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Enterococcus spp. from chicken meat collected 20 years apart overcome multiple stresses occurring in the poultry production chain: Antibiotics, copper and acids

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ABSTRACT

Poultry meat has been a vehicle of antibiotic resistant bacteria and genes. Yet, the diversity of selective pressures associated with their maintenance in the poultry-production chain remains poorly explored. We evaluated the susceptibility of *Enterococcus* spp. from chicken meat collected 20 years apart to antibiotics, metals, acidic pH and peracetic acid-PAA. Contemporary chicken-meat samples ($n = 53$ batches, each including a pool of neck skin from 10 single carcasses) were collected in a slaughterhouse facility using PAA as disinfectant (March–August 2018, North of Portugal). Broilers were raised in intensive farms ($n = 29$) using $CuSO_4$ and organic acids as feed additives. Data were compared with that of 67 samples recovered in the same region during 1999–2001. All 2018 samples had multidrug resistant-MDR isolates, with >45 % carrying *Enterococcus faecalis, Enterococcus faecium* or *Enterococcus gallinarum* resistant to tetracycline, erythromycin, ampicillin, quinupristin-dalfopristin, ciprofloxacin, chloramphenicol or aminoglycosides. Resistance rates were similar ($P > 0.05$) to those of 1999–2001 samples for all but five antibiotics. The decrease of samples carrying vancomycin-resistant isolates from 46 % to 0 % between 1999-2001 and 2018 was the most striking difference. Isolates from both periods were similarly susceptible to acid pH [minimum-growth pH (4.5-5.0), minimum-survival pH (3.0-4.0)] and to PAA (MIC_{90} = 100–120 mg/L/MBC_{90} = 140–160 mg/L; below concentrations used in slaughterhouse). Copper tolerance genes (*tcrB* and/or *cueO*) were respectively detected in 21 % and 4 % of 2018 and 1999-2001 samples. The *tcrB* gene was only detected in *E. faecalis* ($MIC_{CuSO4} > 12$ mM), and their genomes were compared with other international ones of chicken origin (PATRIC database), revealing a polyclonal population and a plasmid or chromosomal location for *tcrB*. The *tcrB* plasmids shared diverse genetic modules, including multiple antimicrobial resistance genes (e.g. to tetracyclines, chloramphenicol, macrolide-lincosamide-streptogramin B-MLS_B, aminoglycosides,

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bacitracin, coccidiostats). When in chromosome, the *tcrB* gene was co-located closely to *merA* (mercury) genes. Chicken meat remains an important vehicle of MDR *Enterococcus* spp. able to survive under diverse stresses (e.g. copper, acid) potentially contributing to these bacteria maintenance and flux among animal-environment-humans.

1. Introduction

Poultry is the most consumed meat type worldwide (OECD/FAO, 2021). It is estimated that 90 % of chicken in Europe, one of the top four chicken-meat producers in the world, are raised in intensive systems with high stocking densities, in indoor rearing and using fast-growing breeds (European Parliament and Augère-Granier, 2019). These conditions, allied to poor hygiene measures, seem to promote transmission of infectious diseases among animals and consequently a high antibiotic consumption (European Parliament and Augère-Granier, 2019).

For many years the use of antibiotics in food-producing animals has been considered the main driver of multidrug resistant (MDR) bacteria selection, with chicken-meat or subproducts potential vehicles for the transmission of such bacteria or their mobile genetic elements to humans and the environment (Berg et al., 2017; Fatoba et al., 2022). However, these MDR bacteria are also under the selective pressure of other stresses during the poultry production chain, namely those related to animal host (e.g. stomach acid pH), feed (e.g. metals, organic acids) or disinfection of facilities (e.g. organic acids) for pathogen control (Gadde et al., 2017; Marmion et al., 2021; Obe et al., 2020). Among them, copper has been used in poultry diets (mostly as $CuSO_4$) as it is an essential nutrient for tissue and bone development and as a cofactor for metabolic enzymes (Gadde et al., 2017) or as a modulating factor of gut microbiota in broilers (Forouzandeh et al., 2021). Dietary supplementation with organic acids to modify intestinal microbiota (e.g. lowering intestinal pH increases acid tolerant beneficial species such as *Lactobacillus* spp.) or increasing nutrient digestibility (Gadde et al., 2017) has also been described. Others, such as peracetic acid (PAA), an "environmentally friendly" strong oxidizing agent, are routinely used to disinfect surfaces in the food/poultry industry (Cano et al., 2021; ECHA, 2015; Wessels and Ingmer, 2013). However, the impact of all of these compounds in the co-selection and maintenance of MDR bacteria in the animal production setting is still unclear and needs to be further explored.

Several *Enterococcus* species are part of the intestinal microbiota of poultry and humans and are able to cause a plethora of infections in these hosts (EFSA AHAW Panel et al., 2021; Guzman Prieto et al., 2016; Shang et al., 2018; Souillard et al., 2022). Their natural ability to overcome different stresses and acquire diverse adaptive traits, enabling their survival and circulation among diverse overlapping ecosystems (e.g. human, animal environment), remains poorly explored (Gaca and Lemos, 2019). Previously, we have shown the presence of genes coding for both antibiotic and metal resistance among *Enterococcus* from the last 60 years (Rebelo et al., 2021). Among them, the acquired efflux system *tcrYAZB* (Hasman and Aarestrup, 2002), which codes for copper tolerance, is overrepresented in *Enterococcus* from the food-chain (Rebelo et al., 2021). The ability of MDR *Enterococcus* spp. to overcome other stresses such as acid pH and/or oxidative ones, however, has been less explored in isolates from animal-food production settings (Aarestrup and Hasman, 2004; Luyckx et al., 2017), precluding understanding which factors could be contributing to MDR strains selection and persistence.

This study evaluated *Enterococcus* susceptibility to different stresses inherent to the poultry production chain (antibiotics, metals, acid pH and peracetic acid) among commercially chicken meat samples collected 20 years apart in Portugal.

2. Material and methods

2.1. Sample collection, processing and bacterial identification

To analyze the susceptibility of *Enterococcus* spp. recovered 20 years apart to different stresses occurring in the poultry production chain, this study included 416 isolates from 120 chicken-meat samples collected in 2018 (here obtained and identified for the first time) and in 1999–2001 [previously published (Novais et al., 2005)].

The 2018 chicken meat samples ($n = 53$) were collected between March and August (spring/summer; ten sampling dates, each covering samples from different batches) in a modern large-scale poultry-production slaughterhouse (supplying diverse supermarkets and restaurants) in the North of Portugal, as described (Mourão et al., 2020). They were recovered after slaughter and chilling processes and immediately before distribution for retail sale. Broilers were raised in intensive farms ($n = 29$) settled in the North and the Center of Portugal, with a similar conventional indoor and floor-raised production system (flocks with 2500–8000 animals per house with age at slaughter between 28 and 42 days). Copper sulfate ($CuSO_4$) and organic acids (unknown composition) were used as additives in poultry feed, and peracetic acid-PAA (in concentrations ranging from 5000 to 30000 mg/L) as a biocide for disinfection of the slaughterhouse surfaces.

