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Linezolid- and Multidrug-Resistant Enterococci in Raw Commercial Dog Food, Europe, 2019–2020

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We describe enterococci in raw-frozen dog food commercialized in Europe as a source of genes encoding resistance to the antibiotic drug linezolid and of strains and plasmids enriched in antibiotic-resistance and virulence genes in hospitalized patients. Whole-genome sequencing was fundamental to linking isolates from dog food to human cases across Europe.

Raw meat–based diets are increasingly popular for feeding dogs, but the extent of antimicrobial-resistant bacteria in raw dog food is rarely addressed globally (*1*). The Centers for Disease Control and Prevention does not recommend feeding raw diets to pets because of frequent contamination with *Salmonella* and *Listeria* (https://www.cdc.gov/healthypets/publications/pet-food-safety.html), but awareness about this issue is not as evident in Europe. Eating raw meat has been considered a risk factor for carriage of clinically relevant ampicillin-resistant (AmpR) *Enterococcus faecium* and *optrA*-positive linezolid-resistant *E. faecalis* in dogs (*2*,*3*), but data for commercial pet food are not available. We evaluated multidrug-resistant (MDR) *Enterococcus* in raw-frozen dog food commercialized in countries in Europe; we focused on transferable linezolid resistance (LinR) genes because linezolid is a last-resort drug to treat gram-positive infections (*4*).

We purchased 14 raw-frozen dog food samples from the 2 commercially available brands in Portugal in specialized stores (September 2019–January 2020). Brand A (produced in Europe) is available in specialized

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Table. Characterization of *Enterococcus* isolates obtained from raw dog food samples, Porto, Portugal, 2019–2020*

Species	cgMLST†	MLST‡	Sample (brand)§	Antimicrobial drug resistance profile#	Antibiotic resistance genotype	MIC LIN, mg/L	Transfer of LinR genes
E. faecalis	CT1206	ST40	Duck (B)	ERY, TET, CHL, LIN	*optrA, fexA, cat, erm(B), lsa(A), tet(M), dfr(G)*	8	–
	CT1207	ST674	Salmon (A)	CIP, ERY, TET, STR, CHL, LIN	*optrA, cfrD, fexA, cat, ant(6)-Ia, aph(3')-III, erm(B), lsa(A), tet(M), tet(L), dfr(G)*	8	++
	CT1205	ST1008	Turkey (A)¶	ERY, TET, GEN, STR, CHL	*optrA, poxtA, fexB, cat, aac(6')-aph(2"), ant(6)-Ia, ant(9)-Ia, aph(3')-III, erm(B), lnu(B), lsa(A), lsa(E), tet(M), tet(L), dfr(G)*	4	–
	CT1205	ST1008	Turkey (A)¶	ERY, TET, STR, CHL	*optrA, poxtA, fexB, cat, aac(6')-aph(2"), ant(6)-Ia, ant(9)-Ia, aph(3')-III, erm(B), lnu(B), lsa(A), lsa(E), tet(M), tet(L), dfr(G)*	4	–
	CT1209	ST1008	Chicken + lamb (A)	ERY, TET, STR, CHL, LIN	*optrA, poxtA, fexB, cat, aac(6')-aph(2"), ant(6)-Ia, ant(9)-Ia, aph(3')-III, erm(B), lnu(B), lsa(A), lsa(E), tet(M), tet(L), dfr(G)*	8	–
	CT1208	ST1009	Turkey + goose (B)	ERY, CHL, LIN	*optrA, cfrD, fexA, cat, erm(B), lsa(A), dfr(G)*	8	–
E. faecium	CT106	ST80	Salmon (A)	AMP (>256 mg/L), CIP, ERY, TET, GEN, STR, QD	*aac(6')-aph(2"), ant(6)-Ia, aph(3')-III, erm(B), msr(C), tet(M), tet(L), dfr(G)*	ND	NA
	CT284	ST25	Beef (A)	AMP (32 mg/L), CIP, ERY, TET, GEN, STR, QD, CHL	*poxtA, fexB, aac(6')-aph(2"), ant(6)-Ia, ant(9)-Ia, aph(3')-III, erm(A), erm(B), msr(C), lnu(B), lsa(E), tet(M), tet(L), dfr(G)*	4	–
	CT374	ST264	Beef (A)	AMP (32 mg/L), CIP, TET, STR, QD	*cat, ant(6)-Ia, lnu(G), tet(M), tet(L), dfr(G)*	ND	NA
	CT272	ST1091	Duck (B)	AMP (>256 mg/L), CIP, ERY, TET, STR, QD	*ant(9)-Ia, erm(A), erm(B), msr(C), tet(M), tet(L), dfr(G)*	ND	NA
	CT3399	ST1263	Deer (B)	AMP, ERY, TET, STR, QD, CHL	*poxtA, fexB, cat, ant(6)-Ia, ant(9)-Ia, aph(3')-III, erm(A), msr(C), lnu(B), lsa(E), tet(L), dfr(G)*	4	+

