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Ready-to-eat meat foods as potential vectors for the transmission of antibiotic-resistant *Enterococcus faecium*

Giorgia Piccioni [a,1], Claudia Gabucci [b,1], Andrea Di Cesare [c,*], Raffaella Sabatino [c], Tomasa Sbaffi [c], Gianmarco Mangiaterra [a], Maria Assunta Meli [a], Carla Roselli [a], Célia M. Manaia [d], Ivone Vaz-Moreira [d], David Savelli [b], Cinzia Lorenzetti [b], Stefania Di Lullo [b], Sara Primavilla [b], Francesca Romana Massacci [b], Eleonora Scoccia [b], Elena Lavinia Diaconu [e], Alessia Franco [e], Giuliana Blasi [b], Barbara Citterio [a,2], Annalisa Petruzzelli [b,2]

[a] *Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy*
[b] *IZSUM, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy*
[c] *IRSA-CNR, Molecular Ecology Group, Verbania, Italy*
[d] *Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005, Porto, Portugal*
[e] *Department of General Diagnostics, CRAB, National Reference Laboratory for Antimicrobial Resistance, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri", Rome, Italy*

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ABSTRACT

Enterococcus faecium, a member of the human gut microbiota and the second most abundant enterococcal species after *E. faecalis*, is an important agent of healthcare-associated infection. Its high capacity to acquire antimicrobial resistance genes (ARGs) makes infections difficult to treat. This study investigated ready-to-eat (RTE) meat products, typical of central Italy, as potential vectors of antibiotic-resistant enterococci, focusing on *E. faecium*. A total of 148 enterococcal strains, 36 of which were identified as *E. faecium*, were isolated. Among 22 distinct clones, eight were resistant to tetracycline (TET), two also to linezolid (LZD), and one showed a multidrug-resistance phenotype. Antibiotic susceptibility was determined by minimum inhibitory concentration testing, supported by whole-genome sequence analysis. Both LZD-resistant strains harbored LZD resistance genes (*optr*A or a combination of *optr*A and *poxt*A), while only one isolate demonstrated horizontal gene transfer *via* conjugation. The isolates were further characterized for virulence factors and biofilm formation capacity, both under basal conditions and following exposure to simulated upper gastrointestinal transit. All eight TET resistant strains presented genotypic virulence traits. Following exposure to simulated upper gastrointestinal transit, these isolates demonstrated high survival rates, maintaining both their antibiotic resistance phenotypes and biofilm-forming capacity. These findings highlight the potential role of the analyzed RTE meat products as vehicles for virulent and antibiotic-resistant *E. faecium* strains capable of surviving upper gastrointestinal exposure. The study underscores the need to implement enterococci surveillance in the food sector to improve monitoring of resistant *E. faecium* and to limit its possible dissemination and association in clinical risks.

1. Introduction

Enterococci are part of the human gut microbial community, with *Enterococcus faecium* being one of the prevalent enterococcal species detected in human feces (Almeida-Santos et al., 2025; Novais et al., 2006). While enterococci are typically commensal and are even used as probiotics (Mechoub et al., 2025), they can become pathogenic in vulnerable hosts, such as patients with prolonged hospital stays (Murray, 2000). Although *E. faecium* shows a lower virulence compared to *E. faecalis* (Rathnayake et al., 2012), it is becoming increasingly

* Corresponding author.
E-mail address: andrea.dicesare@cnr.it (A. Di Cesare).
[1] Co-first author.
[2] Co-last author.

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relevant at clinical level due to its propensity to acquire antimicrobial resistance genes (ARGs) (Dadashi et al., 2021), and in case of bloodstream infections, it is recognized as one of the three most common pathogens among Gram-positive bacteria (Rinaldi et al., 2025). Furthermore, *E. faecium* is included in the ESKAPE list and defined as a priority pathogen (Roque-Borda et al., 2024). This underscores its severity and emphasizes the urgent need for its monitoring, characterization, and treatment across clinical and non-clinical sectors.