Each sample, corresponding to a batch, included 50 g of neck skin from a pool of ten carcasses of broilers raised in the same conditions (same flock of the same house within a specific farm) and slaughtered at the same time. They were collected in sterile plastic bags, transported at 4 °C, and processed on the same day at the laboratory. Twenty-five grams of each sample were pre-enriched in 225 mL of Buffered Peptone Water (Liofilchem, Roseto degli Abruzzi, Italy) and incubated at 37 °C during 18 h after 1 h resuscitation step at room temperature. Then, selective enrichments were performed by adding 1 mL of the previous pre-enrichment to tubes with 9 mL of Brain Heart Infusion (BHI) broth (Liofilchem, Roseto degli Abruzzi, Italy) without antimicrobial agents and supplemented with 16 μg/mL of ampicillin, 16 μg/mL of chloramphenicol (previously described to select linezolid-resistant isolates beside chloramphenicol resistant ones) (Finisterra et al., 2021) or 6 μg/mL of vancomycin (all antibiotics from Sigma-Aldrich®Brand, Taufkirchen, Germany), followed by incubation at 37 °C/18 h. Enrichment suspensions and their dilutions were plated (0.1 mL) in Slanetz-Bartley agar medium (Liofilchem, Roseto degli Abruzzi, Italy) without and with the same antibiotics concentrations previously described, and incubated at 37 °C/48 h. From each plate, at least one colony (up to five) per morphology was isolated in BHI agar (Liofilchem, Roseto degli Abruzzi, Italy) (37 °C, 24 h) and frozen for further studies. Genus identification was performed by standard methods (bile-esculin hydrolysis and catalase test) and species identification by PCR (Novais et al., 2013).

Samples and isolates collected during 1999–2001 in butcheries of the Porto area (North of Portugal) were processed and identified as previously described (Novais et al., 2005).

2.2. Antimicrobial susceptibility

2.2.1. Antibiotics

For the isolates obtained in 2018, susceptibility to 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; all antibiotics from OXOID,

Ireland] was evaluated by the disk diffusion method following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2021) or, when not possible, the Clinical and Laboratory Standards Institute (CLSI) ones (CLSI, 2020). Isolates categorized as "susceptible, increased exposure" (EUCAST guidelines) or as "intermediate resistant" (CLSI guidelines) were grouped as susceptible ones. Multidrug-resistance (MDR) was considered when the isolates were resistant to three or more antimicrobial agents of different families (Magiorakos et al., 2012). *E. faecalis* ATCC 29212 was used as control strain in the different assays. Search of genes encoding phenotypic resistance to tetracyclines [*tet(M)*, *tet(L)*], macrolides-lincosamide-streptogramines of group B [*erm(B)*], aminoglycosides [*aadE*, *aac(6′)-Ie-aph(2″)-Ia*] or chloramphenicol (*catA*_pC221/*catA*7, *catA*_pC223/*catA*8) was done by PCR as described (Freitas et al., 2017; Novais et al., 2013; Rebelo et al., 2021). The genes coding for linezolid resistance (*optrA, poxtA*) (Finisterra et al., 2021; Freitas et al., 2017) were searched in all isolates, as they can be associated with both susceptibility and resistance phenotypes. All PCR reactions included positive (*E. faecium* SN289-*tet(M), tet(L), erm(B), aadE, aac(6′)-Ie-aph(2″)-Ia*; *E. faecalis* PF101-*optrA, poxtA; E. faecalis* SN300- *catA*_pC221/*catA*7, *E. faecalis* SN254 *catA*_pC223/*catA*8) and negative controls (Freitas et al., 2017; Rebelo et al., 2021; Finisterra et al., 2021).

To compare antibiotic resistance rates from 1999-2001 and 2018 periods we only considered isolates (and respective batches) recovered from Slanetz-Bartley agar non-supplemented with antibiotics. Such strategy avoided potential bias on data comparison that could be associated with the use of different antibiotic selective enrichments in sample processing of both studies. Also, we reanalyzed 1999–2001 data using current resistant breakpoints from EUCAST (2021) and, when not possible, the CLSI (2020) guidelines. Thus, from the 1999–2001 collection, we selected 242 isolates from 67 samples for antibiotic resistance comparison with data of 2018 samples. The 2018 and 1999–2001 data comparison was done by sample.

2.2.2. Metals

Copper (*tcrB*, *cueO*) tolerance genes were searched by PCR in all isolates ($n = 174$) selected from the 53 samples of 2018. On those isolates positive for *tcrB* and/or *cueO*, other genes coding for metals (arsenic - *arsA_I*, *arsA_II*; and mercury - *merA_I*, *merA_II*, *merA_III*, *merA_IV* and *merA_V*) considered environmental pollutants in the food-chain (Rensing et al., 2018; Vareda et al., 2019) and often co-occurring with copper ones (Rebelo et al., 2021) were also searched. Primers, amplification conditions and positive controls were described previously (Rebelo et al., 2021). $CuSO_4$ susceptibility was evaluated by the agar dilution method under anaerobiosis (Mourão et al., 2016) in 60 isolates, representing different metal occurrence genotypes, species, poultry farms, time spans (spring/summer) and antibiotic susceptibility profiles, covering most of the samples collected ($n = 41/53$). Briefly, MIC_{CuSO4} values were determined using in all assays freshly prepared Mueller–Hinton 2 agar (bioMerieux, Marcy-l'Étoile, France) supplemented with $CuSO_4$ (0.25 to 36 mM) (Sigma-Aldrich-Merk, Taufkirchen, Germany) and adjusted to pH = 7.2. Incubation was done under anaerobiosis (18–20 h) (GENbox jar with GENbox anaer and an anaerobic indicator, bioMérieux, France). A 0.001 mL suspension of 10^7 cfu/mL bacteria was applied to each plate. The MIC was considered the first concentration without visible growth. The cut-off ≤12 mM was used to classify isolates as wild-type to $CuSO_4$ following tentative Epidemiological Cut Offs (ECOFFs) previously proposed (Mourão et al., 2016). Control strains included *Enterococcus lactis* BM4105RF (no copper tolerance acquired genes) (Novais et al., 2022) and *Escherichia coli* ED8739 (carrying the plasmid pRJ1004 with *pco* + *sil* cluster).

The *tcrB/cueO* genes occurrence in *Enterococcus* spp. from 1999-2001 collection was previously done (Rebelo et al., 2021; Silveira et al., 2014). From those, we selected 80 *Enterococcus* spp. from 45 samples for comparison purposes. Again, we only compared copper tolerance genes occurrence among 2018 and 1999–2001 periods using isolates recovered from Slanetz-Bartley without supplementation to overcome any bias associated with co-selection with particular antibiotics. The 2018 and 1999–2001 data comparison was done by sample.