*AMP, ampicillin; cgMLST, core-genome MLST; CIP, ciprofloxacin; CHL, chloramphenicol; CT, complex type; ERY, erythromycin; GEN, high-level resistance to gentamicin; LIN, linezolid; LinR, linezolid-resistant; MLST, multilocus sequence typing; NA, not applicable; ND, not done: QD, quinupristin/dalfopristin; STR, high-level resistance to streptomycin; ST, sequence type: +, positive (transfer frequency of 10^{-8}); ++, positive (transfer frequency of 10^{-7}); –, negative.
†The *E. faecalis* CT1205-CT1209 and the *E. faecium* CT3399 were identified in this study by submitting them to the cgMLST database (https://www.cgMLST.org) through Ridom SeqSphere+ version 7.2 software (https://www.ridom.de/seqsphere).
‡The novel *E. faecalis* ST1008–ST1009 were submitted to the MLST database (https://www.pubmlst.org).
§Brand A is produced in Europe; Brand B is produced in the United Kingdom.
¶These 2 samples correspond to 2 different batches and were acquired at different times (October 2019 and January 2020).
#QD resistance was tested only against *E. faecium* isolates. Successful transfer of ampicillin resistance is underlined (AMP) and all transconjugants exhibited high values of ampicillin resistance (16–256 mg/L).

stores, brand B (produced in the United Kingdom) in specialized stores and online; both are commercialized across different countries in Europe. We enriched samples (25 g) in buffered peptone water (1:10), then in brain–heart infusion broth with or without different antibiotic drugs (ampicillin [16 μg/mL], vancomycin [6 μg/mL], chloramphenicol [16 μg/mL]), and plated them onto Slanetz-Bartley agar with and without the same drug concentrations. We identified isolates with different morphologies per plate by PCR. We performed antibiotic susceptibility testing by disk diffusion using European Committee on Antimicrobial Susceptibility Testing (EUCAST) (*5*) or Clinical and Laboratory Standards Institute (*6*) guidelines. We used broth microdilution for linezolid and Etest for ampicillin. We searched acquired LinR genes (*optrA/poxtA/cfrA-E*) and typed representative isolates by multilocus sequence typing (n = 20; https://www.pubmlst.org) and whole-genome sequencing (LinR *E. faecalis* [n = 6] and AmpR/LinR *E. faecium* [n = 5]) using the Hi Seq 2500 Sequencing System (Illumina, https://www.illumina.com). We deposited assemblies (SPAdes version 3.11.1; https://cab.spbu.ru/software/spades) in GenBank (Bioproject PRJNA663240) and characterized them using in silico tools (http://www.genomicepidemiology.org) and in-house databases (*7*).

All samples carried enterococci resistant to erythromycin, streptomycin, chloramphenicol, and tetracycline; 93% resistant to ampicillin, ciprofloxacin, and quinupristin/dalfopristin; 79% resistant to gentamicin; and 50% resistant to linezolid. We detected acquired LinR genes among 20 MDR isolates from

64% of samples from both brands and with different types of ingredients (Table): *optrA* (4 *E. faecalis*, 1 *E. faecium*), *poxtA* (2 *E. faecium*), *optrA*+*poxtA* (8 *E. faecalis*, 3 *E. faecium*) or *optrA*+*cfrD* (2 *E. faecalis*). Of those, 15 expressed LinR (MIC = 8 mg/L), whereas 5 were susceptible (MIC = 4 mg/L) (Table).