Thus, following the *One Health* concept, which acknowledges that human, animal, and environmental health are strictly interconnected (Meisner et al., 2024), enterococci, especially antibiotic-resistant and virulent *E. faecium* strains, can be isolated from non-clinical sources, including food. In particular, ready-to-eat (RTE) foods could be vehicles for these bacteria, posing a risk of difficult-to-treat bacterial infections. Previous studies have reported a high prevalence of *E. faecium* in such foods, including meat products, fermented milk products, and seafood (Chajęcka-Wierzchowska et al., 2020; Igbinosa and Beshiru, 2019; Chajęcka-Wierzchowska et al., 2016; Macovei and Zurek, 2007). Moreover, enterococci isolated from these products frequently harbor ARGs, showing multidrug resistance profiles and demonstrating the ability to horizontally transfer these genes (Belloso Daza et al., 2022; Chajęcka-Wierzchowska et al., 2016). Although to a lesser extent compared to clinical isolates, enterococcal strains isolated from RTE foods have also been found to carry different virulence genes (Chajęcka-Wierzchowska et al., 2017). These findings further underscore the importance of identifying and characterizing *Enterococcus spp.* strains isolated from food matrices, as they may be vectors of antibiotic resistant and virulent enterococci, with the potential to spread these pathogenic bacteria to humans through the food chain.

Therefore, this study focused on the characterization of *E. faecium* from RTE meat foods, specifically ciauscolo (variety of Italian not fermented salami), salami and sausages, collected from central Italy. These food products are highly relevant to Italian manufacturing and consumption. Indeed, salami production accounts for nearly 10% of the total meat products manufactured in Italy (Zanardi and Novelli, 2021), and traditional sausages are considered by Italian consumers as an essential part of their dietary habits (Conter et al., 2008). Starting from 35 collected RTE meat samples, we processed them to isolate *E. faecium* strains and to characterize their resistance against linezolid (LZD), an antibiotic used exclusively in clinical settings, as well as to erythromycin (ERY) and tetracycline (TET), which are commonly used in veterinary (Sarkar et al., 2023), together with their virulence profiles. Furthermore, on selected strains, we assessed their ability to survive the upper gastrointestinal transit and to maintain the expression of antibiotic resistance and virulence phenotypes under these stress conditions. Another important aspect evaluated was biofilm formation. This capacity is closely linked to bacterial persistence on food-processing machinery, where difficult-to-sanitize areas and dead spaces promote biofilm growth, acting as a continuous source of food contamination. Understanding this phenomenon is crucial, as biofilm production not only facilitates entry into the food chain but is also tightly linked to enhanced tolerance against gastrointestinal transit and host colonization. Finally, we evaluated the potential of multidrug resistant (MDR) strains to transfer ARGs *via* conjugation. This workflow may provide relevant insights into the risk to human health associated with potential *E. faecium* infections and poses a basis for the future evaluation of such products as vehicles for antibiotic resistant and potentially pathogenic bacteria.

2. Materials and methods

2.1. Food sampling, isolation and identification of Enterococcus spp. strains

This study investigated *Enterococcus* strains isolated in 2025 from short-aged (<30 days) cured and fermented pork-based RTE products. Samples were selected from meat products submitted to the laboratory of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (IZSUM) within the framework of routine official control activities. Sampling was performed randomly, focusing on products from large-scale retail distribution channels within the Marche region (central Italy), in accordance with the predefined eligibility criterion of a ripening period of less than 30 days. The chronological experimental design, illustrating the selection and distribution of isolates across the different phenotypic and genotypic characterizations, is schematically represented in Fig. 1.

Ten grams of each sample matrix were homogenized in 90 mL of Buffered Peptone Water (BPW); the initial suspension and subsequent decimal dilutions in maximum recovery diluent were inoculated by spread method onto Slanetz-Bartley Agar (SBA) and incubated at 37 °C for 48 h. Five typical red or brown colonies from each culture plate counted, in accordance with ISO 7218:2024 (ISO, 2024), were selected and subcultured on Bile Aesculin Azide agar (BEA) to confirm aesculin hydrolysis, incubating the plates 24 h at 30 °C. All culture media were purchased from Biofile Italiana (Monza, Italy). Presumptive *Enterococcus* isolates were further identified to the species level by PCR assays with genus/species-specific primers and their corresponding amplification protocols (Layton et al., 2010; Biavasco et al., 2007; Frahm and Obst, 2003; Knijff et al., 2001; Dutka-Malen et al., 1995; see Supplementary Table 1).

The reactions were performed using 1× Buffer, 10 μM of each primer, and 1 U of Taq polymerase (S.i.a.l. Group, Roma, Italy). The PCR products were visualized by gel electrophoresis run in a 1% agarose gel (GellyPhor, Euroclone, Milan, Italy) with TBE 1×. Molecular size markers and related positive controls (TrackIt 1 Kb Plus DNA Ladder, Thermo Fisher Scientific) were used in each agarose gel.