2.2.3. Acids

Susceptibility to acidic pH and PAA was performed in the same isolates selected for $CuSO_4$ susceptibility study from 2018 collection (Table S1) using an adaptation of the microdilution standard method (ISO 20776-1:2019). The minimum growth pH corresponded to the lowest pH with visible bacterial growth. It was assessed by using Mueller-Hinton II broth (BD BBL™, Franklin Lakes, NJ, USA) adjusted with hydrochloric acid-HCl (Merck, Darmstadt, Germany) to obtain a pH range from 2.0 to 6.5 (with sequential 0.5 intervals) and distributed in a 96-well microtiter plate (freshly prepared for each assay). Then, bacterial suspensions in log-phase growth were adjusted and inoculated in each well with the corresponding pH to reach a final inoculum of 5×10^5 CFU/mL (confirmed for each isolate tested by a colony count in Mueller-Hinton 2 agar) and incubated at 37 °C/20-22 h. The minimum survival pH corresponded to the minimum pH showing at least one colony growth in BHI agar (37 °C/24 h–48 h), after inoculating 10 μL of the wells without visible growth.

Regarding PAA susceptibility, Minimum Inhibitory Concentration (MIC_{PAA}) corresponded to the first concentration of PAA without visible growth. It was determined using Mueller-Hinton II broth supplemented with PAA (15 % stock solution, CAS—No. 79-21-0; PanReac AppliChem, Darmstadt, Germany) at different concentrations (60 to 160 mg/L, with a 10 mg/L interval between them) and distributed in a 96-well microtiter plate (freshly prepared for each assay). Bacterial suspensions in log-phase growth were adjusted and inoculated in each well with the corresponding PAA concentration to reach a final inoculum of 5×10^5 CFU/ml (confirmed for each isolate tested by a colony count in Mueller-Hinton 2 agar), followed by incubation at 37 °C/20-22 h. The Minimum Bactericidal Concentration (MBC_{PAA}) was established as the lowest PAA concentration for which the number of colonies was equal or less than the rejection value defined by CLSI:1999 (former NCCLS:1999) guidelines, based on the final inoculum of each well confirmed by actual count. It was assessed by plating 10 μL of the previous wells without visible growth in BHI agar (37 °C/24-48 h). The pH's of PAA concentrations tested were also determined for each assay (ranging between 5.5 and 6.5, in which non-dissociated PAA were at 99.8–98 %; PAA pKa = 8.2 at 20 °C). *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were used as control strains in all assays. All pH and PAA susceptibility assays were performed at least in duplicate.

From the 1999–2001 collection, 31 isolates (15 *E. faecalis* and 16 *E. faecium*) from 22 samples, representing different brands and antibiotic resistance profiles, were selected and characterized for pH and PAA susceptibility, according to the description above. To compare the data from both collections (1999–2001 and 2018) we only performed the analysis by isolates and not by sample as in other parameters, based on the fact that there are no ECOFF values or specific acquired genotypes separating wild type and tolerant populations.

2.3. Molecular comparative studies from isolates recovered 20 years apart and from public databases

To elucidate if the *tcrB* ± *cueO* genes were associated with the expansion of particular clonal lineages within the chicken production chain, we analyzed the diversity of clonal backgrounds of the genomes from the samples of 1999–2001 and 2018 ($n = 15$) (Pöntinen et al., 2021), and some selected from PATRIC database ($n = 14$; last update on the 30th of November 2021). From the latter only genomes from feces and meat chicken samples were considered. A multilocus sequence typing (MLST; Sequence Type-ST) (http://pubmlst.org) and core genome MLST [(cgMLST; Complex Type-CT; Ridom SeqSphere+, version 8.2.0) (https://www.ridom.de/seqsphere)] were done. All genomes were included in a minimum-spanning tree based on the cgMLST

data of *E. faecalis* (1972 genes) performed in Ridom SeqSphere+. Then, to assess if *tcrB* ± *cueO* carrying platforms were hotspots of other metals tolerance genes as well as antibiotic resistance ones, we analyzed 25 out of the 29 *E. faecalis* used in the clonality analysis, being associated with diverse farms, brands, STs or antimicrobial resistance profiles. The synteny and nucleotide identity of *tcrB* genetic contexts were compared using the BLASTN option of Easyfig v2.2.2 (Sullivan et al., 2011). The criteria used to select the boundaries of the analyzed sequences was the limit of contigs where the *tcrB* gene was inserted, but when the contig was too short and the *tcrYAZB* was not complete the sequence was excluded from comparison studies.

2.4. Statistical analysis

Differences in antimicrobial resistance, copper, acid pH and PAA tolerance among *Enterococcus* species and samples were analyzed by the Fisher exact test (α = 0.05) using Prism software, version 8.1.1 (GraphPad).

3. Results

3.1. Bacterial identification and antimicrobial susceptibility of *Enterococcus spp.* recovered 20 years apart

3.1.1. Antibiotics

One hundred and seventy-four *Enterococcus* from the 2018 collection were selected from the 53 samples and farms studied and identified as *E. faecalis* (51 %, *n* = 88/174), *E. faecium* (44 %, *n* = 76/174) or *E. gallinarum* (6 %, *n* = 10/174). More than 45 % of the samples presented at least one *Enterococcus* spp. resistant to tetracycline (100 %, *n* = 53/53), erythromycin (98 %, *n* = 52/53), ampicillin (85 %, *n* = 45/53), quinupristin-dalfopristin (75 %, *n* = 40/53), ciprofloxacin (74 %, *n* = 39/53), streptomycin (74 %, n = 39/53), chloramphenicol (62 %, *n* = 33/53) or gentamicin (49 %, *n* = 26/53). Only one sample presented an isolate resistant to nitrofurantoin (2 %, n = 1/53). All isolates were susceptible to glycopeptides and linezolid. MDR isolates were identified in all samples.

Antibiotic resistance rates of the clinically relevant species *E. faecium* and *E. faecalis,* the most recovered from plates supplemented or not with antibiotics, are represented in Table 1. *Enterococcus* spp. resistant to ampicillin (all identified as *E. faecium*; 33 %, *n* = 57/174) or chloramphenicol (all identified as *E. faecalis*; 21 %, *n* = 36/174) were better recovered in Slanetz-Bartley agar media supplemented with these antibiotics (86 %, *n* = 49/57 or 89 %, *n* = 32/36, respectively). Ciprofloxacin resistance was higher for *E. faecium* (53 %, *n* = 40/76) than for *E. faecalis* (33 %, *n* = 29/88; $P < 0.05$). *E. faecium* and *E. faecalis* showed similar ($P > 0.05$) resistance rates to tetracycline (97 %, *n* = 74/76; 94 %, *n* = 83/88), erythromycin (93 %, *n* = 71/76; 84 %, n = 74/88), gentamicin (11 %, n = 8/76; 20 %, *n* = 18/88) or streptomycin (38 %, n = 29/76; 24 %, *n* = 21/88). MDR was higher among *E. faecium* (93 %, n = 71/76) than in *E. faecalis* (57 %, *n* = 50/88) ($P < 0.05$). The most frequently MDR phenotype acquired by *E. faecium* was ampicillin, tetracycline, erythromycin, quinuspristin-dalfopristin and ciprofloxacin (28 %, *n* = 20/71; 17 samples) and by *E. faecalis* was tetracycline, erythromycin and chloramphenicol (24 %, *n* = 12/50; 11 samples).

