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Uncovering the effects of copper feed supplementation on the selection of copper-tolerant and antibiotic-resistant *Enterococcus* in poultry production for sustainable environmental practices

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ABSTRACT

The use of antibiotics in animal production is linked to the emergence and spread of antibiotic-resistant bacteria, a threat to animal, environmental and human health. Copper (Cu) is an essential element in poultry diets and an alternative to antibiotics, supplementing inorganic or organic trace mineral feeds (ITMF/OTMF). However, its contribution to select multidrug-resistant (MDR) and Cu tolerant *Enterococcus*, a bacteria with a human-animal-environment-food interface, remains uncertain. We evaluated whether feeding chickens with Cu-ITMF or Cu-OTMF contributes to the selection of Cu tolerant and MDR *Enterococcus* from rearing to slaughter. Animal faeces [2–3-days-old ($n = 18$); pre-slaughter ($n = 16$)] and their meat (n = 18), drinking-water ($n = 14$) and feed (n = 18) from seven intensive farms with ITMF and OTMF flocks (10.000–64.000 animals each; 2019–2020; Portugal) were sampled. *Enterococcus* were studied by cultural, molecular and whole-genome sequencing methods and Cu concentrations by ICP-MS. *Enterococcus* ($n = 477$; 60 % MDR) were identified in 80 % of the samples, with >50 % carrying isolates resistant to tetracycline, quinupristin-dalfopristin, erythromycin, streptomycin, ampicillin or ciprofloxacin. *Enterococcus* with Cu tolerance genes, especially *tcrB* ± *cueO,* were mainly found in faeces (85 %; *E. faecium/E. lactis*) of ITMF/OTMF flocks. Similar occurrence and load of *tcrB* ± *cueO Enterococcus* in the faeces was detected throughout the chickens' lifespan in the ITMF/OTMF flocks, decreasing in meat. Most of the polyclonal MDR *Enterococcus* population carrying *tcrB* ± *cueO* or only *cueO* (67 %) showed a wild-type phenotype ($MIC_{CuSO4} \leq 12$ mM) linked to absence of *tcrYAZB* or truncated variants, also detected in 85 % of *Enterococcus* public genomes from poultry. Finally, < 65 μg/g Cu was found in all faecal and meat samples. In conclusion, Cu present in ITMF/OTMF is not selecting Cu tolerant and MDR *Enterococcus* during chickens' lifespan. However, more studies are needed to assess the minimum concentration of Cu required for MDR bacterial selection and horizontal transfer of antibiotic resistance genes, which would support sustainable practices mitigating antibiotic resistance spread in animal production and the environment beyond.

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1. Introduction

Intensive poultry farming is widely recognized as one of the most efficient animal husbandry production systems, with standards evolving in recent decades to meet the nutritional requirements of animals and the high demand from consumers around the world (Gržinić et al., 2023; Hafez and Attia, 2020). However, this intensive production has a significant environmental footprint often associated with waste materials with impact in the animal-environment-human ecosystems (Gržinić et al., 2023). The European Union (EU) is one of the worlds' leading producers of poultry meat (European Parliament and Augère-Granier, 2019), with approximately 13.2 million tonnes available for consumption in 2021 (Eurostat, 2022). Most poultry raised in the EU is reared in intensive farming systems with flocks comprising thousands of fast-growing animals (European Parliament and Augère-Granier, 2019) which might be under antibiotics to treat infections or, in exceptional cases, used as prophylactic or metaphylactic agents [Regulation (EU), 2019a]. Several efforts are being made to meet the EU goal of reducing sales of antibiotics to farm animals by 50 % by 2030, helping to prevent or reduce consumer exposure to antibiotic-resistant bacteria and the associated environmental impact (European Commission, 2021). Among such efforts is the replacement of antibiotics with alternative compounds, such as metals (Gadde et al., 2017). Copper (Cu) is an essential trace element widely used in poultry diets, acting as a critical cofactor and enzyme component for several biological processes (Shannon and Hill, 2019), including at cell membranes or in ATP production (Dalecki et al., 2017). Its antimicrobial properties have also been associated with poultry growth promotion, modulating the gut microbiota profile (Forouzandeh et al., 2021).

Cu has been recommended at 8 mg Cu/kg in poultry diet to fulfil the nutritional requirements of the animals (National Research Council, 1994), although it can be used until a maximum allowed concentration of 25 mg Cu/kg according to EU legislation [Commission Implementing Regulation (EU) 2018/1039, 2018]. It can be provided to poultry through inorganic trace mineral feed (ITMF) or organic trace mineral feed (OTMF) formulations. ITMF is a cost-effective solution conventionally used for decades in poultry production (da Cruz Ferreira Júnior et al., 2022; Lu et al., 2020), but less bioavailable than OTMF, requiring higher concentrations in the feed to avoid animal deficiencies in Cu (De Marco et al., 2017). Furthermore, when using inorganic Cu forms, a higher amount of the metal is expected to be released in the birds' faeces (De Marco et al., 2017), which could create a serious environmental footprint, with impact on different compartments (air, water, manured soil) (Gržinić et al., 2023). The OTMF formulations, including Cu chelated with amino acids, peptides or proteins, are described to be more efficiently absorbed by animals (da Cruz Ferreira Júnior et al., 2022; Lu et al., 2020) and represent a promising alternative to improve feed conversion, increasing nutrient digestibility and poultry growth (Lu et al., 2020), while decreasing the excretion of minerals into the environment (De Marco et al., 2017). Despite the benefits of Cu feed supplementation for poultry growth and well-being, whether with organic or inorganic formulations, it can also potentially select for Cu tolerant bacteria and co-select for antibiotic-resistant bacteria, as metal and antibiotic resistance genes are often co-located on the same mobile genetic elements (Fang et al., 2016; Mourão et al., 2016a; Rebelo et al., 2023; Rebelo et al., 2021).

Recent metagenomic studies have shown that Bacillota (former Firmicutes) are one of the main taxa identified in the poultry gut (Feng et al., 2021). Among them, *Enterococcus* spp. are ubiquitous bacteria that can be found at the human-animal-environment-food interface, and are highly adapted to various stressors such as metals, pH, and temperature (Gaca and Lemos, 2019). They are also major carriers of antibiotic resistance genes in poultry, and certain species are associated with infections in both humans and poultry (EFSA AHAW Panel, 2021; Guzman Prieto et al., 2016; Souillard et al., 2022). In a previous study (Rebelo et al., 2021), the widespread occurrence of a single variant of the *tcrB* Cu tolerance (CuT) gene, which encodes an efflux pump and is part of the *tcrYAZB* operon, has been reported among MDR *Enterococcus* spp. strains from various environments, particularly the food chain. It was also shown that poultry meat available to consumers over the last 20 years carried *Enterococcus faecalis* with CuT genes on variable genetic platforms, often co-located with multiple genes encoding resistance to antibiotics used in animal production for decades (Rebelo et al., 2023, Rebelo et al., 2021). However, the contribution of the amount of Cu and the type of feed to the selection of Cu tolerant and MDR bacteria in animal production remains uncertain, especially in poultry production. In this work we assessed whether the amount and type of Cu supplemented feed (OTMF vs ITMF) used in chicken production could have an impact on the selection and expansion of Cu tolerant and MDR *Enterococcus* from the first chickens' rearing stages to the final slaughter process.

2. Material and methods

2.1. Farms' characterization and sampling stages

A total of seven intensive farms settled in the North and Centre of Portugal, with similar conventional indoor and raised-floor production systems (flocks with 10.000–64.000 animals each; Ross 308 strain) and complying with key practices and requirements of the European Union legislation (according to the operators) were included in the study (October 2019 to November 2020). Farms were selected based on the presence of grow-out poultry houses with individual feed silos to independently fed chicken flocks (one-day-old chicks divided in half between two houses upon arrival) with ITMF or OTMF throughout their lifespan. On all farms, Cu is routinely used as a feed additive in the poultry daily diet, mainly in the inorganic form. Additionally, specifically for this study, feed supplemented with OTMF was also used. It was previously demonstrated that both types of feed formulations could maintain chicken performance under commercial conditions (Tavares et al., 2013). Organic and inorganic minerals were added to identical commercial feed formulations, with metal concentrations adapted to different periods of chickens' life (0–7 days; 7–16 days; 16–23 days; 23 days to slaughter; Cu concentrations decreased from 9.2 to 8.0 mg/kg in ITMF and 5.3 to 4.6 mg/kg in OTMF over these periods according to operators' information).

