M.C., Hazegh-Azam, M., 1996. Copper biochemistry and molecular biology. Am. J. Clin. Nutr. https://doi.org/10.1093/ajcn/63.5.797.

Liu, Y., Espinosa, C.D., Abelilla, J.J., Casas, G.A., Lagos, L.V., Lee, S.A., Kwon, W.B., Mathai, J.K., Navarro, D.M.D.L., Jaworski, N.W., Stein, H.H., 2018. Non-antibiotic feed additives in diets for pigs: a review. Anim. Nutr. 4, 113–125. https://doi.org/10.1016/J.ANINU.2018.01.007.

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.

Lu, X.M., Lu, P.Z., Zhang, H., 2014. Bacterial communities in manures of piglets and adult pigs bred with different feeds revealed by 16S rDNA 454 pyrosequencing. Appl. Microbiol. Biotechnol. 98, 2657–2665. https://doi.org/10.1007/S00253-013-5211-4/FIGURES/3.

Medardus, J.J., Molla, B.Z., Nicol, M., Morrow, W.M., Rajala-Schultz, P.J., Kazwala, R., Gebreyes, W.A., 2014. In-feed use of heavy metal micronutrients in U.S. Swine production systems and its role in persistence of multidrug-resistant salmonellae. Appl. Environ. Microbiol. 80, 2317–2325. https://doi.org/10.1128/AEM.04283-13.

Muloi, D., Ward, M.J., Pedersen, A.B., Fèvre, E.M., Woolhouse, M.E.J., Van Bunnik, B.A.D., 2018. Are food animals responsible for transfer of antimicrobial-resistant Escherichia coli or their resistance determinants to human Populations? A Systematic Review. Foodborne Pathog. Dis. 15, 467–474. https://doi.org/10.1089/fpd.2017.2411.

Munk, P., Knudsen, B.E., Lukjacenko, O., Duarte, A.S.R., Van Gompel, L., Luiken, R.E.C., Smit, L.A.M., Schmitt, H., Garcia, A.D., Hansen, R.B., Petersen, T.N., Bossers, A., Ruppé, E., Graveland, H., van Essen, A., Gonzalez-Zorn, B., Moyano, G., Sanders, P., Chauvin, C., David, J., Battisti, A., Caprioli, A., Dewulf, J., Blaha, T., Wadepohl, K., Brandt, M., Wasyl, D., Skarzyńska, M., Zajac, M., Daskalov, H., Saatkamp, H.W., Stärk, K.D.C., Lund, O., Hald, T., Pamp, S.J., Vigre, H., Heederik, D., Wagenaar, J.A., Mevius, D., Aarestrup, F.M., 2018. Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine european countries. Nat. Microbiol. 3, 898–908. https://doi.org/10.1038/s41564-018-0192-9.

Muurinen, J., Richert, J., Wickware, C.L., Richert, B., Johnson, T.A., 2021. Swine growth promotion with antibiotics or alternatives can increase antibiotic resistance gene mobility potential. Sci. Rep. 11, 1–13. https://doi.org/10.1038/s41598-021-84759-9.

Nishino, K., Honda, T., Yamaguchi, A., 2005. Genome-wide analyses of Escherichia coli gene expression responsive to the BaeSR two-component regulatory system. J. Bacteriol. 187, 1763–1772. https://doi.org/10.1128/JB.187.5.1763-1772.2005.

Nishino, K., Nikaido, E., Yamaguchi, A., 2007. Regulation of multidrug efflux systems involved in multidrug and metal resistance of salmonella enterica serovar typhimurium. J. Bacteriol. 189, 9066–9075. https://doi.org/10.1128/JB.01045-07.

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. Reports 2015 51 (5), 1–7. https://doi.org/10.1038/srep09938.

Nybroe, O., Brandt, K.K., Ibrahim, Y.M., Tom-Petersen, A., Holm, P.E., 2008. Differential bioavailability of copper complexes to bioluminescent Pseudomonas fluorescens reporter strains. Environ. Toxicol. Chem. 27, 2246–2252. https://doi.org/10.1897/08-025.1.