The tested acquired genes were greatly associated with the antibiotic resistance phenotypes found, namely erythromycin [*erm(B)*-97 %, *n* = 140/145 resistant isolates], tetracycline [*tet(M)*-98 %, *n* = 154/157; *tet (L)*-89 %, n = 140/157] gentamicin [*aac(6′)-Ie-aph(2″)-Ia*-85 %, *n* = 22/26), streptomycin (*aadE*-54 %, *n* = 27/50) and chloramphenicol [*cat (pC223)*-92 %, *n* = 33/36; *cat(pC221)*-8 %, n = 3/36] (Table 1). The genes coding for linezolid resistance (*optrA*, *poxtA*) were not detected in any of the isolates.

The evolution rates of antibiotic resistance by sample between 1999-2001 and 2018 studies is shown in Fig. 1. For the following antibiotics no differences ($P > 0.05$) were observed, respectively: tetracycline (82

Table 1
Antibiotic susceptibility of *E. faecalis* (*n* = 88) and *E. faecium* (*n* = 76) recovered from different poultry meat batches (2018) against 12 antibiotics.

Isolate selection	Species (n isolates)	Antimicrobial-resistance (n isolates)												
		VAN	TEC	AMP	TET	ERY	CIP	Q/D	CHL	GEN	STR	NIT	LIN	MDR
Without antibiotics	*E. faecalis* (53)	0	0	0	48	41	14	ND	3	7	7	1	0	15
	E. faecium (27)	0	0	8	25	23	4	24	0	2	13	ND	0	22
	Total (80)	0	0	8 (10 %)	73 (91 %) *tet(M)*-72; *tet(L)*-58	64 (80 %) *erm(B)*-61	18 (23 %)	24 (30 %)	3 (4 %) *cat(pC221)*-1 *cat(pC223)*-3	9 (11 %) *aac(6′)-Ie-aph(2″)-Ia*-9	20 (25 %) *aadE*-8	1 (1 %)	0	37 (46 %)
Vancomycin (6 μg/mL)	*E. gallinarum* (10)	ND	ND	0	10	10	6	ND	0	7	10	ND	0	10
	Total (10)	ND	ND	0	10 (100 %)	10 (100 %)	6 (60 %)	ND	0	7 (70 %)	10 (100 %)	ND	0	10 (100 %)
Ampicillin (16 μg/mL)	*E. faecalis* (3)	0	0	0	3	3	2	ND	1	1	0	0	0	3
	E. faecium (49)	0	0	49	49	48	36	34	0	6	16	ND	0	49
	Total (52)	0	0	49 (94 %)	52 (100 %) *tet(M)*-51; *tet(L)*-50	51 (98 %) *erm(B)*-50	38 (73 %)	34 (65 %)	1 (2 %) *cat(pC223)*-1	7 (13 %) *aac(6′)-Ie-aph(2″)-Ia*-6	16 (31 %) *aadE*-12	0	0	52 (100 %)
Chloramphenicol(16 μg/mL)	*E. faecalis* (32)	0	0	0	32	30	13	ND	32	10	14	0	0	32
	Total (32)	0	0	0	32 (100 %) *tet(M)*-31; *tet(L)*-32	30 (94 %) *erm(B)*-29	13 (41 %)	0	32 (100 %) *cat(pC221)*-2; *cat(pC223)*-29	10 (31 %) *aac(6′)-Ie-aph(2″)-Ia*-7	14 (44 %) *aadE*-7	0	0	32 (100 %)
All isolates	*E. faecalis* (88)	0	0	0	83 (94 %)	74 (84 %)	29 (33 %)	ND	36 (41 %)	18 (20 %)	21 (24 %)	1 (1 %)	0	50 (57 %)
	E. faecium (76)	0	0	57 (75 %)	74 (97 %)	71 (93 %)	40 (53 %)	58 (76 %)	0	8 (11 %)	29 (38 %)	ND	0	71 (93 %)
	E. gallinarum (10)	ND	ND	0	10 (100 %)	10 (100 %)	6 (60 %)	ND	0	7 (70 %)	10 (100 %)	ND	0	10 (100 %)

Abbreviations: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; LIN, linezolid; MDR, multidrug-resistance (resistance to 3 or more antimicrobial agents from different families); ND, not determined (*E. gallinarum* are intrinsically resistant to vancomycin; *E. faecalis* are intrinsically resistant to Q/D; in EUCAST breakpoints to nitrofurantoin are only available for *E. faecalis*); NIT, nitrofurantoin; Q/D, quinupristin-dalfopristin; STR, streptomycin; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin.

%, $n = 55/67$ versus 91 %, $n = 48/53$), erythromycin (78 %, $n = 52/67$ versus 83 %, $n = 44/53$), chloramphenicol (16 %, $n = 11/67$ versus 6 %, n = 3/53), gentamicin (15 %, $n = 10/67$ versus 17 %, $n = 9/53$) or nitrofurantoin (7 %, n = 5/67 versus 2 %, n = 1/53). The most significative difference between both studies was the disappearance of vancomycin resistance in 2018, when in 1999–2001 study 46 % ($n = 31/67$) of samples had *E. faecium* or *E. faecalis* with acquired resistance to vancomycin associated with the *vanA* gene (Novais et al., 2005). Samples of 1999–2001 had also higher rates of resistance to ciprofloxacin (52 %, $n = 35/67$ versus 32 %, $n = 17/53$ in 2018) and streptomycin (58 %, $n = 39/67$ versus 32 %, n = 17/53) ($P < 0.05$). On the other hand, samples from 2018 showed higher rates of resistance to ampicillin (17 %, $n = 9/53$ versus 3 %, $n = 2/67$ from 1999-2001) and quinupristin-dalfopristin (43 %, $n = 23/53$ versus 24 %, $n = 16/67$ ($P < 0.05$).

3.1.2. Metals

Copper (*tcrB*, *cueO*) tolerance genes were observed in isolates from almost half of the 2018 samples (49 %, $n = 26/53$, from which 96 % had *cueO* and 77 % *tcrB* genes). The *cueO* and/or *tcrB* were identified in 18 % ($n = 31/174$) of the tested isolates, 65 % with both genes (20 *E. faecalis;* 19 samples), 29 % with only *cueO* (9 *E. faecium;* 8 samples) and 6 % with only *tcrB* (2 *E. faecalis*; 2 samples). Isolates carrying only *cueO* or *tcrB* were recovered from Slanetz-Bartley not supplemented with antibiotics, while all but one *E. faecalis* with both genes were recovered from Slanetz-Bartley supplemented with chloramphenicol. The mercury tolerance gene *merA_IIA* was identified in 39 % ($n = 12/31$) of the *tcrB/cueO* positive isolates, all of them *E. faecalis* recovered from 11 samples. The *arsA* genes were not identified in any of the *tcrB/cueO* positive isolates. *E. faecalis* with *tcrB* ($n = 22$; with/without *cueO*) showed higher rates of resistance to chloramphenicol or streptomycin than those without this gene (91 %, $n = 20/22$ and 41 %, $n = 9/22$ versus 24 %, $n = 16/66$ and 18 %, n = 12/66; $P < 0.05$).