On the seven farms participating in the study (two included twice) we followed eighteen flocks raised in separated poultry-houses and fed with ITMF or OTMF (nine flocks each). Pooled faecal samples were collected from the floor (~50 g), using a zig-zag pattern, at 2–3 days [Stage 1 (P1); $n = 18$] and 28–30 days [Stage 2 (P2); $n = 16$; two samples missed due to COVID-19 pandemic restrictions] of chickens' life, the latter one day before being slaughtered. Chicken meat samples [Stage 3 (P3); $n = 18$] from the same raised chickens were also collected after the slaughter and chilling processes and immediately before distribution for retail sale. Each meat sample included approximately 50 g of neck skin from a pool of ten carcasses from the same batch of chickens raised under the same conditions (same flock from the same house within a specific farm). To assess whether the production environment could have an impact on the introduction of Cu tolerant *Enterococcus* into the chicken flocks, water ($n = 14$; each ~1 L collected at the tap supplying the drinking water lines) and feed ($n = 18$; each ~50 g collected at the silo supplying feeding lines) samples were also collected at P1 on each house/farm. All samples were collected in sterile plastic bags/containers, transported at 4 °C, and processed on the same day in the laboratory.

2.2. Sampling processing, bacterial count, and identification

Twenty-five grams of each sample (faeces, meat, or feed) were diluted in 225 mL of Buffered Peptone Water and left for 1 h at room temperature for resuscitation. Serial dilutions of the previous suspension

were performed, and 0.1 mL was plated onto Slanetz-Bartley (SB) agar and SB agar supplemented with 1 mM of $CuSO_4$ under anaerobic conditions (37 °C/48 h), an atmosphere that has previously been shown to select CuT phenotypes (Mourão et al., 2016a, 2016b; Rebelo et al., 2021). The assays were firstly optimised with a subset of laboratory isolates carrying or not CuT genes and with known Minimum Inhibitory Concentration (MIC) to $CuSO_4$ to assess the most adequate Cu concentration (1, 2, 4, 8 and 12 mM were tested) to select strains with CuT genotypes in this medium. The water samples were processed using the membrane filter technique (100 mL filtered through cellulose membranes; 0.45 μm; ∅ 47 mm) and the filters placed in the same medium (SB and SB + 1 mM of $CuSO_4$) under anaerobiosis (37 °C/48 h).

Whenever possible, a minimum of five to ten colonies of different morphologies were selected for genus and species identification by standard methods (bile-esculin hydrolysis and catalase test) and PCR (*Enterococcus faecium*, *Enterococcus lactis* and *Enterococcus faecalis*) (Belloso Daza et al., 2022; Novais et al., 2013). These species were selected for identification because: i) *E. faecium* and *E. faecalis* are the most clinically relevant in human and poultry infections (Guzman Prieto et al., 2016; Souillard et al., 2022) and have been described as the ones that more often carry CuT genes (Rebelo et al., 2021); ii) *E. faecium* of clade B has been recently reclassified as *E. lactis* (Belloso Daza et al., 2021) and the contribution of *E. lactis* to CuT gene's dissemination is unknown. All isolates classified as *Enterococcus* but not belonging to these three species will be mentioned within the group *Enterococcus* spp. An estimate (CFU/g) of each species and of the total *Enterococcus* spp. (including unidentified species) detected per plate in relation to the total count of typical *Enterococcus* colonies in SB was calculated for faeces (P1, P2) and chicken meat (P3) samples. Only isolates recovered from non-supplemented medium were considered to avoid any species-associated bias introduced by Cu selective pressure in the culture medium. Plates selected for counting had a maximum of 300 colonies.

2.3. Antimicrobial susceptibility

2.3.1. Copper

CuT genes (*tcrB* and *cueO*) were searched by PCR in all recovered isolates, as described by Rebelo et al. (2021). In those with the *tcrB* gene, a long PCR was also performed to identify the presence of the complete *tcrYAZB* operon as described by Mourão et al. (2016b). An estimate (CFU/g) of *Enterococcus* species and of the total *Enterococcus* spp. (including unidentified species) with the *tcrB* ± *cueO* in relation to the total count of typical *Enterococcus* colonies in SB + 1 mM of $CuSO_4$ was calculated for faeces and chicken meat samples.

For representative isolates carrying or not CuT genes, susceptibility to $CuSO_4$ was evaluated by the agar dilution method under anaerobiosis (Mourão et al., 2016b). The isolates selected for these assays were chosen to ensure representation of diverse species, sample types, poultry houses that use either ITMF or OTMF and presence or absence of CuT genotypes. Briefly, the MIC to $CuSO_4$ was determined using Mueller-Hinton 2 agar, freshly prepared for each assay, supplemented with different $CuSO_4$ concentrations (0.25 to 36 mM), and adjusted to pH = 7.2. After inoculation of a 0.001 mL suspension (10^7 CFU/mL) of each isolate in the different $CuSO_4$ concentrations, the plates were incubated under anaerobiosis (18 h–20 h). The first concentration without visible growth was considered the MIC, using a cut-off ≤ 12 mM to classify the isolates as wild-type for $CuSO_4$ following previously proposed tentative Epidemiological Cut Offs (ECOFFs) (Mourão et al., 2016b). Control strains included *E. lactis* BM4105RF (without acquired CuT genes) (Novais et al., 2023) and *Escherichia coli* ED8739 carrying the plasmid pRJ1004 with *pco* + *sil* cluster (Mourão et al., 2016a).

2.3.2. Antibiotics

Among all the *Enterococcus* spp. recovered, antibiotic susceptibility tests were conducted on representative isolates of each identified species per sample. These assays included all isolates that were also tested in $CuSO_4$ susceptibility assays. Antibiotic susceptibility against 12 antibiotics (vancomycin-5 μg, teicoplanin-30 μg, ampicillin-2 μg, tetracycline-30 μg, erythromycin-15 μg, quinupristin-dalfopristin-15 μg, ciprofloxacin-5 μg, chloramphenicol-30 μg, nitrofurantoin-100 μg, linezolid-10 μg, gentamicin-30 μg and streptomycin-300 μg), was evaluated by the disk diffusion method following the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022) or, when not possible, the Clinical and Laboratory Standards Institute (CLSI, 2022) guidelines. Isolates categorised as "susceptible, increased exposure" (EUCAST guidelines) or as "intermediate resistant" (CLSI guidelines) were classified as susceptible. Multidrug-resistant (MDR) was considered when the isolates were resistant to three or more antimicrobial agents from different families (Magiorakos et al., 2012). *E. faecalis* ATCC 29212 was used as a control strain in the different assays.

2.4. Whole genome sequencing

The whole genome of 23 *Enterococcus* spp. (12 *E. faecium*, 9 *E. lactis* and 2 *E. faecalis*) was sequenced to assess clonality and study CuT genotypes in depth. Isolates were chosen to ensure representation of strains of different species, CuT phenotypes and genotypes, antibiotic resistance profile, farms, sampling stages (P1, P2 and P3) and flocks fed ITMF or OTMF.

Genomic DNA was extracted (Wizard Genomic DNA purification kit, Promega Corporation, USA) and the final concentration measured with a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). DNA was sequenced with Illumina NovaSeq 6000 S4 PE150 XP (Illumina, USA) at Eurofins Genomics (https://eurofinsgenomics.eu/). Sequencing data were analysed using FastQC to test the quality of the raw and pre-processed data. SPAdes version 3.1 genome assembler was used for *de novo* assembly of the paired-end reads [Bacterial and Viral Bioinformatics Resource Center (BV-BRC), https://www.bv-brc.org/], and QUAST to evaluate the quality of genome assembly (BV-BRC). RAST (Rapid Annotation Using Subsystem Technology, 2.0; https://rast.nmpdr.org/) was used to annotate the genomes.