2.2. Pulsed-Field Gel Electrophoresis (PFGE) and phenotypic antimicrobial resistance test

To evaluate the main spreading clones of *E. faecium* strains ($n = 36$) Pulsed-Field Gel Electrophoresis (PFGE) was performed following the protocol previously described by Vignaroli et al., 2013.

The antibiotic resistance phenotype was assessed against LZD, ERY and TET (all purchased from Sigma Aldrich, Milan, Italy). Initially, bacterial strains were inoculated ($OD_{600} = 0.1$, approx. 1×10^8 CFU/mL) onto Muller-Hinton (MH) agar plates supplemented with antibiotic concentrations corresponding to the resistance breakpoints for each drug, as indicated in the CLSI guidelines (CLSI, 2025 M100), specifically, 8 μg/mL for LZD, 8 μg/mL for ERY and 16 μg/mL for TET. Plates were then incubated overnight at 37 °C.

Strains showing resistance based on the initial agar screening tests were further characterized through Minimum Inhibitory Concentrations (MICs), using the broth microdilution method in cation-adjusted Mueller-Hinton II (MHII) broth. Each strain was tested at least two times. MIC values were interpreted based on CLSI clinical breakpoints; the MIC plates were incubated for 20 h at 37 °C.

2.3. Whole genome sequencing and bioinformatic analysis

Genomic DNA from strains resistant to at least one of the tested antibiotics (*i.e.*, eight *E. faecium* strains) was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's instructions. DNA concentration and purity were assessed using a Qubit fluorometer and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Purified DNA samples were subjected to whole genome sequencing (WGS, Macrogen Inc., Seoul, South Korea) on Illumina platform with a 151 bp paired-end approach.

Raw sequencing reads (FASTQ files) were received and uploaded to the Galaxy platform (https://usegalaxy.eu/) for downstream analyses. The quality of raw reads was assessed using FastQC (Galaxy version 0.74 + galaxy1). Adapter sequences and low-quality bases were