When only considering isolates from non-supplemented Slanetz-Bartley plates, a lower number of samples with isolates carrying copper tolerant genes were identified in 1999–2001 (4 %, n = 2 out of 45) compared to those of 2018 (21 %, $n = 11$ out of 53; $P < 0.05$), the latter with a great contribution of *E. faecium* only carrying *cueO* genes. The *merA* and *arsA* genes were not found in any isolates carrying *tcrB* or *cueO* genes from 1999-2001.

Copper phenotypic assays were performed in 38 *E. faecalis* and 22 *E. faecium* (80 %, $n = 48/60$ isolates tested were MDR) from 2018 carrying or not copper tolerance genes (Table S1). It was observed that all *E. faecalis* tested carrying the *tcrB* gene (42 %, $n = 16/38$) exhibited MICs to $CuSO_4$ between 16 and 28 mM and were considered as copper tolerant accordingly with the ECOFF values previously proposed for this species (Mourão et al., 2016). These data contrast with that of *Enterococcus* spp. without acquired copper tolerance genes, showing MIC to $CuSO_4$ of 4–12 mM. All isolates with only *cueO* gene had a MIC to $CuSO_4 \leq 12$ mM. The MICs to $CuSO_4$ of isolates from 1999-2001 were similar to those identified in 2018 isolates (Mourão et al., 2016; Rebelo et al., 2021).

3.1.3. Acids

Regarding HCl, similar minimum growth pH of 4.5–5.0 was observed for all isolates from 2018, with no differences among *E. faecium* and *E. faecalis* ($P > 0.05$). The minimal pH growth at pH = 4.5 was observed for 68 % ($n = 26/38$) of *E. faecalis* and 50 % ($n = 11/22$) of *E. faecium,* and at pH = 5 for 32 % ($n = 12/38$) of *E. faecalis* and 50 % (n = 11/22) of *E. faecium.* The minimum survival pH ranged between 3.0 and 4.0, with most isolates surviving at pH = 4.0 (all *E. faecalis* and 27 %-$n = 6/22$ of *E. faecium*), followed by pH = 3.5 and 3.0 (73 %, $n = 16/22$ of *E. faecium*). Similarly, the minimum growth pH of all 1999–2001 *E. faecalis* and *E. faecium* varied between 4.5 and 5.0, again with no differences among both species ($P > 0.05$) (Table S1, Fig. 2). Despite a higher number of *E. faecalis* from 2018 (68 %, n = 26/38) grown at lower pH (4.5) compared to *E. faecalis* (40 %, n = 6/15) from 1999-2001, no statistical difference was observed ($P > 0.05$), as well as for *E. faecium* isolates between these time spans (50 %, $n = 11/22$ from 2018 vs 56 %, $n = 9/16$ from 1999-2001; $P > 0.05$). Regarding the minimum survival pH, isolates from 1999-2001 showed similar phenotypes (pH = 3.0: 10 %, $n = 3/31$, *E. faecium;* pH = 3.5: 26 %, $n = 8/31$, 6 *E. faecium* and 2 *E. faecalis;* pH = 4.0: 65 %, $n = 20/31$, 7 *E. faecium* and 13 *E. faecalis*) to those detected in 2018 collection ($P > 0.05$) (Table S1, Fig. 2). As in 2018 isolates, *E. faecium* from 1999-2001 presented a better ability to survive at lower pHs (pH <4.0) (56 %, n = 9/16) compared to *E. faecalis* (13 %, n = 2/15) ($P < 0.05$).

The MIC to PAA ranged between 70 and 120 mg/L among isolates from 2018, with *E. faecalis* presenting higher MICs (MIC_{range} = 100–120 mg/L; MIC_{90} = 120 mg/L) compared to *E. faecium* (MIC_{range} = 70–120 mg/L; MIC_{90} = 100 mg/L). The MBC to PAA ranged between 80 and 150 mg/L, with *E. faecalis* showing slightly higher values (MBC_{range} = 120–150 mg/L; MBC_{90} = 140 mg/L) than *E. faecium* (MBC_{range} = 80–140 mg/L; MBC_{90} = 130 mg/L). Similarly, the MIC_{PAA} of isolates from 1999-2001 ranged between 80 and 120 mg/L (MIC_{90} = 120 mg/L), with *E. faecalis* also showing higher MICs (MIC_{range} = 90–120 mg/L; MIC_{90} = 120 mg/L) than *E. faecium* (MIC_{range} = 80–100 mg/L; MIC_{90} = 100 mg/L) (Table S1, Fig. 3). The MBC in isolates from 1999-2001 were also similar to those observed in 2018 isolates, ranging between 100 and 160 mg/L, with *E. faecium* presenting slightly higher values (MBC_{range} = 100–160 mg/L and MBC_{90} = 160 mg/L) than *E. faecalis* (MBC_{range} = 120–150 mg/L; MBC_{90} = 150 mg/L) (Table S1, Fig. 3).

3.2. Comparative genomics of *E. faecalis* carrying *tcrB*±*cueO* recovered 20 years apart and from public databases

Based on the cgMLST analysis, *E. faecalis* genomes carrying *tcrYAZB* operon from our 1999–2001 and 2018 collections as well as from PATRIC database were clonally diverse, with ten isolates distributed in four clusters (each cluster including isolates with ≤7 alleles of difference) (Neumann et al., 2019) and 19 not included in any particular cluster (Fig. 4). Cluster 1, 2 and 4 were composed by *E. faecalis* from Portugal. Cluster 1 was composed of three isolates identified as ST843/CT1571 and recovered between April and May 2018 from meat of chicken raised in three farms. Cluster 2 included two isolates identified as ST21/CT1568 and one as ST21/CT1577 recovered between March and July 2018 from meat of chicken from three farms. Cluster 4 was composed of two isolates identified as ST288/CT1629 recovered in 2001 from meat of chicken from two brands. Cluster 3 was composed of two isolates identified as ST476/CT2136 recovered in 2017 from the market of two different cities in Tunisia (Freitas et al., 2020).

The genetic environment of *tcrYAZB* operon was variable among *E. faecalis* recovered from chicken over the last 20 years. In Fig. S1 we divided the *tcrYAZB* genetic contexts (A to D groups) accordingly with their similarity. In most cases, isolates belonging to the same ST, but not necessarily to the same cgMLST complex type, showed similar *tcrB* genetic contexts (Fig. S1, A, B and C groups). In the closed genomes it was possible to assess the *tcrYAZB* location, with some isolates presenting this operon in plasmids of diverse sizes (Fig. S1, group B) and others in the chromosome (Fig. S1, group C).