For all genomes, Sequence Types (ST) were determined by multilocus sequence typing (MLST; http://pubmlst.org) using the Centre of Genomic Epidemiology - CGE services (MLST 2.0; https://cge.food.dtu.dk/services/MLST/). The phylogeny of *E. faecium* and *E. lactis* was inferred by constructing a maximum likelihood phylogenetic tree for each species (CSI Phylogeny 1.4 from CGE)] based on Single Nucleotide Polymorphisms-SNPs, using *E. faecium* F17E0263 (GenBank accession no. CP040849.1; complete genome from chicken origin) and *E. lactis* KCTC21015 (GenBank accession no. CP065211.1; complete genome, type strain) as reference strains. Closely related isolates were considered when presenting a SNPs difference ≤ 16 (de Been et al., 2015). For each genome a screening of antibiotic resistance genes was performed using the ResFinder 4.1 tool available at CGE. Also, other metal tolerance (MeT) genes (arsenic – 2 variants of *arsA* and mercury - 5 variants of *merA*) were searched using MyDBfinder 2.0 and an in-house genes database previously described (Rebelo et al., 2021).

A correlation between *tcrYAZB* operon variability and CuT phenotypes was analysed. The *tcrYAZB* genes identified in each genome were translated into the corresponding amino acid sequences by the DNA Translate Tool of ExPASy SIB Bioinformatics Resource Portal (https://web.expasy.org/translate/), and the occurrence of incomplete or deleted TcrYAZB proteins was evaluated. To assess whether the detected TcrYAZB modifications were associated only with isolates recovered from farms in this study, we also analysed 267 annotated genomes from the NCBI database (retrieved as of February 11, 2023) of poultry origin and other types of food-producing animals for which Cu is given in the feed. The epidemiological information of the isolates was retrieved from the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) database.

2.5. Total and bioavailable Cu

Selected chicken samples ($n = 33$; 23 faeces, 10 meat), representative of the two types of chicken production (OTMF, $n = 17$; ITMF, $n = 16$) from the three sampling stages (P1, $n = 11$; P2, $n = 12$; P3, $n = 10$) and covering all 7 farms, were stored in the freezer (−20 °C) until their total and bioavailable Cu content was analysed. Before treatment, the samples were manually thawed and homogenised.

Considering the two types of Cu to be determined (total and bioavailable), different sample treatments were applied. Bioavailable Cu was considered here as the water-soluble fraction (dissolved Cu species) capable of being toxic and responsible for inhibitory effects in bacteria. For this purpose, a simple extraction was performed by dissolving 1 g of sample (wet weight) in 10 mL of ultrapure water (Mili-Q system, Millipore, Billerica, MA) and homogenised in a pendular shaker for 1 h at room temperature (21 ± 2 °C), followed by centrifugation at 4000*g* for 10 min. The supernatant (4.9 mL) was then filtered through a 0.45 μm PTFE syringe filter membrane, 0.1 mL of high-purity nitric acid ($HNO_3 \geq 69$ %, TraceSELECT™, Fluka, Germany) was added, and the solution was stored at 4 °C until analysis. Several blanks were prepared by filtering ultrapure water under the same conditions.

To determine the total Cu content, samples were solubilized by closed-vessel microwave-assisted acid digestion in an ETHOS EASY microwave oven (Milestone, Sorisole, Italy) equipped with a SK-15 easyTEMP high pressure rotor. A sample mass between 0.5–1 g (wet weight) was weighed directly into the microwave modified polytetrafluorethylene (PTFE-TFM) vessels and 9 mL of high-purity nitric acid ($HNO_3 \geq 69$ %, TraceSELECT™, Fluka, Germany) plus 1 mL of high-purity hydrogen peroxide (H_2O_2 30 %, Suprapur®, Supelco, Germany) were added. Samples digestion was performed using the following microwave oven program: gradual increase in temperature for 20 min to 210 °C, followed by 15 min at 210 °C. After cooling to room temperature, the vessels were opened and the sample solutions were transferred to decontaminated 50 mL polypropylene tubes, and the volume adjusted to 25 mL with ultrapure water. Sample blanks (one in each series of digestions) were obtained using the same procedure. The obtained solutions were stored at 4 °C until analysis. Each sample was processed in triplicate in both sampling treatments (simple water extraction and acid digestion) and mean values for blanks were subtracted from sample values. Additionally, 1 g (wet weight) of each sample was dried in an incubator at 100 °C for 24–48 h until reaching a constant weight, in order to allow expression of Cu concentrations as μg/g dry weight.

Cu determination was carried out by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP™ Q instrument (Thermo Fisher Scientific, Bremen, Germany). All blanks and sample solutions were diluted with a diluent solution containing 2 % v/v HNO_3 and 10 μg/L Ga (Ga Standard for AAS, 1000 mg/L, Fluka) as an internal standard (IS). An 8-point calibration curve (1, 5, 10, 25, 50, 100, 250 and 500 μg/L) was generated with standard solutions prepared by appropriate dilution of a multi-element stock solution (ICP multi-element standard solution XVI, 100 mg/L, Certipur®, Supelco) in 2 % HNO_3. The calibration solutions were then diluted with the diluent solution like the samples. The elemental isotope ^{65}Cu was measured for analytical determination and the elemental isotope ^{71}Ga was monitored as IS. After complete mixing on a vortex mixer, the diluted samples and calibration standards were presented to the ICP-MS instrument using a CETAC ASX-520 autosampler (Teledyne CETAC Technologies, Omaha, NE).

For analytical quality control purposes and considering the type of sample analysed in this study, four certified reference materials (CRM) were analysed, including Hay Powder (BCR-129), Cabbage Powder (BCR-679), Fish Muscle (ERM-BB422) and Mussel Tissue (ERM-CE278k), all from the EC Joint Research Centre. The CRM were subjected to the same pre-treatment used to determine the total Cu content in the samples (microwave-assisted acid digestion). The values obtained proved the adequacy of the analytical procedure (Table S1).

2.6. Statistical tests

Differences in Cu concentrations and bacterial counts, as well as the occurrence of antimicrobial phenotypes and genotypes between flocks fed with ITMF or OTMF and during the animals' lifespan (P1, P2 and P3 stages) were analysed by Fisher exact, Wilcoxon or Mann-Whitney tests ($\alpha = 0.05$) using IBM SPSS Statistics software, version 28.0.0.0.

3. Results

3.1. Enterococcus occurrence and load

Enterococcus spp. isolates ($n = 477$) were identified in 80 % ($n = 67/84$) of the tested samples, representing all flocks and farms studied. Isolates were selected from SB plates supplemented with 1 mM of $CuSO_4$ ($n = 217$) and from SB without $CuSO_4$ ($n = 260$). They were recovered mainly in faecal samples (97 %, $n = 33/34$; 345 isolates) and chicken meat (83 %, $n = 15/18$; 80 isolates), but also in feed (61 %, $n = 11/18$; 21 isolates) and water (57 %, $n = 8/14$; 31 isolates). *E. faecium,* the predominant species ($n = 231$ isolates), and *E. lactis* ($n = 41$ isolates) occurred at higher rates among faecal samples (94 %, $n = 32/34$ and 44 %, n = 15/34, respectively), while *E. faecalis* ($n = 38$ isolates) were more frequently identified in chicken meat (61 %, $n = 11/18$) ($P < 0.05$). In faeces and meat samples no differences were observed in the species occurrence between feed type (OTMF vs ITMF). *E. faecium* was also identified in water (43 %, $n = 6/14$) and feed (6 %, $n = 1/18$) samples, while *E. faecalis* was recovered only from water (14 %, $n = 2/14$) and *E. lactis* from feed (17 %, $n = 3/18$). Other unidentified *Enterococcus* species also occurred in feed (44 %, $n = 8/18$) and water samples (29 %, $n = 4/14$).

Regarding *Enterococcus* spp. load in samples, it was variable between farms and sampling stages, although a significantly decreased was observed between faecal samples (P1 and P2) from both ITMF or OTMF flocks, as well as between faeces from P2 and meat in OTMF flocks, especially *E. faecium* ($P < 0.05$; Fig. 1-A and 1-B). Analysing separately the *Enterococcus* load in each farm, a decrease over time was also observed for most farms (Fig. S1-A and S1-B).