The *E. faecium* isolates (n = 39) were mostly MDR (70%), expressing resistance to tetracycline (85%), quinupristin/dalfopristin (72%), erythromycin (64%), ciprofloxacin (59%), streptomycin (57%), ampicillin (56%), gentamicin (23%), chloramphenicol (21%), or linezolid (10%). We compared selected dog food AmpR *E. faecium* genomes with 7,660 available GenBank *E. faecium* genomes by complex types (CTs) through core-genome multilocus sequence typing (Ridom SeqSphere+ version 7.2, https://www.ridom.de/seqsphere). Those data (Figure) and data from single-nucleotide polymorphisms (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/27/8/20-4933-App1.pdf) showed different clusters grouping related isolates obtained from dog food and hospitalized patients (sequence type [ST] 80/CT106; ST264/CT374) or from pet food and livestock or wastewaters (ST1091/CT284; ST1263/CT3399) in different countries. Dog food *E. faecium* was enriched in acquired antibiotic-resistant and virulence genes as strains from different sources (Appendix Figure 1). ST80 *E. faecium* from brand A was phylogenetically related to other strains from Germany and Netherlands; ST1091 and ST1263 from brand B were phylogenetically related to UK strains (Figure). By filter-mating (*8*), we found that 3 (ST25, ST80, ST1263) of 5 AmpR *E. faecium* isolates transferred a chromosomal genetic platform containing *pbp5* to GE1 *E. faecium* strain (Table). Following our previous description of a large transferable *pbp5*-containing platform in a clinical isolate (*8*), we partly identified highly similar genetic platforms carrying different adaptive features including virulence genes (e.g., *sgrA*) in ST80 and ST1263 dog food AmpR *E. faecium* (Appendix Figure 2). ST1263 *E. faecium* was able to transfer *poxtA* by conjugation (Table).

The *E. faecalis* isolates (n = 52) recovered were mostly MDR (75%), resistant to chloramphenicol (83%), tetracycline (79%), erythromycin (75%), streptomycin (63%), gentamicin (31%), linezolid (21%), or ciprofloxacin (10%). ST40, ST674, ST1008, and ST1009 sequences corresponded to novel complex types carrying antimicrobial resistance (*aac(6′)-aph(2″)/ant(6)-Ia/aph3″-III/erm(B)/tet(M),tet(L),dfr(G)*) and virulence (*ace/gelE/elrA*) genes linked to clinically relevant MDR lineages (Table) (*7*,*9*). ST674 *E. faecalis* carried *optrA* on a pheromone-responsive plasmid (pAPT110) identical to others from non–clonally related *E. faecalis* in hospitalized patients in Spain and China (Appendix Figure 3). Similarly to pAPT110 in this study transferring *optrA* in high rates (Table), pEF10748 (China) is an *optrA* highly transferable plasmid with a complete sex-pheromone response module (*10*).

In conclusion, the diversity and rate of *E. faecium* and *E. faecalis* with linezolid-resistance genes (*optrA*/*poxtA*/*cfrD*) we identified were unexpectedly high. Our data suggest that raw dog food could be a sentinel of emerging antimicrobial resistance traits because this type of food may accumulate raw ingredients of different origins, namely from animals associated with intensive farming, adding a new concern to the global health burden of antimicrobial resistance.

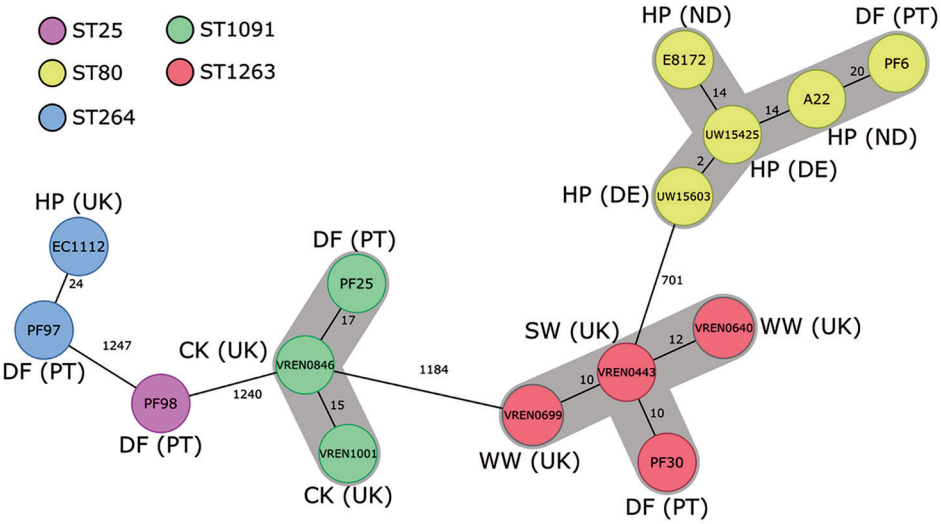

Figure. Minimum-spanning tree based on the core-genome multilocus sequence typing (cgMLST) data from *Enterococcus faecium* isolates (n = 15) from different sources in Europe. The tree is based on cgMLST (1,423 genes) analyses made with Ridom SeqSphere+ version 7.2 software (https://www.ridom.de/seqsphere). Each circle represents 1 allele profile. The numbers on the connecting lines represent the number of cgMLST allelic differences between 2 isolates. Sequence types are shown in colored circles (see key); numbers in circles are isolate identifications. Gray shading around nodes indicates clusters of closely related isolates (≤20). CK, chicken; DE, Denmark; DF, dog food; HP, hospitalized patient; PT, Portugal; ST, sequence type; SW, swine; UK, United Kingdom; WW, wastewater.