In most cases (Fig. S1, A to D groups) the *tcrYAZB* operon was adjacent to the *cueO*-response regulator-histidine kinase cluster of genes. Insertion sequences or recombinases, associated with the recombinogenic and modular structures of *tcrYAZB* genetic platforms, were often observed, gathering modules related to antibiotic resistance, bacteriocin production, diverse types of metabolism (e.g. carbohydrates, acetoin, amino acids), UV damage repair, associated with colonization or plasmid stability. Particularly, ST843 *E. faecalis* (Fig. S1, group A; Fig. 4, cluster 1) from our 2018 collection showed the *tcrYAZB* operon near to clusters of genes conferring resistance to antibiotics often used in the animal production (tetracyclines, phenicols, MLS_B and aminoglycosides) and related to class IIa bacteriocin production (100 % homology with that of *E. faecalis* MC4) that presents activity against different

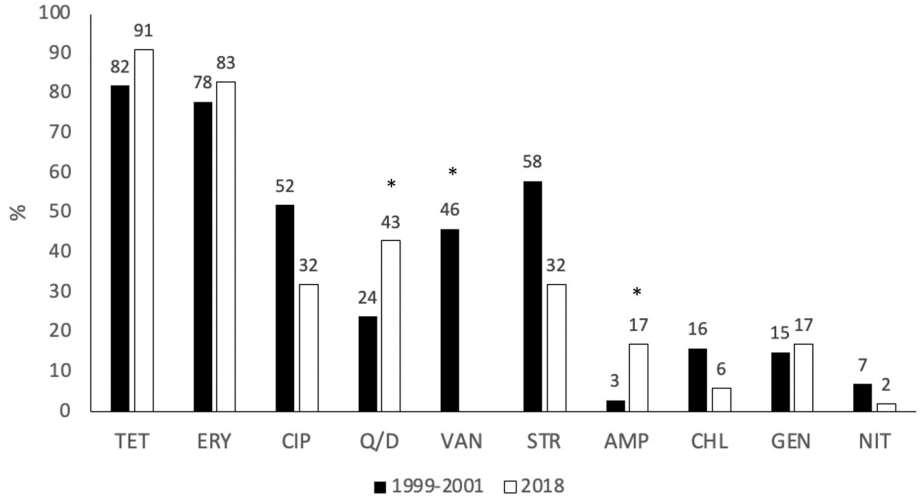

Fig. 1. Percentage of chicken meat batches collected in 1999–2001 and 2018 with antibiotic resistant *Enterococcus* spp.. For this comparison only isolates recovered from non-supplemented Slanetz-Bartley plates were considered. Abbreviations: AMP, ampicillin, CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NIT, nitrofurantoin; TET, tetracycline; Q/D, quinupristin-dalfopristin; STR, streptomycin; VAN, vancomycin. *, $P < 0.05$ (Fisher exact test).

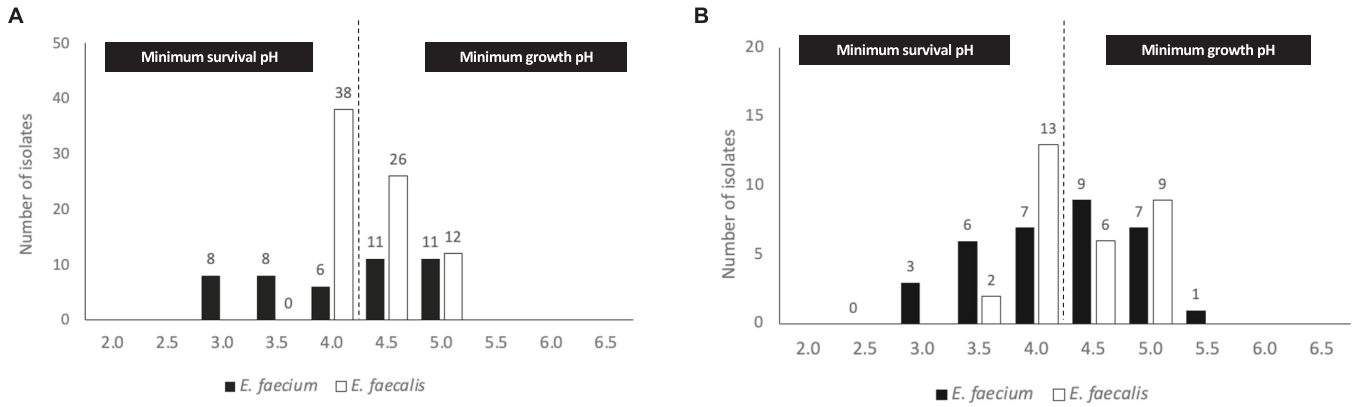

Fig. 2. Minimum survival and minimum growth pH of *E. faecium* and *E. faecalis* from chicken meat batches collected in 2018 (A) and 1999–2001 (B).

Enterococcus species and *Listeria monocytogenes* (Flannagan et al., 2008). Similar antibiotic resistance and bacteriocin coding genes were also found in a ST476 isolate from South Korea (2016), and part of this *tcrYAZB* platform in ST476 isolates from Tunisia (2017), all from chicken meat (Fig. S1, group A; 2 from cluster 3 and 1 related to this). Antibiotic resistance genes co-located in the same platform as *tcrYAZB* operon were also detected in Portuguese *E. faecalis* from the 1999–2001 collection from diverse brands (Fig. S1, group B; ST53 and ST288; Fig. 4, cluster 4 or no cluster). Antibiotic resistance genes found in this group included those to bacitracin and/or polyether ionophores (e.g. narasin, salinomycin and maduramicin) resistance or, in one case, to MLS_B and aminoglycosides. Genes related to acetoin, 2,3-butanediol and sucrose metabolism were also common in these platform variants. Acetoin is a neutral product formed to overcome acid pH occurring from sugar metabolism, that can itself be used as a source of energy by acetoin dehydrogenases when sugars are depleted (Ould Ali et al., 2001). Our recent ST21/ST22-*E. faecalis* showed the *tcrYAZB* in the chromosome region (studied for 1 isolate) near to a *merR-merA*_IIA, being this genetic environment also detected in recent USA and Tunisia strains, all ST21 (Fig. S1, group C; Fig. 4, cluster 4 or related to this). Finally, in Fig. S1, group D, we gathered the *tcrB* genetic environments which were more dissimilar, including isolates belonging to diverse ST not identified in the former groups.

4. Discussion

This study shows that a high rate of recent chicken meat batches available for consumers in Portugal are vehicles of MDR *Enterococcus* spp. with ability to overcome other stresses occurring on the poultry production chain, such as copper and acids.