3.2. Antimicrobial susceptibility

3.2.1. Copper

Enterococcus with CuT genes (33 %, $n = 158/477$) were better recovered from Slanetz-Bartley agar supplemented with $CuSO_4$ (87 %, $n = 138/158$; $P < 0.05$) than without supplementation. Isolates with CuT genes were identified in 85 % ($n = 28/33$) of faecal samples with *Enterococcus*, most carrying *tcrB* ± *cueO* (86 %, $n = 24/28$) genotype compared to only *cueO* (57 %, $n = 16/28$) ($P < 0.05$). The *tcrB* ± *cueO* genotype was similarly found among P1 and P2 faeces samples ($P > 0.05$), while *cueO* was mostly identified in P1 samples ($P < 0.05$). Both genotypes (*tcrB* ± *cueO* or only *cueO)* occurred alike at OTMF and ITMF flocks ($P > 0.05$) (Figs. 1 and 2). Lower rates of *Enterococcus* carrying CuT genes were found in chicken meat (40 %, $n = 6/15$ samples with *Enterococcus* spp.), and included batches of the same OTMF or ITMF flocks (3 of each) in which CuT genes were also found in P1 and P2 stages. Regarding the load of *Enterococcus* spp. carrying *tcrB* ± *cueO* in samples, it was similar between the P1 and P2 sampling stages for ITMF or OTMF flocks, with no differences observed among species ($P > 0,05$) (Fig. 1-C and 1-D). However, a higher load of *Enterococcus tcrB*+ was found in faeces from P2 of ITMF flocks compared to meat samples of the same flocks ($P < 0.05$), but not in OTMF flocks (Fig. 1-C and 1-D). The load of *Enterococcus tcrB*+ over time in each farm was variable, with increasing or decreasing trends, as well as no changes, observed between faeces of P1 and P2 but decreasing in meat in most flocks (Fig. S1-C and S1-D).

Diverse CuT genotypes were found among *Enterococcus* species from faeces and meat samples, namely the *tcrB* + *cueO* genes (53 *E. faecium*,

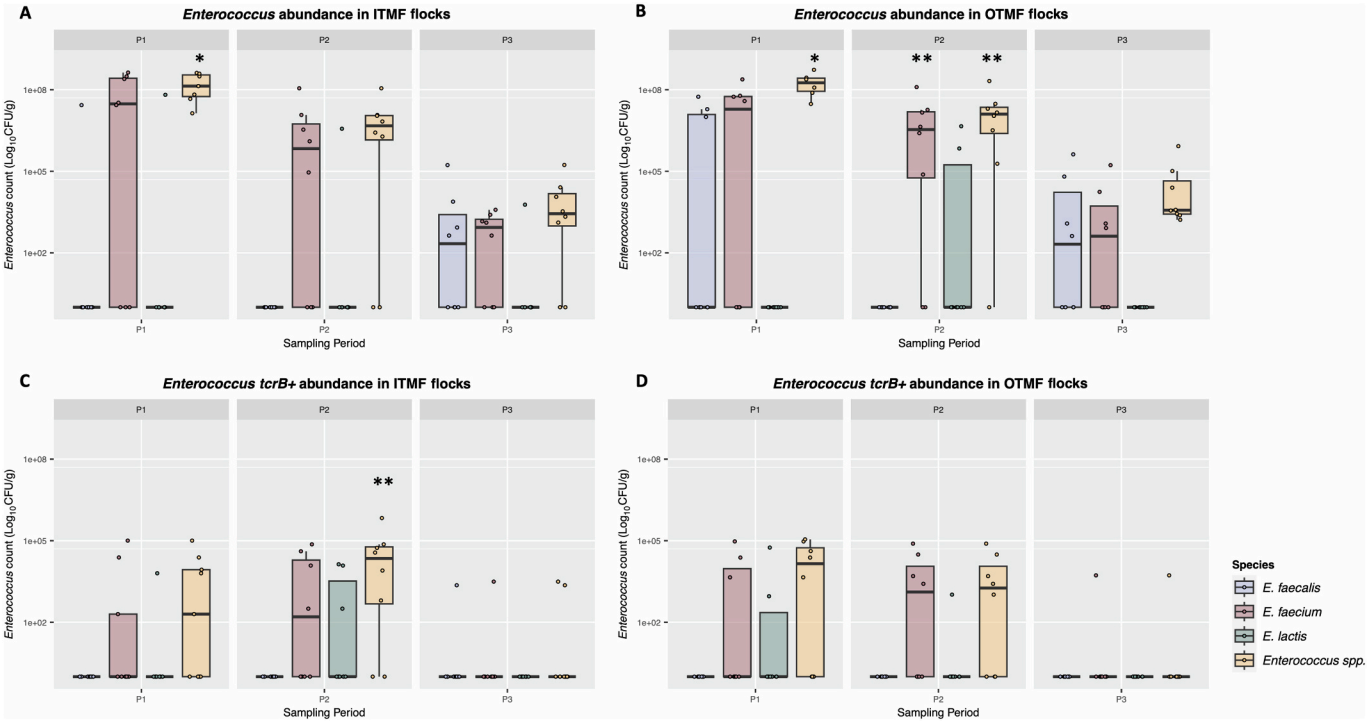

Fig. 1. *Enterococcus* species [ITMF (A) and OTMF (B)] and *Enterococcus tcrB*+ species [ITMF (C) and OTMF (D)] abundance across different sampling stages (P1, P2, P3). *Enterococcus* spp. represent all the isolates recovered (with or without species identified). The dots represent individual observations in each boxplot and the thick lines the median. Samples from flocks E1 were excluded as no samples were collected from P2 sampling stage due to COVID-19 constraints. Significant statistical differences ($P < 0.05$) were observed for *Enterococcus* spp. abundance between P1 and P2 (*) for ITMF and OTMF samples (Wilcoxon test), for *Enterococcus* spp. and *E. faecium* abundance between P2 and P3 (**) for OTMF samples and for *Enterococcus tcrB*+ abundance between P2 and P3 (**) for ITMF samples (Mann-Whitney test).

20 *E. lactis*, 4 *E. faecalis*, 11 *Enterococcus* spp.), only *cueO* (55 *E. faecium*, 8 *E. lactis*, 3 *Enterococcus* spp.), or only *tcrB* (3 *E. faecium*, 1 *Enterococcus* spp.) (Fig. 2). Additionally, *E. faecium* with CuT genes were also observed in 25 % of water samples with *Enterococcus* spp. ($n = 2/8$), contrasting with feed samples in which no isolates with CuT genes were detected (Fig. 2).

Cu phenotypic assays were performed on 103 isolates out of the 477, with and without CuT genotypes and covering all species (70 *E. faecium*, 18 *E. faecalis* and 15 *E. lactis*), farms and sample types ($n = 77$ from faeces; $n = 21$ from chicken meat; $n = 5$ from water) (Table 1). High rates (75 %, $n = 27/36$) of *Enterococcus* carrying the *tcrB* ± *cueO* genes showed a MIC for $CuSO_4 = 12$ mM, not complying with the previously proposed breakpoint to separate *Enterococcus* with or without *tcrYAZB* operon (> 12 mM) (Mourão et al., 2016b). This unique CuT phenotype was observed in *E. faecium* ($n = 16$) and *E. lactis* ($n = 5$) from faeces of P1 (44%, $n = 4/9$) and P2 samples (85 %, $n = 11/13$), in *E. faecium* ($n = 3$) and *E. faecalis* ($n = 1$) from all positive chicken meat samples ($n = 3$), and in *E. faecium* from water ($n = 1$) from both OTMF and ITMF flocks. Of note, all isolates but one *E. faecalis* with *tcrB* and $MIC_{CuSO4} = 12$ mM were negative for the *tcrYAZB* operon screened by long-PCR, unlike those with MIC_{CuSO4} between 16 and 28 mM, being mostly positive for *tcrYAZB* (91 %, $n = 10/11$). All isolates with only the *cueO* gene had a $MIC_{CuSO4} = 12$ mM and those without CuT genes a MIC of 4–16 mM (91 %, $n = 39/43$ with a $MIC_{CuSO4} = 4$-8 mM).

