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

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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.

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 (CuSO4; 250 μg Cu g−1 feed), and monovalent copper oxide (Cu2O; 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 by 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.
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 microorganisms 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; Medaruds 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 Cu starting at 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 (Cu2+) and reduced cuprous (Cu+; Linder and Hazegh-Azam, 1996). Divalent copper (II) sulfate (CuSO4) is a common growth promoter in pig diets (Park and Kim, 2016), but monovalent copper oxide (CuO2) 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 CuSO4 and Cu2O 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 CuSO4 and Cu2O 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 resistance 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. Offspring 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 CuSO4 g−1 feed (control group), which is the minimal recommended dose of Cu for growing-finishing pigs, control diet supplemented with 250 μg CuSO4 g−1 feed (CuSO4 group), or control diet supplemented with 250 μg Cu2O g−1 feed (Cu2O 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 (GTGYCAGCMGCCGCGGTA A) 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 (Ct) of 31 was used as the detection limit for the individual PCR reactions. Ct 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\(^{(Ct_{target}−Ct_{reference})}/10^{30}/2\) as described previously (Looff 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 (Rlavi 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 plotordination. Indicator genera (Streptococcus, Lactobacillus, and Bifidobacterium) affected by dietary Cu treatments were selected based on previous studies (Hu et al., 2017). ANOVA was used to examine differences in Cu concentration in dietary treatments (Table 1). In addition, [Cu\(_{bio}\)] levels were slightly higher for the CuSO\(_4\) group compared with the CuO 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 treatment groups 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 g \(\mu\)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>
<thead>
<tr>
<th>Treatment</th>
<th>[Cu(_{bio})] (g (\mu)g(^{-1}))</th>
<th>[Cu(_{total})] (g (\mu)g(^{-1}))</th>
<th>[Cu(<em>{bio})]/[Cu(</em>{total})] (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;7 × 10(^{-4})</td>
<td>295 ± 74</td>
<td>&lt;2.49 × 10(^{-4})</td>
</tr>
<tr>
<td>CuSO(_4)</td>
<td>0.47 ± 0.25</td>
<td>2900 ± 438</td>
<td>0.016 ± 0.007</td>
</tr>
<tr>
<td>CuO</td>
<td>0.70 ± 0.50</td>
<td>2356 ± 357</td>
<td>0.029 ± 0.019</td>
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a [Cu\(_{bio}\)] of control samples was below the detection limit (<7 × 10\(^{-4}\) g \(\mu\)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, CuSO₄ (copper sulfate, 250 μg g⁻¹), and Cu₂O (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. 1).

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. 1; 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, but increased between the first time point (d 26) and later time points (d 61, 96, and 116) (\( P < 0.001 \)) (Fig. 2).

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treated 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 0.97. The most abundant ARGs conferred resistance to aminoglycosides (primarily *aph3-IIIa*, *aph3-III*, *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 *Tn614* 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 \( \mu \)g g\(^{-1}\)) (triangles), and CuO (monovalet copper oxide, 250 \( \mu \)g 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).](image-url)
The relative abundance of genes encoding insertional sequences (primarily \textit{IS91}, \textit{IS26}, and \textit{IS1247}), plasmids (primarily \textit{trn-C}), and integrases (primarily \textit{int2}) varied between approx. $6 \times 10^{-4}$ and $9 \times 10^{-3}$ 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 P-CoA, 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 \textit{ars3}, \textit{ermX}, \textit{aac(6)-ir}, \textit{pAKD1-incP1}, and \textit{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 \textit{tetM}, \textit{lnuA}, and \textit{lnuB}, which all were reduced by dietary Cu during multiple time points. Moreover, a gene associated with enterococci (i.e., a group of bacteria containing \textit{Enterococcus} and \textit{Streptococcus}) was reduced by the Cu treatments throughout multiple time points, which corresponds to the data obtained for the \textit{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, \textit{trpA-5}, was correlated with the beta-lactamase marker, \textit{ampC}. In addition, the IS element, \textit{IS630}, correlated with the tetracycline resistance gene, \textit{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 \textit{Bifidobacterium} and the tetracycline resistance gene, \textit{tetM}, was observed. This corresponds well with the other data, indicating that Cu treatments reduce the abundance of \textit{Bifidobacterium}, and thereby, also the \textit{tetM} gene. In addition, \textit{Streptococcus} and \textit{Lactobacillus} were positively correlated ($P < 0.05$) with the lincomycin resistance gene, \textit{lnuA}. In addition, \textit{Escherichia} and \textit{Shigella} genera were highly correlated ($P < 0.05$) with numerous multidrug resistance genes (\textit{acrB}, \textit{acrF isoC}, \textit{IS26}, \textit{mdrA}, \textit{mdrE}, \textit{mdrH}, \textit{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 microorganisms were dominated by ASVs belonging to the phyla \textit{Firmicutes} and \textit{Bacteroidetes} and the genus \textit{Prevotella} spp., in accordance with previous studies on the swine gut (Kim et al., 2011; Lamendella et al., 2011; Niu et al., 2015).
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, CuSO₄ (copper sulfate, 250 μg g⁻¹), and Cu₂O (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.
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 (Loofte 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 CuSO₄ (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, Cu₂O was a more efficient pig growth promoter than CuSO₄, but CuSO₄ resulted in greater Cu concentrations in the liver and spleen confirming different bioavailability of the two Cu sources when fed to pigs. Likewise, Cu₂O was more effective than CuSO₄ 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...
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 CuSO₄ or Cu₂O. 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; Yazdankah 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 CuSO₄ (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 environmental samples as reviewed previously (Yazdankah 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, MGEs, 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’ (Pål 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 resistance 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; Looff 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.

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**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); CuSO₄, 250 μg g⁻¹ (red triangles); Cu₂O, 250 μg g⁻¹ (black circles).
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 (Muloti et al., 2018; Zhao et al., 2021). The combined use of metallic growth promoters (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 (Kümmerl 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


Appendix A. Supplementary data

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References