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Highly Pathogenic Avian Influenza A(H5N8) Virus Clade 2.3.4.4b, Western Siberia, Russia, 2020

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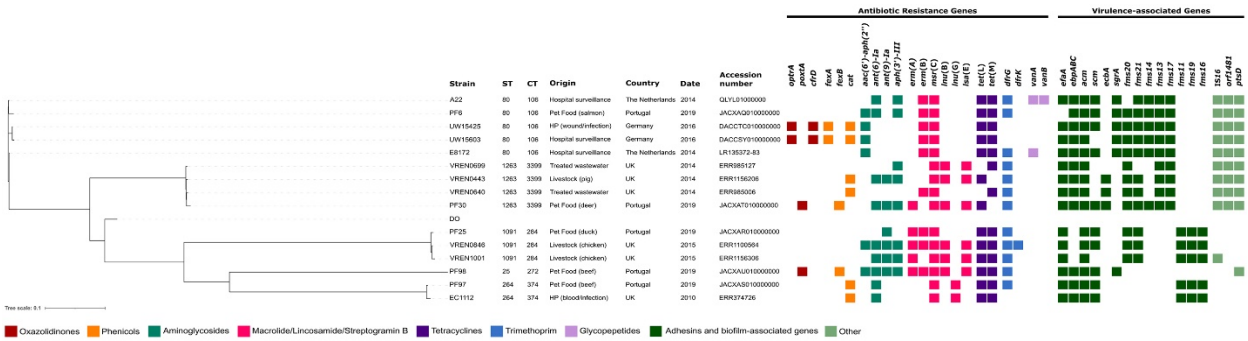
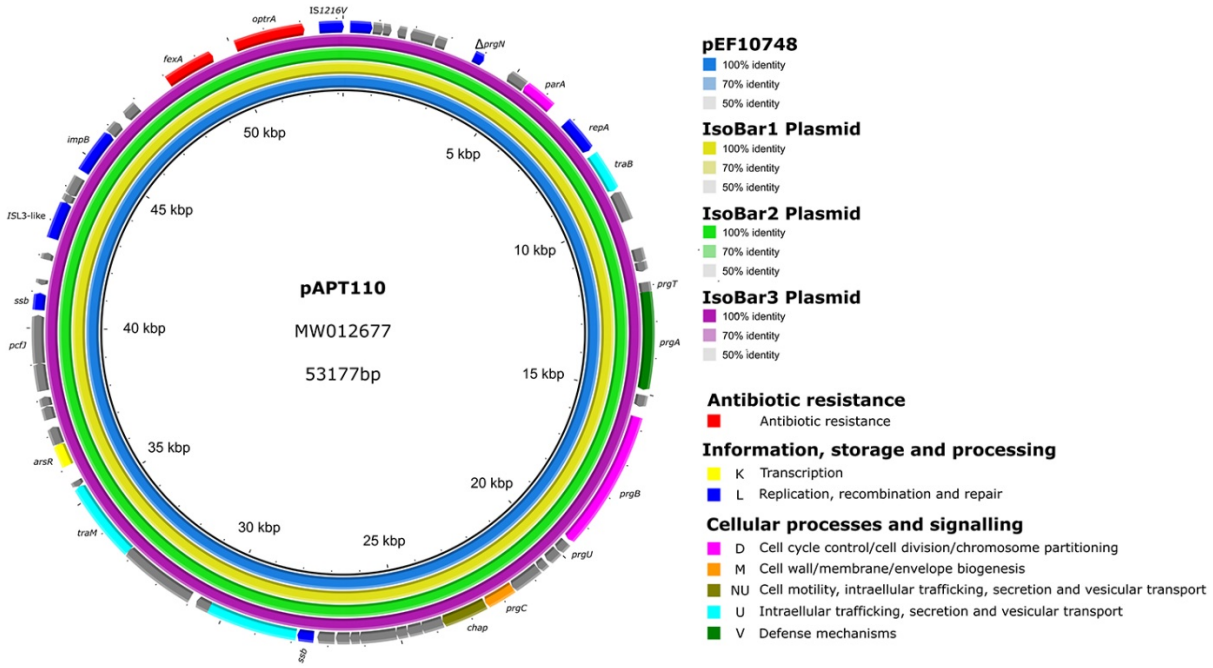
Linezolid- and Multidrug-Resistant Enterococci in Raw Commercial Dog Food, Europe, 2019–2020