3.2.2. Antibiotics

Antibiotic susceptibility was studied for 182 isolates encompassing all species identified per sample (131 *E. faecium*, 27 *E. faecalis* and 24 *E. lactis*). These isolates covered all flocks (OTMF, ITMF) and farms studied, and carried ($n = 89$) or not ($n = 93$) CuT genes. More than 50 % of faeces samples with *Enterococcus* spp. presented at least one isolate resistant to tetracycline (100 %, $n = 33/33$), quinupristin-dalfopristin (97 %, $n = 31/32$, only for samples with *E. faecium*), erythromycin (94 %, $n = 31/33$), streptomycin (76 %, $n = 25/33$), ampicillin and/or ciprofloxacin (55 %, $n = 18/33$, each). The same was observed in chicken meat samples, which > 50 % carried isolates resistant to tetracycline or erythromycin (91 %, $n = 10/11$ each), quinupristin-dalfopristin (88 %, $n = 7/8$, only for samples with *E. faecium*) or ciprofloxacin (64 %, $n = 7/11$). Analysing the antibiotic resistance rates of each species by sampling stage and feed type (Fig. S2), the only difference observed was a significant decrease in samples carrying *E. faecium* resistant to tetracycline, erythromycin or quinupristin-dalfopristin between faeces from P2 and meat recovered from OTMF flocks ($P < 0.05$). Additionally, more than half of the animal drinking water and feed samples also showed isolates with resistance to erythromycin or quinupristin-dalfopristin. A total of 60 % ($n = 110/182$) of the isolates were MDR (Table S2), occurring at similar rates in faeces (85 %, $n = 28/33$) and chicken meat samples (82 %, $n = 9/11$) ($P > 0.05$) but at lower rates in water and feed samples (30 %, $n = 3/10$) ($P < 0.05$). Comparing antibiotic resistance rates by species, *E. faecium* (73 %, $n = 95/131$) and *E. lactis* (79 %, $n = 19/24$) were more resistant to erythromycin than *E. faecalis* (48 %, $n = 13/27$; $P < 0.05$). Also, MDR was higher among *E. faecium* (69 %, $n = 90/131$) than in *E. faecalis* (37 %, $n = 10/27$) or *E. lactis* (42 %, $n = 10/24$) ($P < 0.05$). Tetracycline, erythromycin, ciprofloxacin or streptomycin resistant isolates were better recovered from Cu-supplemented plates ($P < 0,05$) (Table S2). The most frequently observed MDR phenotype in *E. faecium* was tetracycline, erythromycin, quinupristin-dalfopristin and streptomycin (13 %, $n = 17/128$) and in *E. faecalis* or *E. lactis* was tetracycline, erythromycin and streptomycin (19 %, $n = 5/27$ and 33 %, $n = 8/24$, respectively).

Enterococcus with *tcrB* ± *cueO* genes showed higher rates of MDR (85 %, $n = 51/60$) compared to isolates without these genotypes (48 %, $n = 59/122$; $P < 0.05$). *E. faecium* with *tcrB* ± *cueO* was more resistant to tetracycline, erythromycin or streptomycin and *E. faecalis* to

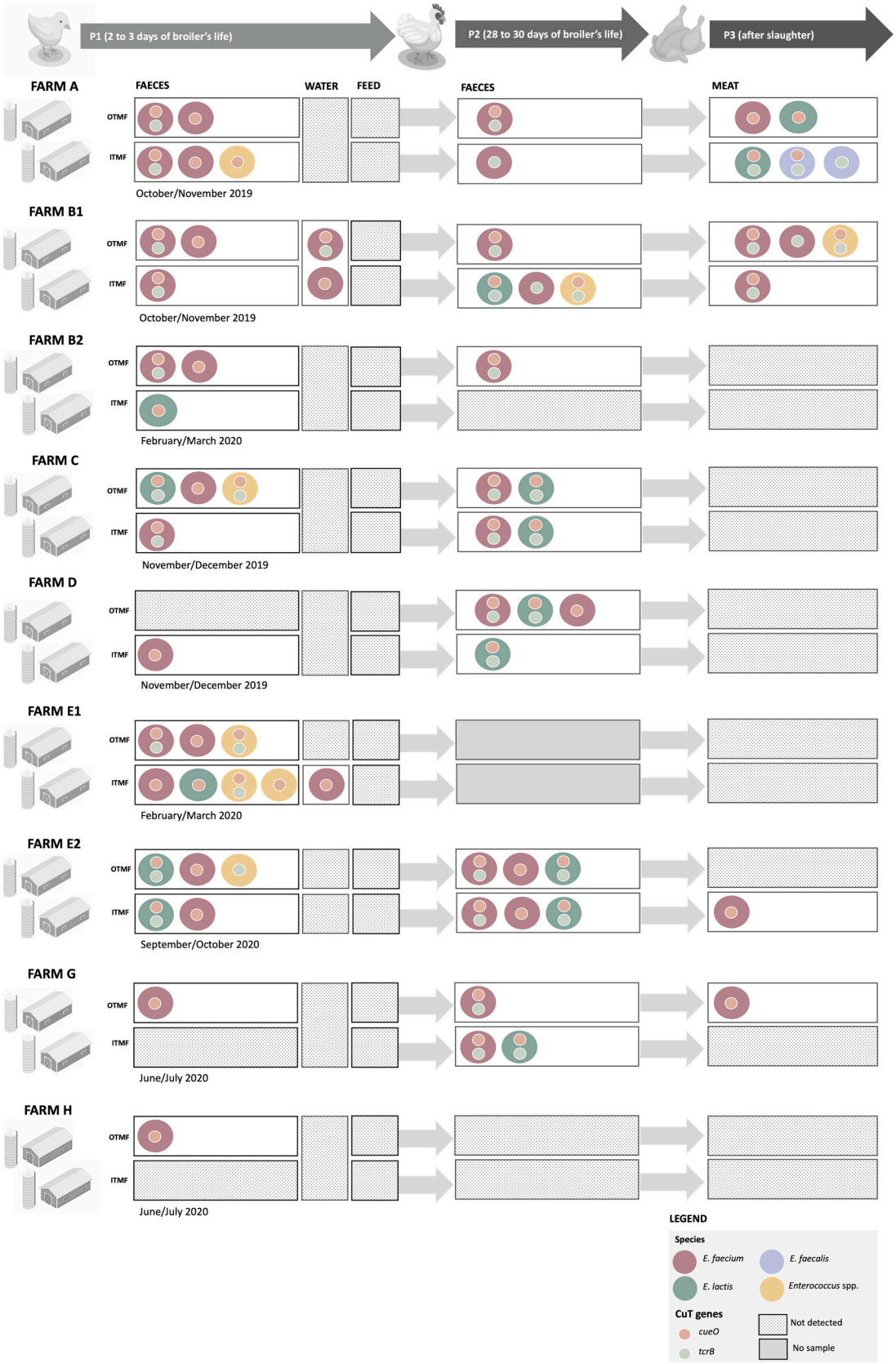

Fig. 2. Distribution of *Enterococcus* species with CuT genes in OTMF and ITMF samples by farm and across the three sampling stages (P1, P2, P3). In the Farm E1 no samples were collected in P2 sampling stage due to COVID-19 constraints. Farms A, B (B1 and B2), C, D, G and H had only one water source for OTMF and ITMF flocks (single box) and farm E (E1 and E2) had a different water source for each OTMF and ITMF flock (separated boxes). *Enterococcus* spp. represents the isolates with no species identified.

Table 1
Minimum inhibitory concentrations of $CuSO_4$ against *Enterococcus* spp. isolates ($n = 103$) by type of feed and species.

Feed	Species	CuT gene	No. Isolates	MIC $CuSO_4$ (mM)												
				0.25	0.5	1	2	4	8	12*	16	20**	24**	28**	32	36
OTMF[a]	*E. faecium*	*tcrB* ± *cueO*	14	–	–	–	–	–	–	11	–	–	2	1	–	–
		cueO	12	–	–	–	–	–	2	10	–	–	–	–	–	–
		None	12	–	–	–	–	4	8	–	–	–	–	–	–	–
		tcrB ± *cueO*	5	–	–	–	–	–	–	3	–	–	1	1	–	–
		cueO	0	–	–	–	–	–	–	–	–	–	–	–	–	–
	E. lactis	None	1	–	–	–	–	1	–	–	–	–	–	–	–	–
		tcrB ± *cueO*	0	–	–	–	–	–	–	–	–	–	–	–	–	–
		cueO	0	–	–	–	–	–	–	–	–	–	–	–	–	–
	E. faecalis	None	10	–	–	–	–	5	3	2	–	–	–	–	–	–
		tcrB ± *cueO*	9	–	–	–	–	–	–	8	–	–	–	1	–	–
		cueO	6	–	–	–	–	–	–	6	–	–	–	–	–	–
	E. faecium	None	13	–	–	–	–	3	10	–	–	–	–	–	–	–
		tcrB ± *cueO*	5	–	–	–	–	–	–	2	–	–	–	3	–	–
		cueO	3	–	–	–	–	–	–	3	–	–	–	–	–	–
	E. lactis	None	1	–	–	–	–	–	–	1	–	–	–	–	–	–
		tcrB ± *cueO*	2	–	–	–	–	–	–	2	–	–	–	–	–	–
		cueO	0	–	–	–	–	–	–	–	–	–	–	–	–	–
ITMF[a]	*E. faecalis*	None	6	–	–	–	–	2	3	–	1	–	–	–	–	–
		tcrB ± *cueO*	1	–	–	–	–	–	–	1	–	–	–	–	–	–
		cueO	3	–	–	–	–	–	–	3	–	–	–	–	–	–
OTMF/ITMF[b]	*E. faecium*	None	0	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	All species	*tcrB* ± *cueO*	36	–	–	–	–	–	–	27	–	–	3	6	–	–
		cueO	24	–	–	–	–	–	2	22	–	–	–	–	–	–
		None	43	–	–	–	–	**15**	**24**	**3**	**1**	–	–	–	–	–