Oksanen, A.J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., Hara, R.B.O., Wagner, H., 2019. vegan: Community Ecology Package. R package version 2.5-4.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16, 1–14. https://doi.org/10.1186/s12864-015-2153-5.

Pál, C., Papp, B., Lázár, V., 2015. Collateral sensitivity of antibiotic-resistant microbes. Trends Microbiol. 23, 401–407. https://doi.org/10.1016/J.TIM.2015.02.009.

Park, C.S., Kim, B.G., 2016. In vitro solubility of Copper(II) sulfate and dicopper chloride trihydroxide for pigs. Asian-Australasian J. Anim. Sci. 29, 1608–1615. https://doi.org/10.5713/ajas.16.0189.

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. Reports 2020 101 (10), 1–10. https://doi.org/10.1038/s41598-020-58659-3.

Poole, K., 2017. At the nexus of antibiotics and metals: the impact of cu and zn on antibiotic activity and resistance. Trends Microbiol. 25, 820–832. https://doi.org/10.1016/j.tim.2017.04.010.

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.

Saphier, M., Silberstein, E., Shotland, Y., Popov, S., Saphier, O., 2018. Prevalence of monovalent copper over divalent in killing Escherichia coli and Staphylococcus aureus. Curr. Microbiol. 75, 426–430. https://doi.org/10.1007/S00284-017-1398-4/FIGURES/1.

Segura Munoz, R.R., Mantz, S., Martínez, I., Li, F., Schmaltz, R.J., Pudlo, N.A., Urs, K., Martens, E.C., Walter, J., Ramer-Tait, A.E., 2022. Experimental evaluation of ecological principles to understand and modulate the outcome of bacterial strain competition in gut microbiomes. ISME J. 2022 166 (16), 1594–1604. https://doi.org/10.1038/s41396-022-01208-9.

Song, J., Klümper, U., Riber, L., Dechesne, A., Smets, B.F., Sørensen, S.J., Brandt, K.K., 2020. A converging subset of soil bacterial taxa is permissive to the IncP-1 plasmid pKJK5 across a range of soil copper contamination. FEMS Microbiol. Ecol. 96. https://doi.org/10.1093/FEMSEC/FIAA200.

Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., Gunturu, S., Tift, L., Wang, F., Howe, A., Chai, B., Yin, D., Cole, J.R., Tiedje, J.M., Hashsham, S.A., 2018. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. FEMS Microbiol. Ecol. 94. https://doi.org/10.1093/FEMSEC/FIY130.

Tom-Petersen, A., Hosbond, C., Nybroe, O., 2001. Identification of copper-induced genes in Pseudomonas fluorescens and use of a reporter strain to monitor bioavailable copper in soil. FEMS Microbiol. Ecol. 38, 59–67. https://doi.org/10.1111/j.1574-6941.2001.tb00882.x.

U.S. Food and Drug Administration, 2018. Summary report on antimicrobial sold or distributed for use in food-producing animals.

Wales, A.D., Davies, R.H., 2015. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics 4, 567–604. https://doi.org/10.3390/antibiotics4040567.

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/SUPPL_FILE/SYS001160029ST4.DOCX.

WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance 2014. World Health Organization, Geneva, Switzerland.

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

Wright, E.S., 2015. DECIPHER: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinformatics 16, 1–14. https://doi.org/10.1186/S12859-015-0749-Z/FIGURES/6.

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. Heal. Dis. 25. https://doi.org/10.3402/mehd.v25.25862.

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.

Zhao, Y., Su, J.Q., An, X.L., Huang, F.Y., Rensing, C., Brandt, K.K., Zhu, Y.G., 2018. Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. Sci. Total Environ. 621, 1224–1232. https://doi.org/10.1016/J.SCITOTENV.2017.10.106.

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.