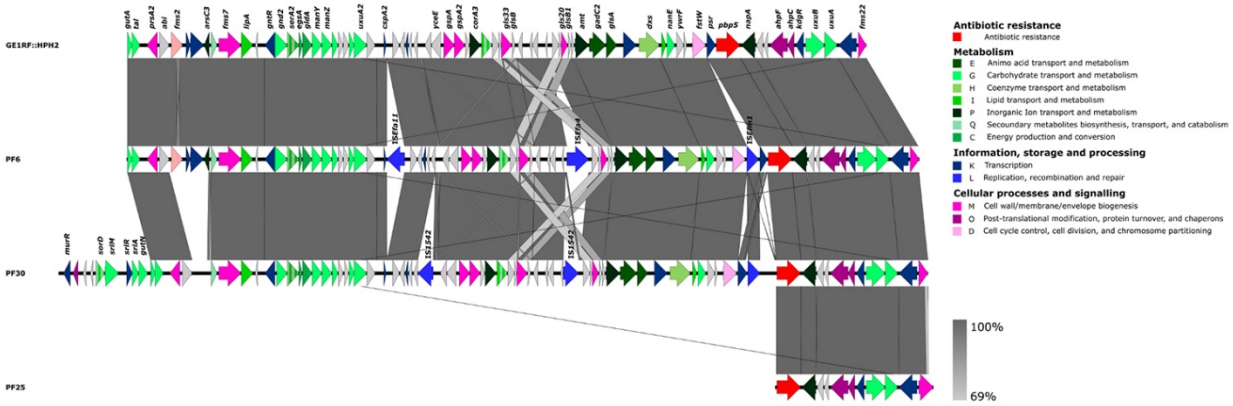
Appendix



Appendix Figure 1. SNP-based phylogenetic tree comparing *E. faecium* isolates (n = 15) of different sources. SNP alignment was obtained using CSI Phylogeny (https://cge.cbs.dtu.dk/services/CSIPhylogeny) and *E. faecium* DO (GenBank accession no. CP00358) as reference strain. Input parameters were 10x minimum depth and minimum relative depth at SNP positions; SNPs were filtered out if they were called within the vicinity of 300 bp of another SNP (pruning); minimum SNP quality of 30; minimum read mapping quality of 25; minimum Z-score of 1.96. Data were analyzed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to test the quality of the raw and preprocessed data; SPAdes v.3.11.1 (https://cab.spbu.ru/software/spades) for de novo assembling the paired-end reads; and QUAST (http://quast.bioinf.spbau.ru) for evaluating the quality of genome assembly. Colored cells represent the presence of acquired antimicrobial drug resistance or virulence genes with each color indicating the correspondent family, as indicated in the keys.



Appendix Figure 2. Representation of partial transferable chromosomal genetic platforms containing *pbp5*. Mapping and annotation (Geneious Prime version 2020.2.2; http://www.geneious.com) of partial transferable *pbp5* chromosomal genetic platforms of selected AmpR-*Efm* PF6/ST80 (68209 bp), PF30/ST1263 (74804 bp) and PF25/ST1091 (13613 bp) were performed using *pbp5*-containing contig of the ampicillin-resistant transconjugant TCGEHPH2 (63575 bp; GenBank accession no. MBRI01000000). The contigs identified were assembled using Vector NTI advance v.11 and the platform annotated using eggNOG-mapper.



Appendix Figure 3. BLAST Ring Image Generator (BRIG) alignment of 5 *optrA*-carrying plasmids from different isolation sources and geographic regions. The novel *optrA*-carrying plasmid pAPT110 (deposited under GenBank accession no. MW012677) obtained from a raw pet food ST674 *E. faecalis* was annotated using Vector NTI advance v.11 (https://www.thermofisher.com) and eggNOG-mapper

(http://eggnog-mapper.embl.de), and used as a reference plasmid. The outermost circle is an annotation of the reference plasmid and shows the direction of transcriptional open-reading frames. Genes encoding for antibiotic resistance (red), information, storage, and processing (yellow and dark blue), and cellular processes and signaling (pink, orange, greens, and light blue) are indicated. pEF10748 (GenBank accession no. MK993385) was identified in a ST480 *E. faecalis* from a hospitalized patient in China (2015) and Isobar1 (European Nucleotide Archive [ENA] accession no. ERX2067873), Isobar2 (ENA accession no. ERX2067874), and Isobar3 (ENA accession no. ERX2067875) plasmids were recovered from closely related ST585 strains in hospitalized patients in Spain (2016).