Correspondence between $CuSO_4$ μg/g and mM values tested: 0.25 mM - 39.9 μg/g, 0.5 mM - 79.8 μg/g, 1 mM - 159.6 μg/g, 2 mM - 319.2 μg/g, 4 mM - 638.4 μg/g, 8 mM - 1276.9 μg/g, 12 mM - 1915.3 μg/g, 16 mM - 2553.8 μg/g, 20 mM - 3192.2 μg/g, 24 mM - 3830.6 μg/g, 28 mM - 4469.1 μg/g, 32 mM - 5107.5 μg/g, 36 mM - 5746 μg/g.

[a] Isolates obtained in P1, P2 and P3 sampling stages.

[b] Isolates from a water sample collected from a reservoir supplying both pavilions (OTMF/ITMF).

* All isolates with *tcrB* gene and MIC = 12 mM were negative for *tcrYAZB* operon using long-PCR.

** All isolates with *tcrB* gene and MIC >12 mM were positive for *tcrYAZB* operon using long-PCR.

chloramphenicol than isolates without these genes ($P < 0.05$) (Fig. 3). *Enterococcus* with only *cueO* did not show higher rates of MDR compared to isolates without CuT genes ($P > 0.05$), despite being more resistant to tetracycline, ciprofloxacin or streptomycin, mainly *E. faecium* ($P < 0.05$) (Fig. 3). In contrast, a higher rate of ampicillin resistance was observed in *E. faecium* lacking CuT genes compared to isolates with CuT genes (*tcrB* ± *cueO* or only *cueO*) ($P < 0.05$) (Fig. 3).

3.3. Clonality and genomic analysis of antibiotic resistance and CuT gene clusters

Twenty-three *Enterococcus* covering different species (12 *E. faecium*, 9 *E. lactis,* 2 *E. faecalis*), farms, OTMF/ITMF flocks, sample types (15 faeces, 4 meat), CuT genotypes (*tcrB* ± *cueO,* only *cueO*) and antibiotic resistance profiles were selected for whole genome sequencing to assess

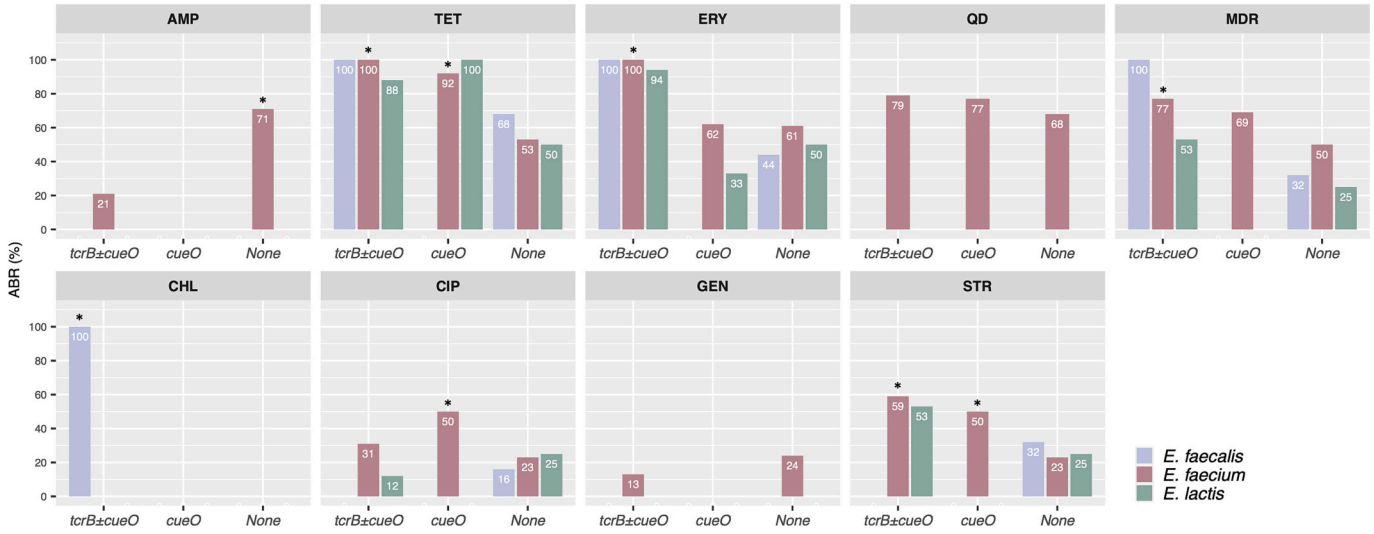

Fig. 3. Antibiotic resistance rates of *E. faecalis* ($n = 27$), *E. faecium* ($n = 131$) and *E. lactis* ($n = 24$) with and without CuT genes. Isolates are from diverse sources (faeces-132, meat-35, water-11 and feed-4) and represent all farms/flocks studied. The star (*) represents a statistically significant result ($P < 0.05$, Fisher exact Test) when comparing the antibiotic resistance phenotype between isolates with CuT genes (*tcrB* ± *cueO* or only *cueO*) and isolates without CuT genes. Abbreviations: ABR, antibiotic resistance; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; QD, quinupristin-dalfopristin; MDR, multidrug-resistant; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin.

clonality and conduct an in-depth study of CuT genotypes. *E. faecium* and *E. lactis* with CuT genes of our field isolates belonged to a polyclonal population (Fig. 4). *E. faecium* belonged to 8 STs with isolates differing between 6 and 7.774 SNPs, *E. lactis* between 6 and 36.403 SNPs and the *E. faecalis* were from the same clone (ST288; 0 SNPs). Clones were shared among different farms, sampling stages (P1/P2; P1/P3) or feed type (Fig. 4).The sequenced isolates carried several antibiotic resistance genes, namely related to resistance to tetracyclines [*tet(M)*, *tet(L)*], macrolides, lincosamides, and/or streptogramins [*erm(A)*, *erm(B)*, *lsa(A)*, *lsa(E)*, *lnu(A)*, *lnu(B)*, *msr(C)*, *vatE*], aminoglicosides [*aac(6′)-Ie-aph(2″)-Ia*; *aph(3′)-III*; *aadE*, *ant(9)-Ia*], chloramphenicol [*cat(pC194)*, *cat(pC221)*] or trimethoprim (*dfrG*; *dfrK*). The mercury tolerance genes (*merA*_IIA, *merA*_IV) were associated only with *E. faecium* or *E. lactis*, respectively, showing a complete *tcrYAZB* operon. Correlation between CuT phenotype and genotypes showed that all but one isolate (*E. faecalis*) with $MIC_{CuSO4} \geq 16$ mM carried the complete *tcrYAZB* operon (3 *E. faecium*; 4 *E. lactis*) and those with a $MIC_{CuSO4} < 16$ mM had a complete *tcrYAZB* with a truncated TcrA with a stop codon after amino acid 396 (1 *E. faecalis*), the absence of *tcrYAZB* (4 *E. faecium*; 1 *E. lactis* with *cueO* only) or deletion of *tcrY* and of part of *tcrA* (5 *E. faecium*; 4 *E. lactis*). *E. faecium* or *E. lactis* phylogenetically more closer shared the same CuT genotypes and phenotypes (Fig. 4).