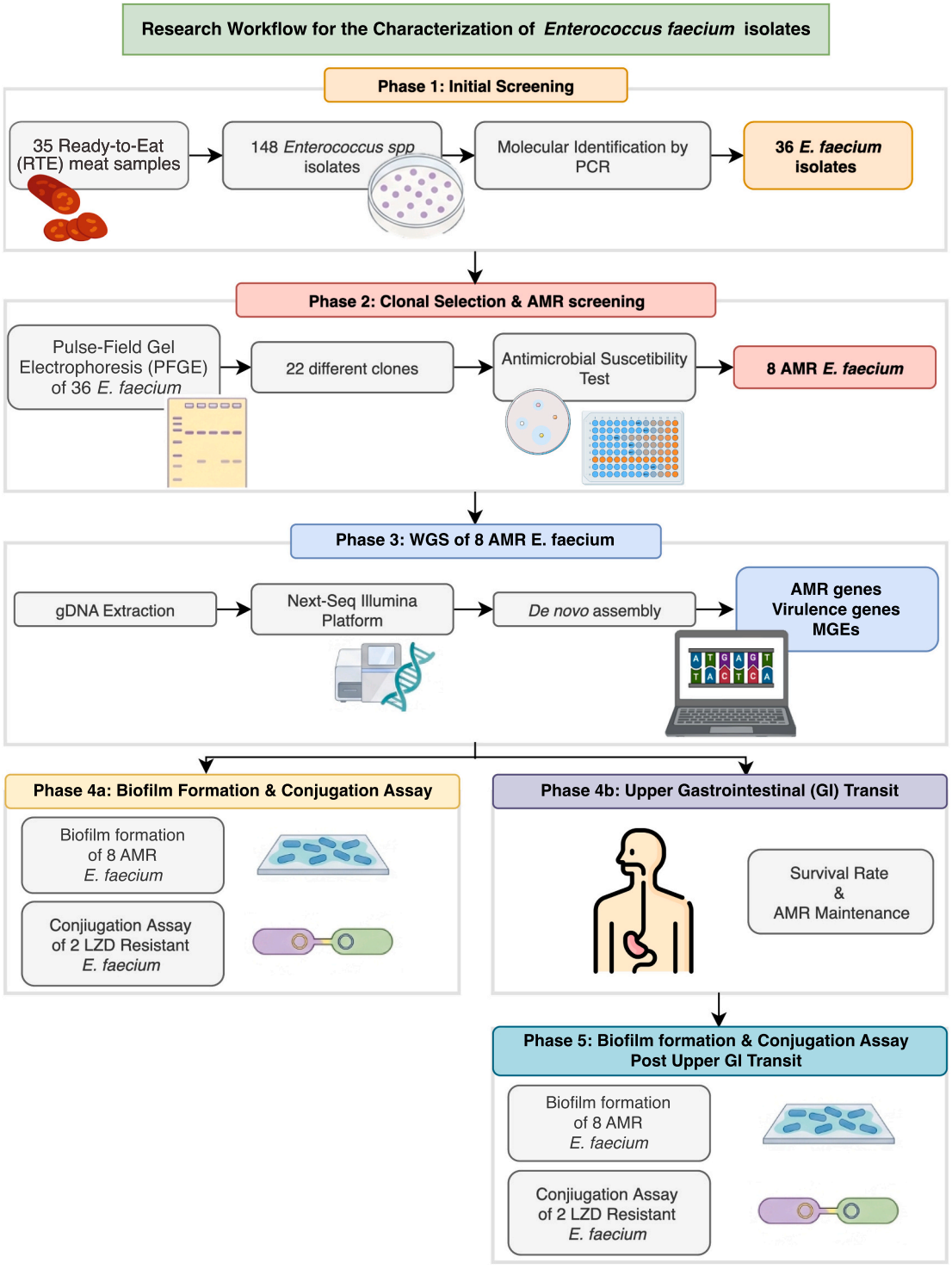

Fig. 1. Schematic research workflow for the characterization of *E. faecium* strains.

removed using Cutadapt (Galaxy version 5.0 + galaxy0) and Prinseq (Galaxy version 0.20.4 + galaxy2), with the following parameters: sliding window size = 4, minimum quality score = 20, and minimum read length = 20. High-quality reads were *de novo* assembled using SPAdes (Galaxy version 4.1.0 + galaxy0) using default parameters, and contigs shorter than 500 bp filtered out using the Filter Sequences tool (Galaxy version 1.2). Assembly quality was evaluated with QUAST (version 4.4), and genome completeness, contamination, and coverage were assessed with CheckM (Galaxy version 1.2.3 + galaxy0). The *de novo* assembly metrics for the eight sequenced genomes showed a high sequencing depth (100×), with a number of contigs per genome (≥500 bp) spanning from 55 to 156, and N50 values ranging between 51,815 bp and 164,717 bp. Furthermore, CheckM analysis confirmed high genome completeness (99.63% for all assemblies) with negligible contamination levels (≤0.37%).

Species identification was confirmed using the SpeciesFinder 2.0 tool (https://cge.food.dtu.dk/services/SpeciesFinder/) and the multilocus sequence typing (MLST) determined through the PubMLST web platform (https://pubmlst.org/organisms), doing a comparison against the *Enterococcus faecium* MLST scheme database. Average Nucleotide

Identity (ANI) was calculated on the EzBioCloud platform (https://www.ezbiocloud.net) by comparing each assembly with the genome of the *E. faecium* type strain ATCC 19434^T. For pangenome analysis and phylogenetic reconstruction, each genome assembly was first annotated using Prokka (Galaxy Version 1.14.6 + galaxy1) to generate standardized GFF3 files. A core genome alignment was then produced using Roary (Galaxy Version 3.13.0 + galaxy3) with default parameters, based on the annotated genomes. The core gene alignment output by Roary was used to infer genetic distances among isolates. A pairwise distance matrix derived from the core genome alignment was generated using IQ-TREE (Galaxy Version 2.4.0 + galaxy1), and a neighbour-joining phylogenetic tree was constructed based on these distances. The resulting tree was visualized and annotated using the Interactive Tree Of Life (iTOL, https://itol.embl.de/).

ARGs were identified using AMRFinderPlus (Galaxy version 3.12.8 + galaxy0), ResFinder (version 3.2), and the Resistance Gene Identifier (RGI, version 5.1.0) against the Comprehensive Antibiotic Resistance Database (CARD, version 3.0.9). The results from these analyses were merged and further confirmed by BLASTn against the NCBI database.

Plasmids were identified using the PlasmidFinder version 2.0.1 (https://cge.food.dtu.dk/services/PlasmidFinder/), while mobile genetic elements (MGEs) were identified using MobileElementFinder version 1.0.3 (https://cge.food.dtu.dk/services/PlasmidFinder/). Virulence factors were screened using ABRicate (Galaxy version 1.0.1) with the Virulence Factor Database (VFDB).