The decrease of antibiotic use at farm level in Europe during the last decade (ECDC/EFSA/EMA, 2021; EMA, 2021) was not enough to reduce antibiotic resistance rates associated with samples recovered >20 years apart. High levels of resistance to macrolides, tetracyclines, chloramphenicol or gentamicin were observed in samples recovered in 1999–2001 and 2018, with genes coding for resistance to these antibiotics widely spread in *Enterococcus* and other *Bacillota* (former *Firmicutes*) (Oren and Garrity, 2021) from diverse origins, for decades (this study; Lanza et al., 2015; Rebelo et al., 2021). This gene's abundance and flow, often in clusters (e.g.*Tn5405*-like) (Kambarev et al., 2018; Lanza et al., 2015; Schwarz et al., 2014) might facilitate the co-selection and maintenance of genetic platforms or clones with multiple adaptive features under diverse and co-occurring antibiotic selective pressures, namely in the poultry production. In fact, sales of tetracyclines or macrolides were still high in 2018 for the animal food-production in Portugal (EMA, 2021), which can explain the maintenance of resistance rates detected during this long-time span of 20 years. It has been recently shown that among antibiotic resistant bacteria to which consumers are at most risk to be exposed by poultry meat are *E. faecium* and *E. faecalis*

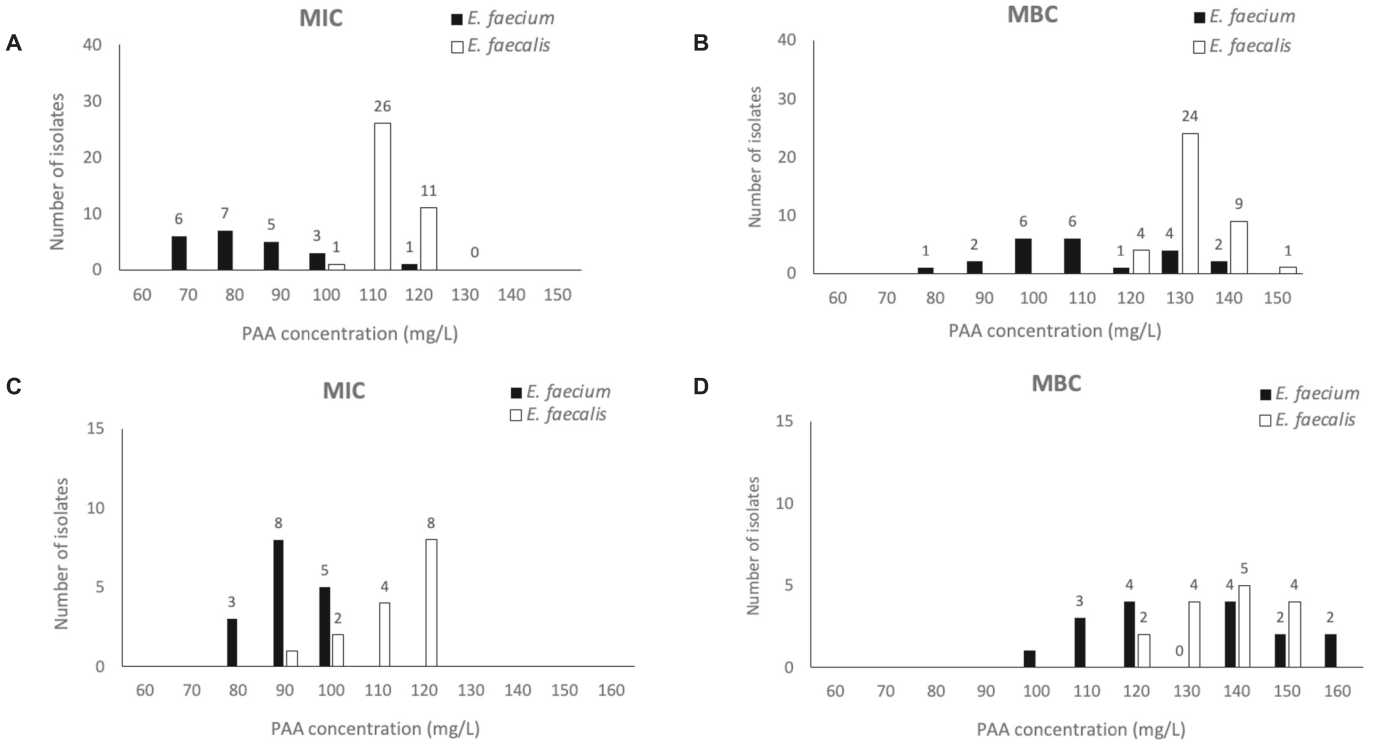

Fig. 3. Minimum Inhibitory Concentration (MIC) of Peracetic Acid (PAA) against *E. faecium* and *E. faecalis* from chicken meat batches collected in 2018 (A) and 1999–2001 (C). Minimum Bactericidal Concentration (MBC) of PAA against *E. faecium* and *E. faecalis* from chicken meat batches collected in 2018 (B) and 1999–2001 (D).

with resistance to tetracyclines or macrolides (Collineau et al., 2018). Encouraging was the absence of resistance to the clinically relevant antibiotics linezolid and vancomycin in 2018 samples, despite the use of antibiotic selective methodological steps. In fact, vancomycin resistance significantly decreased from 1999-2001 to 2018 samples, >20 years after avoparcin ban, data also supported by other European studies (Borck et al., 2015; de Jong et al., 2019; Pesavento et al., 2014; Rivera-Gomis et al., 2021; Simm et al., 2019). Still, European data comparison is difficult as studies including *Enterococcus* from chicken meat over the last 10 years are very scarce (Borck et al., 2015; EFSA/ECDC, 2015; Leinweber et al., 2018) limiting the analysis of antibiotic resistance features of strains reaching humans during meat handling, preparation and/or consumption in this geographical context.

The more restricted use of antibiotics as metaphylactic and prophylactic agents in Europe from January 2022 Regulation (EU) 2019/6 of the European Parliament] and the European goal for 2030 to reduce the antibiotics sells for farm animals to 50 % (European Commission, 2020) creates new expectations on antimicrobial resistance reduction in the next few years but also on the role of other antimicrobial compounds (e.g. metals, disinfectants) for antimicrobial resistance selection and maintenance. This is particularly relevant for *Enterococcus* spp., recently pointed out as one of the major bacterial hosts of antibiotic resistance genes in chicken microbiota (Yang et al., 2022). Poultry are among the animal species in which copper can be used as growth promoter (up to 25 mg/kg) (EFSA FEEDAP Panel, 2016). The detection of the Cu tolerant *tcrYAZB* operon in plasmids carrying genes coding for antibiotics often used in the poultry European production as therapeutic agents or that were once used as growth promoters (e.g., tetracyclines, lincosamides, macrolides, aminoglycosides, bacitracin) (Castanon, 2007; ECDC/EFSA/EMA, 2021) in strains collected 20 years apart, stresses the importance of multiple compounds contributing to antibiotic resistance co-selection events among diverse *E. faecalis* clones. The co-occurrence of other variable genetic features related to metabolism, UV damage repair or colonization, highlights chicken *tcrB*-plasmids as "survival tool kits" against several environmental challenges, being the importance of their maintenance in *E. faecalis* supported by the often occurrence of toxin-antitoxin systems. Moreover, despite *tcrYAZB* operon being generally described in plasmids (Rebelo et al., 2021), here it was also detected in ST21 *E. faecalis* chromosomes adjacent to the mercury tolerance gene *merA*, modules often found on *E. faecium* plasmids (Rebelo et al., 2021), also suggesting fixation of *tcrYAZB* operon. If such fixation is to achieve lower fitness cost or is due to segregational instability of such plasmids in the *E. faecalis* hosts conducting to plasmid lost in *E. faecalis* population remains to clarify (Carroll and Wong, 2018). Although there is still no clear evidence of copper impact in the selection and persistence of MDR bacteria (EFSA BIOHAZ Panel et al., 2021), our data deserves reflection and support detailed future studies to assess copper use implications.