In public genomes, the analysis of the *tcrYAZB* operon from isolates of animal origin showed a truncated TcrA with a stop codon after amino acid 393 or a partial deletion of the TcrA often present among *E. faecalis* from chicken origin (at least from 2001), with the former genotype also often found in other animal sources (59 % of 101 *E. faecalis* genomes analysed) (Table S3). However, most genomes of *E. faecium* of chicken and turkey origin (98 %/59 and 100 %/14, respectively; at least from 2002) showed deletion of part of the *tcrA* and of the *tcrY* genes, as we observed in our isolates. These data contrast with *E. faecium* from other sources, especially from pig origin, in which the *tcrYAZB* operon was intact in most genomes (96 %, $n = 3/68$). For other non-*E. faecalis* and non-*E. faecium* species, *tcrYAZB* also appeared intact in most of the analysed genomes, including those of chicken origin. In those *Enterococcus* with complete genomes, the *tcrYAZB* operon was plasmid located in most cases, with a lower size for *E. faecalis* than for *E. faecium*.

3.4. Copper concentrations over the three sampling stages

Cu concentrations (total and bioavailable Cu) found in chicken faeces and meat samples are shown in Fig. 5. At P1, faecal samples showed varying concentrations of total and bioavailable Cu under OTMF [20.03–50.66 μg/g (0.32–0.80 mM) and 10.24–25.09 μg/g (0.16–0.40 mM), respectively] or ITMF [25.95–48.45 μg/g (0.41–0.76 mM) and 14.08–30.47 μg/g (0.22–0.48 mM)] with no differences between feed type ($P > 0.05$). No significant differences ($P > 0.05$) were observed when comparing data from P1 with total or bioavailable Cu concentrations found in P2 faeces, either from OTMF [33.17–47.10 μg/g (0.52–0.74 mM) and 13.74–20.86 μg/g (0.22–0.33 mM)] or ITMF [44.46–64.47 μg/g (0.70–1.01 mM) and 18.93–29.89 μg/g (0.30–0.47 mM)] flocks ($P > 0.05$). However, lower Cu concentrations (total and bioavailable) were observed in P2 faeces from OTMF flocks when compared to ITMF ones ($P < 0.05$) (Fig. 5). As expected, total and bioavailable Cu concentrations significantly decrease in meat samples [all <0.81 μg/g (0.01 mM); $P < 0.05$], although no differences were detected between meat from OTMF or ITMF flocks ($P > 0.05$).

4. Discussion

This study shows that Cu used in OTMF or ITMF formulations for intensive chicken production systems does not have a major impact on the expansion of Cu tolerant and MDR *Enterococcus* spp., despite the high occurrence of MDR strains found during chickens' lifespan and in derived meat. It also shows that worldwide *Enterococcus* spp. of poultry origin have a predominance of CuT truncated genotypes associated with a loss of CuT, possibly related to the low concentration of Cu used in this setting that does not seem to be a stressful factor for the bacteria.

Even with EU efforts to decrease the use of antibiotics in animal production [Regulation (EU), 2019a; Regulation (EU), 2019b], our data still point to intensively farmed chickens and their meat as important sources of MDR *Enterococcus* spp., regardless of OTMF or ITMF type used. Resistance to antibiotics widely available for veterinary use (e.g., tetracyclines, macrolides, beta-lactams, aminoglycosides and/or quinolones) was observed, but not to those reserved for the treatment of human infections caused by Gram-positive MDR bacteria, such as vancomycin or linezolid, similar to other European studies with samples of poultry origin (de Jong et al., 2018; Nowakiewicz et al., 2017; Rebelo et al., 2023; Rivera-Gomis et al., 2021). The high rates of antibiotic resistance already found in the 2/3-day-old chicks suggest that they could be a major vehicle for MDR strains, persisting through the chickens' life until their meat is available to consumers (Coppola et al.,

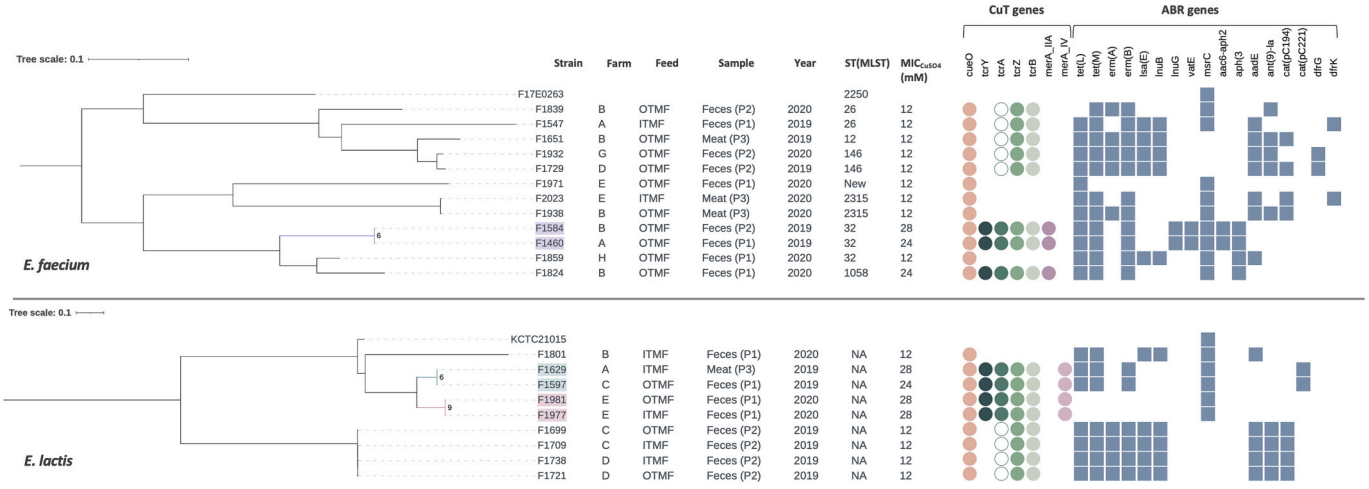

Fig. 4. Phylogeny of *E. faecium* and *E. lactis* strains with copper tolerance (CuT) genes (*tcrB* and/or *cueO*) from faeces (P1/P2) and meat samples (P3). Whole-genome single nucleotide polymorphisms (SNPs) from 12 *E. faecium* and 9 *E. lactis* genomes were used to construct a maximum likelihood phylogenetic tree [CSI Phylogeny 1.4 from Center for Genomic Epidemiology (CGE)] for each species using as reference strains *E. faecium* F17E0263 (GenBank accession no. CP040849.1; complete genome from chicken origin) and *E. lactis* KCTC21015 (GenBank accession no. CP065211.1; complete genome, type strain). Isolates with a SNPs difference ≤ 16 are identified with the same colours and represented in each branch. Strains individual data and CuT and antibiotic resistance (ABR) genes were included using the iTol software (https://itol.embl.de). CuT genes represented by an empty circle mean that the gene is truncated.

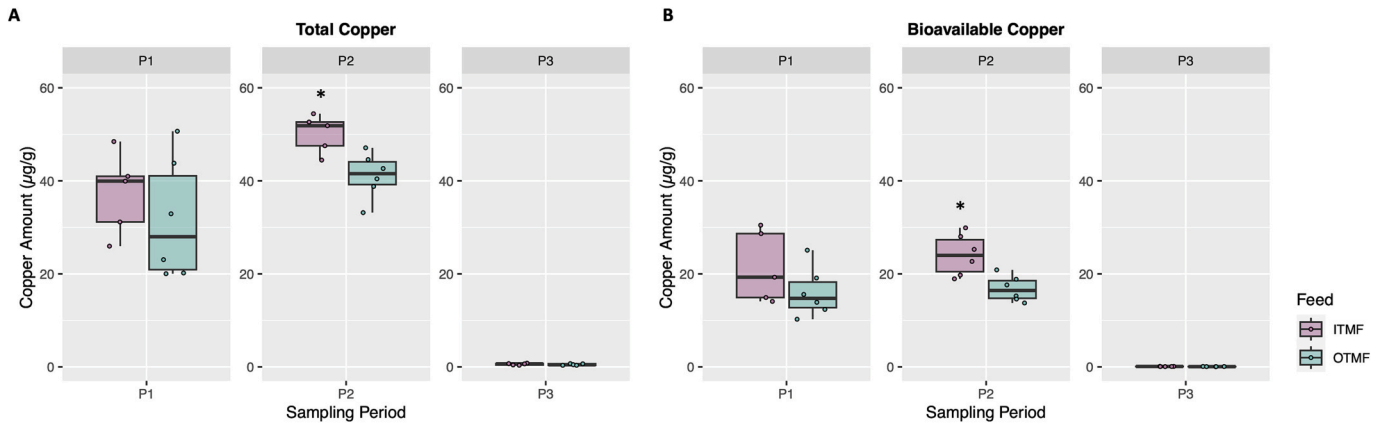

Fig. 5. Total (A) and bioavailable (B) copper concentrations (µg/g) over the three sampling stages (P1, P2, P3) from ITMF and OTMF flocks. The dots represent individual observations in each boxplot and the thick lines the median. Significant statistical differences ($P < 0.05$) were observed for total copper and bioavailable copper between ITMF and OTMF in P2 sampling stage (Mann-Whitney test).