2.4. *In vitro* conjugation and simulated upper gastrointestinal transit assay

The two LZD- and multidrug resistant strains, *E. faecium* 106 and *E. faecium* 133, were tested for their ability to transfer ARGs *via* conjugation using filter mating assays, as previously described (Vignaroli et al., 2011). Ten random colonies of transconjugants were selected on BHI (Brain Heart Infusion; Sigma-Aldrich, St. Louis, Missouri, USA) agar plates containing fusidic acid, rifampicin and LZD (10 μg/mL fusidic acid, 10 μg/mL rifampicin and 6 μg/mL of LZD). Conjugation frequency was calculated as the number of transconjugants per recipient cell, determined by counting colonies on selective plates for transconjugants and on recipient control plates. Each mating experiment was performed in technical triplicate. All transconjugants were confirmed using PFGE. Moreover, the LZD resistance transfer was confirmed by MIC determination and by PCR using primers targeting the *optr*A and *poxt*A genes using protocols already described (Cinthi et al., 2022). The significance of the difference in conjugation frequency was tested by a paired *t*-test in R environment v4.4.0 (R Core Team, 2024).

The eight antibiotic resistant *E. faecium* strains, selected based on WGS analysis, were subjected to *in vitro* upper gastrointestinal transit assays to evaluate their survival and the persistence of antibiotic resistance after simulated gastrointestinal transit. The transit simulation process was performed using previously described digestion solutions (Desideri et al., 2018) and digestion times and procedures (Citterio et al., 2020), with slight modifications concerning the food matrix used for the upper transit simulation (ciauscolo) and the antibiotics used to test resistance persistence (LZD, TET, and ERY).

Each strain was inoculated intoBHI broth and incubated overnight at 37 °C. The following day, the optical density at 600 nm (OD_{600}) was measured and bacterial suspensions were standardized to an OD_{600} of 0.1 (corresponding to approximately 1×10^8 CFU/mL).

In the meantime, 4 g of ciauscolo were sterilized to evaluate exclusively the survival rate and the persistence of antibiotic resistance in pure cultures of the strains under investigation. The test was carried out using three technical replicates for each strain.

Each 4 g of sterile food matrix was inoculated with 4 mL of bacterial suspension standardized to $OD_{600} = 0.1$ (approx. 1×10^8 CFU/mL). From the same bacterial culture, six replicates were prepared: three replicates were subjected to the *in vitro* upper gastrointestinal process, while three replicates were used as controls and stored at 4 °C for the entire duration of the transit simulation, in accordance with commercial storage conditions for the food matrix.

The three replicates subjected to simulated upper transit were incubated at 37 °C under agitation (55 rpm) using a planetary shaker to simulate peristaltic movements. Simulated gastrointestinal fluids were added sequentially as follows: 6 mL of salivary solution (pH 6.5 ± 0.2, 5 min incubation), 12 mL of gastric solution (pH 1.07 ± 0.07, 2 h incubation), followed by 6 mL of bile solution and 12 mL of duodenal solution (pH 8.0 ± 0.2, 2 h incubation) (Desideri et al., 2018).

At the end of the upper transit simulation, 1 mL was collected from each treated replicate and from the control replicates stored at 4 °C. Serial dilutions were performed, and 10 μL of each dilution were plated on Brain Heart Agar (BHA) plates and on BHA plates supplemented with antibiotics at the following concentrations: LZD (6 μg/mL), ERY (8 μg/mL), and TET (16 μg/mL), one plate for each replicate.

Bacterial counts were determined after 24 h of incubation at 37 °C and expressed as log10 CFU/mL. The survival rate and the persistence of antibiotic resistant populations were evaluated by comparing bacterial counts obtained before and after simulated transit and between treated and control samples.

To evaluate post-transit phenotypic traits, surviving colonies recovered from BHA plates were harvested and subsequently tested for biofilm formation capacity and LZD conjugation frequency, following the protocols described below.

Differences in strain abundance before and after digestion, with and without antibiotics, were evaluated using a paired *t*-test in the R environment (v4.4.0), performed on the technical replicates. Differences were considered significant for $p < 0.05$. Data were log-transformed prior to statistical analysis as they represented count data.

2.5. *Biofilm formation assay*

The biofilm plate assay was carried out by crystal violet staining, following the protocol described by Baldassarri et al. (1996) with minor modifications, including incubation of the plates at 37 °C for 20–24 h. Crystal violet absorbance was quantified at 600 nm; the values were normalized by the OD_{600} of the supernatant of each biofilm samples. Each strain was tested in technical triplicates. The significance of the difference in biofilm formation of each strain, was tested by a paired t-test in R environment v4.4.0 (R Core Team, 2024), applied exclusively to these technical replicates. Differences were considered significant for $p < 0.05$.