Disinfectants have also been pointed out as possible selectors of MDR bacteria in the food chain due their wide use and occurrence of residues in subinhibitory concentrations in surfaces and other matrices (Davies and Wales, 2019). All *E. faecalis* and *E. faecium,* including the MDR isolates, were susceptible to concentrations far below those used in the slaughterhouse where the 2018 samples were collected. The *in vitro* good activity of PAA against *Enterococcus* spp. is also supported by other studies, as no tolerant isolates have been described (Sahulka et al., 2021; Turolla et al., 2017). Finally, despite *Enterococcus* spp. are known to survive a wide range of pH values (Fisher and Phillips, 2009), the specific acid susceptibility phenotypes of isolates from poultry production were scarcely described and usually only involved a few strains (Shi et al., 2020). The acid tolerance found was similar to other *Enterococcus* spp. from chicken or other origins (Shi et al., 2020; Singhal et al., 2019). Such tolerance is advantageous for their survival and spread through the food chain, where extreme fluctuations in external pH occur (chicken gastrointestinal system: 2.5-8.0; chicken meat/skin: 5.8-6.3; human gastrointestinal system: 1–7.5; immune system macrophages: 4.5-5.5), in particular mild to acidic pH (Koziolek et al., 2015; Marmion et al., 2021; Park et al., 2021; Salze et al., 2020).

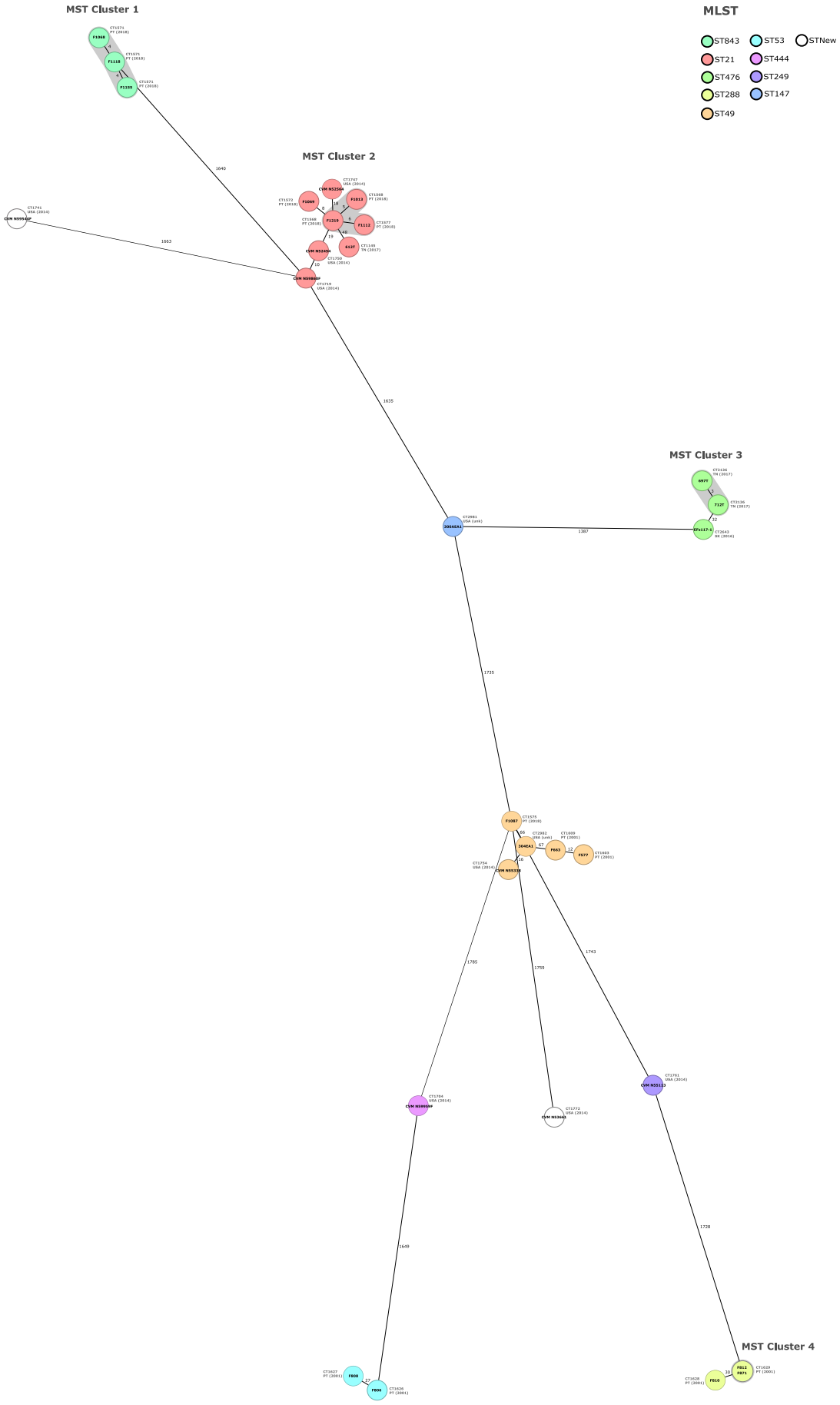

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Fig. 4. Minimum-spanning tree based on the core-genome multilocus sequence typing (cgMLST) data from *Enterococcus faecalis* isolates ($n = 29$) carrying the copper tolerance gene *tcrB* from chicken meat/feces from this study and PATRIC database. The analysis was performed with Ridom SeqSphere^{+} version 7.2 software (https://www.ridom.de/seqsphere). Each circle represents a complex type and the code inside them the isolates number. The numbers on the connecting lines represent the cgMLST allelic differences between 2 isolates. Sequence types (MLST) are shown in colored circles (see key). Gray shading around nodes indicates clusters of closely related isolates (<7 alleles of difference) (Neumann et al., 2019). CT, complexe type; PT, Portugal; SK, South Korea; TN, Tunisia; USA, United States of America.

In summary, despite the European policies to reduce the use of antibiotics in the last decades, poultry meat still remains a vehicle of MDR *Enterococcus* spp. to the consumers. Their ability to overcome other stresses (e.g. copper, acids) present in the poultry food chain might contribute to the successful flux of MDR strains among animal hosts, poultry production environment, meat and ultimately the human host. Identification of environmental transmission routes of MDR bacteria to chickens and their meat, and of the minimum concentrations of stressors (e.g. copper, antibiotics) selecting and maintaining MDR strains or their genetic elements, are gaps needed to be addressed in future One Health studies to mitigate the antibiotic resistance spread associated with the food chain.

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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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