2022). Colonisation of chicks with antibiotic-resistant isolates might occur through laying hens or during transport, in which MDR *Enterococcus* has been previously described (EFSA BIOHAZ Panel, 2022; Lanza et al., 2022; Rivera-Gomis et al., 2021; Van Hoorebeke et al., 2011). This study also shows water and feed as sources of MDR *Enterococcus* spp. (EFSA BIOHAZ Panel, 2021), despite the lower rates of antibiotic resistance found compared to chicken faeces. Other sources of contamination of chickens with MDR *Enterococcus* described in literature (e.g., lack of efficient disinfection of animal houses, workers or wild animals or the inadequate downtime between flocks) (EFSA BIOHAZ Panel, 2021; Luyckx et al., 2015) cannot be excluded.

The influence of various antimicrobial agents (e.g., metals) on the selection and persistence of MDR bacteria in the animal production setting has been largely discussed in recent years (Gullberg et al., 2014; Poole, 2017; Yazdankhah et al., 2014). Evidence that Cu supplemented feed contributes to the increase of Cu tolerant and antibiotic-resistant *Enterococcus* spp. is available (Hasman et al., 2006; Rensing et al., 2018), but mostly related to pig production (Amachawadi et al., 2011; Hasman et al., 2006), where allowable concentrations of Cu in feed are higher than those in poultry [Commission Implementing Regulation (EU) 2018/1039, 2018]. In this study we found high rates of *Enterococcus* carrying CuT genes in samples of chickens fed either OTMF or ITMF, although they were associated with a range of genotypes, mostly with deleted or mutated *tcrYAZB* operons, coding for $CuSO_4$ phenotypes lower than those expected to isolates carrying the intact operon (Mourão et al., 2016b). Still, most of them were recovered from the Cu-supplemented culture medium, probably justified by the slightly higher Cu phenotypes observed (12 mM) compared to isolates without CuT genes (mostly 4-8 mM). The dominance of deleted/mutated CuT genotypes combined with similar load rates of *Enterococcus* spp. with *tcrB* during the chickens' lifespan, and the very low concentrations of total and bioavailable Cu in animals' faeces from OTMF and ITMF flocks, point to a scenario in which Cu-supplemented chicken feed does not have an important role in the selection of Cu tolerant and MDR *Enterococcus* spp. in such producing environments. In fact, the concentration of Cu found in faecal samples was < 1.0 mM, a value much lower than the MIC_{CuSO4} (4 mM - 28 mM) values for *Enterococcus* either carrying or not any kind of CuT genotypes. Previous studies based on wet lab experiments or mathematical models suggest that the Cu concentrations required to select CuT bacteria could be much lower than the respective MIC (Arya et al., 2021; Gullberg et al., 2014) and that the presence of multiple pollutants even decreases the value of minimum selective concentration (MSC) estimated for a single antimicrobial (Arya et al., 2021; Gullberg et al., 2014). However, studies in *Enterococcus* spp. from pigs fed with Cu showed that 2.8 mM (175 µg/g) efficiently selected for *tcrB*+ *Enterococcus* spp. with MIC = 20 mM but not at lower concentrations, such as 0.09 mM (6 µg/g), which maintained similar levels of strains with *tcrB-Enterococcus* spp. from day zero to day 28 (Hasman et al., 2006), as in our study with poultry samples. Other studies in pigs and cattle also showed that higher levels of Cu (1.57–2.8 mM = 100–177 µg/g) than those found in this study are necessary to select for *tcrB*+ *Enterococcus* spp. with MIC > 12 mM (Amachawadi et al., 2011; Amachawadi et al., 2013, Zou et al., 2017). It has also been suggested that, in addition to Cu concentrations given to the animals, the exposure time is important for the expansion of *tcrB*+ *Enterococcus* (Amachawadi et al., 2013; Zou et al., 2017), limited by the short lifespan of chickens (30–35 days).

The *tcrYAZB* operon is often located on megaplasmids that carry a variety of metal, metabolic and/or particular antibiotic (e.g., tetracycline, erythromycin, streptomycin, etc.) resistance genes (Amachawadi et al., 2011; Hall et al., 2022; Rebelo et al., 2023; Rebelo et al., 2021), contributing to their environmental maintenance by co-selection events due to antibiotics use, combined with the frequent presence of toxin-antitoxin systems that prevent plasmid loss (Rebelo et al., 2023, Rebelo et al., 2021). However, deletion of *tcrYA* genes in *E. faecium* and *E. lactis* (close genome species exchanging genetic elements) (Belloso Daza et al., 2021; Novais et al., 2023) or *tcrA* mutations in *E. faecalis* might occur to alleviate species-specific plasmids burden in an environment without heavy Cu selection, as described for plasmids carrying antibiotic resistance genes when selective pressures were removed (Dorado-Morales et al., 2021; Hall et al., 2022). Data on *tcrYAZB* fitness cost are still lacking and would be of interest to better support this hypothesis. The occurrence of truncated genotypes among 2/3-day old chicks suggests that the deletion/mutation of *tcrYAZB* operon genes is already occurring early in the chicken production chain.

Similar truncated *tcrYAZB* genotypes found in public genomes from chicken and turkey origins point to a global phenomenon. These data contrast with those of other animals, such as pigs, in which most *E. faecium* maintain the complete *tcrYAZB* operon, pointing to beneficial trait for the bacteria when higher amounts of Cu are used (e.g., up to 150 mg/kg in the EU; up to 250 mg/kg in the USA as a growth promoter) [Commission Implementing Regulation (EU) 2018/1039, 2018; Espinosa and Stein, 2021] and animals are more exposed to Cu as they live longer. Comparison of data from chickens and pigs can facilitate the design of future studies for the clarification of Cu MSC, fundamental to better understand the impact of Cu in-use concentrations in Cu tolerant and MDR bacteria, not only in the food chain but also in all environments other than those linked to animal production (e.g., aquatic setting, manured soils) (Yin et al., 2017; Zhang et al., 2018).

Finally, despite the apparent lack of effect of the OTMF and ITMF Cu forms in the selection and expansion of Cu tolerant and MDR *Enterococcus,* other Cu-associated phenomena, namely the promotion of

horizontal transfer of antibiotic resistance genes (e.g., due to ROS bacterial production, increasing cell permeability) after exposure to very low Cu concentrations (e.g., 0.1–0.00016 mM) (Zhang et al., 2019; Zhang et al., 2018), as well as the emergence of mutations in genes associated with antibiotic resistance (Li et al., 2019), have been described in wet lab studies and cannot be ruled out in the chicken production setting and in other environments.

5. Conclusions

This study demonstrated that the levels of Cu present in OTMF or ITMF used in chicken production do not appear to selectively promote Cu tolerant and MDR *Enterococcus*, despite the lifetime spread of MDR isolates in chickens and their meat. For future surveillance studies of Cu tolerant and MDR *Enterococcus* spp. within a One Health perspective, it is essential to integrate both phenotypic and genomic approaches to identify functional CuT genotypes, as relying solely on PCR screening for the *tcrB* gene may lead to an overinterpretation of CuT selection and MDR co-selection events. To better understand the impact of Cu on the selection and spread of MDR bacteria among animals, humans and the environment (such as air, farms, slaughterhouse wastewater or manured soils), as well as on the *de novo* acquisition of antibiotic resistance, field studies are necessary to identify the minimum Cu concentrations that can promote such events. These studies are critical for a One Health strategy to promote sustainable practices and mitigate the spread of antibiotic resistance in animal production and environments beyond.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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