3. Results

3.1. *Isolation, identification and clonal relatedness of E. faecium strains*

A total of 148 *Enterococcus* strains were isolated from the analyzed RTE pork-based products, which included 18 ciauscolo, 1 salami and 16 sausage samples. Molecular identification of the 148 isolates by species-specific PCR revealed a predominance of *E. faecalis* ($n = 74$, 50.0%), followed by *E. faecium* ($n = 36$, 24.3%), *E. hirae* ($n = 5$, 3.4%), *E. casseliflavus* (n = 3, 2.0%), *E. durans* (n = 3, 2.0%), and unidentified *Enterococcus spp.* ($n = 27$, 18.2%).

Regarding the distribution of the 36 *E. faecium* isolates, they were recovered across all three food matrices, specifically distributed as follows: 30 isolates from ciauscolo, 4 from sausages and 2 from salami. *E. faecium* was frequently co-isolated alongside other enterococcal species from the same samples. Specifically, a multi-species co-existence was documented in 7 out of the 18 examined ciauscolo samples, where *E. faecium* co-existed with *E. faecalis*, and, occasionally, with *E. hirae* or unidentified *Enterococcus spp.* Moreover, *E. faecium* co-occurred with *E. hirae* in the single salami sample (2 *E. faecium* and 1 *E. hirae*), and with *E. durans* and *E. hirae* in one sample of raw sausages (4 *E. faecium*, 3 *E. durans* and 2 *E. hirae*).

Clonal relatedness among the 36 *E. faecium* isolates was assessed by PFGE, which allowed their subdivision into 22 non-repetitive clones and highlighting the high genome plasticity of the microbial pool analyzed.

3.2. Phenotypic antimicrobial resistance profile of non-repetitive clones

The subsequent characterization of antibiotic resistance profile for TET, ERY and LZD was performed on these 22 non-repetitive *E. faecium* clones to assess the prevalence of phenotypic resistance within the distinct genetic lineages. The screening revealed the presence of 8 resistant strains, named *E. faecium* 44, *E. faecium* 52, *E. faecium* 78, *E. faecium* 90, *E. faecium* 106, *E. faecium* 125, *E. faecium* 133 and *E. faecium* 152, resistant at least to one of the tested antibiotics, representing an antimicrobial resistance prevalence of 36.4% (8/22) among unique clones. All eight strains exhibited resistance to TET (MICs 32–256 μg/mL). Strains 106 and 133 were additionally resistant to LZD (MIC 8 μg/mL). Notably, strain 133 displayed a MDR phenotype, exhibiting resistance to all three tested antibiotics, belonging to different classes of antibiotic, including ERY (MIC >128 μg/mL).

The phylogenetic relationship among the eight resistant strains was assessed based on MLST and subsequent phylogenetic tree analysis.

3.3. WGS genotypic characterization and resistome analysis

A more in-depth analysis of the eight antibiotic resistant strains by WGS highlighted high-quality genomic assemblies. The WGS characterization highlighted the presence of different ARGs, with the eight strains harbouring genes conferring resistance to Macrolides – Lincosamides – Streptogramins (MLS), aminoglycosides, and tetracyclines underling the potential MDR nature of all strains.

Concerning tetracycline resistance, seven out of eight strains harbored the *tet*M gene, encoding ribosomal protection. Interestingly, strain 52 was the only isolate to harbor *tet*S, while *tet*L was also frequently detected (Fig. 2). These genes were predominantly associated with *Tn916*-like transposons (Table 1).

All tested strains carried genes conferring resistance to MLS, such as *eatA*, *msrC*, and *efmA*, alongside various aminoglycoside-modifying enzymes (*e.g.*, *aac(6′)-Ii*, *ant(6)-Ia*).

In addition, genes conferring resistance to phenicols (*cat*A8, *fex*A, *fex*B) were detected in three strains, *i.e. E. faecium* 90, *E. faecium* 106, and *E. faecium* 133. Regarding oxazolidinones resistance, both strains 106 and 133 carried the *optr*A gene, while *poxt*A was detected only in *E. faecium* 133. These identified genes were often associated with MGEs, such as transposons, plasmids and insertion sequences (IS) (Table 1). More details about ARGs detected are reported in Fig. 2.

3.4. Survival of resistant strains and biofilm modulation under simulated upper gastrointestinal conditions

The survival of eight *E. faecium* strains was evaluated using an *in vitro* upper gastrointestinal transit model, simulating the oral, gastric and intestinal phases. The transit simulation significantly affected cell survival across all tested strains ($p < 0.006$, Supplementary Table 2), resulting in a 3 to 5-log reduction, from initial density of approximately 10^7 CFU/mL, before gastrointestinal transit, to ~10^2–10^4 CFU/mL, after simulated transit (Fig. 3A). To further assess the maintenance of antibiotic resistance following *in vitro* upper gastrointestinal transit, bacterial cultures were plated on media supplemented with antibiotics (*e.g.* TET and a combination of LZD, TET and ERY for the MDR strain and LZD and TET for *E. faecium* 106 strain) (Fig. 3B). When compared to the same bacterial cultures kept at 4 °C, temperature required by law for storage and sale of the tested products (Regulation (EC) No 853/2004), which

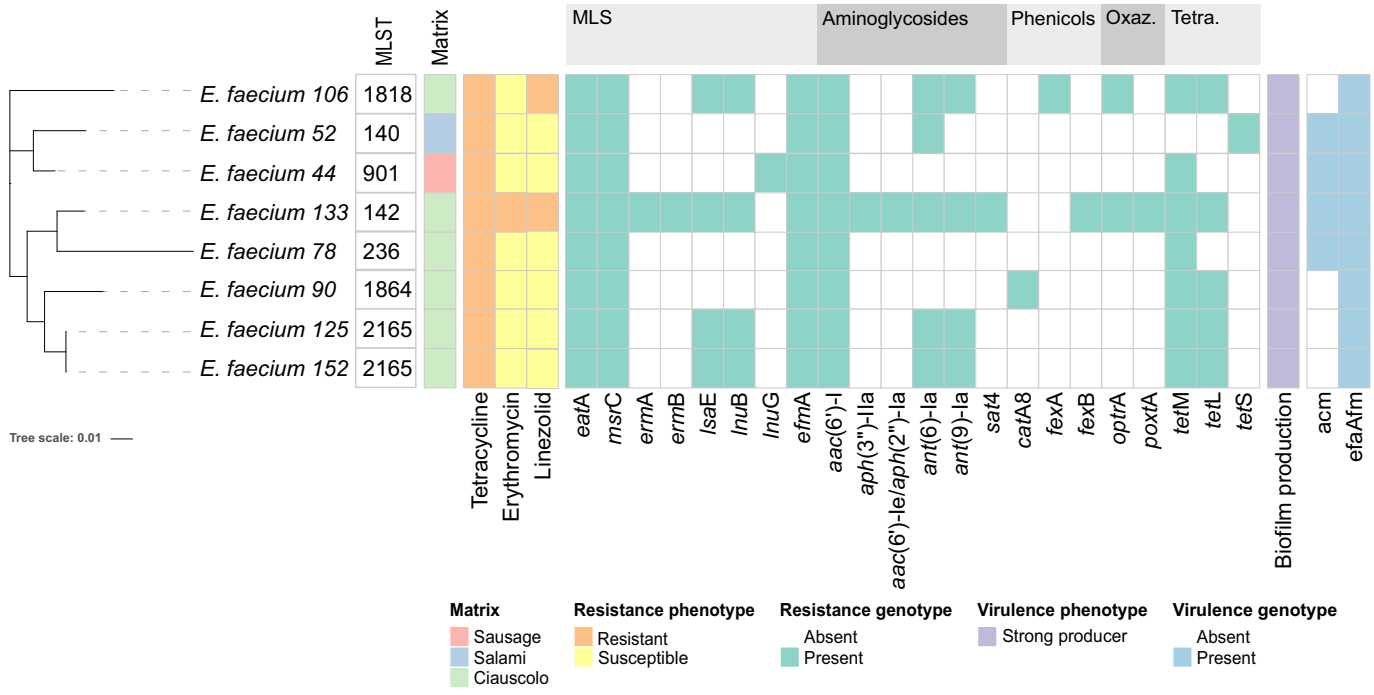

Fig. 2. Neighbour-joining phylogenetic tree showing the relationships among the eight *E. faecium* genomes analyzed in this study, together with antimicrobial resistance and virulence profiles. Genomes were *de novo* assembled from Illumina short reads and annotated using Prokka. A core genome alignment was generated with Roary based on the annotated GFF3 files, and a pairwise distance matrix derived from the core gene alignment was used to construct the tree with IQ-TREE. The resulting tree was visualized and annotated in iTOL and further customised in RStudio. Isolate codes are indicated at the tree tips, together with multilocus sequence type (ST) and sample matrix (sausage, salami, ciauscolo). The scale bar represents substitutions per site. Antimicrobial resistance genes (ARGs), identified using AMRFinderPlus, ResFinder and RGI (CARD database), and confirmed by BLASTn, are shown as coloured squares (presence/absence). Genes are grouped by antimicrobial class (MLS, aminoglycosides, phenicols, oxazolidinones, tetracyclines). Phenotypic resistance to tetracycline, erythromycin and linezolid, determined by MIC testing, is reported as resistant or susceptible. Biofilm production phenotype (strong producer) and selected virulence genes, detected using ABRicate with the VFDB (Virulence Factor Database) database, are also indicated.

Table 1
Genetic context of ARGs identified in the *E. faecium* isolates.

Isolate	Contigs	ARGs	MGEs		
			Transposons	Plasmid replicons	IS
E. faecium 44	Node_19	–	–	repUS15	–
	Node_33	*tet*M	Tn6006 (916-like)	repUS43	–
	Node_49	*lnu*G	Tn6260-like	–	–
E. faecium 52	Node_25	*tet*S	Tn6000 (916-like)	–	
	Node_38	–	–	rep1	–
E. faecium 78	Node_14	–	–	repUS15	ISEfa4
	Node_41	*tet*M	Tn916-like	–	ISfm1
E. faecium 90	Node_57	*cat*A8, *tet*M, *tet*L	–	repUS47	–
	Node_68	–	–	rep2	–
E. faecium 106	Node_15	*optr*A, *fex*A	Tn558	–	–
	Node_39	*tet*M, *tet*L, *ant6-Ia*	Tn916-like	repUS43	–
	Node_85	–		rep2	–
E. faecium 125	Node_28	*tet*M, *tet*L, *ant6-Ia*, *ant9* *lnu*B, *lsa*E	Tn916-like	repUS43	–
	Node_68	–	–	rep2	–
E. faecium 133	Node_34	–	–	repUS15	–
	Node_36	*erm*A, *optr*A	Tn6261	–	IS256
	Node_55	–	–	rep1	ISEfa4
	Node_75	–	–	rep2	–
	Node_76	*erm*B	Tn917	–	–
E. faecium 152	Node_28	*tet*M, *tet*L, *ant6-Ia*, *ant9* *lnu*B, *lsa*E	Tn916	repUS43	–
	Node_71	–	–	rep2	–

were not subjected to the transit process, a decrease (on average of 10^3 CFU/mL) in resistant bacterial cell numbers were observed for all the strains ($p < 2.5 \times 10^{-4}$, Supplementary Table 2) (Fig. 3B).

However, since these statistical comparisons are based on technical replicates, the biological relevance of this decrease should be interpreted with caution, as it primarily reflects the reproducibility of the experimental setup under these specific *in vitro* conditions.

Notability, the baseline counts for the untreated controls in the antibiotic-supplemented media (Fig. 3B) were lower compared to the total viability counts on non-selective media (Fig. 3A). This difference reflects the additional fitness cost, and the longer adaptation phase required by the cells to recover from cold storage (4 °C) and simultaneously initiate growth under selective antibiotic pressure.

The ability to form biofilms was assessed for the eight strains that were resistant to at least one antibiotic, before and after *in vitro* gastrointestinal transit. All tested strains were classified as biofilm producers, as the measured OD_{600} consistently exceeded the 0.240 threshold. According to the classification criteria proposed by Baldassarri et al. (1996), all strains were categorized as strong biofilm producers (Fig. 3C). Although a heterogeneous trend was observed across the samples, every strain demonstrated a high capacity for biofilm formation. Specifically, *E. faecium* 90, 106 e 133 resulted in the strongest biofilm producers, with a mean absorbance of 1.85 OD_{600} (Fig. 3C). After the transit simulation, all the strains maintained the ability to produce biofilm (Fig. 3C). Five out of eight strains significantly increased the production of biofilm ($p < 4.6 \times 10^{-2}$, Supplementary Table 2) (Fig. 3C), this suggests a specialized adaptive response. One strain, *E. faecium* 90, showed a significant decrease in the ability to form biofilm ($p = 3.8 \times 10^{-5}$, Supplementary Table 2), while remaining a strong producer (Fig. 3C). For two strains (*E. faecium* 52 and 106), there was no difference in biofilm production before and after the digestion ($p > 2.6 \times 10^{-1}$, Supplementary Table 2) (Fig. 3C).

3.5. Horizontal transfer of linezolid resistance genes post digestion-stress

To evaluate the horizontal transferability of resistance genes, two specific strains were selected for filter mating experiments: the MDR strain *E. faecium* 133 and *E. faecium* 106. Regarding the strain *E. faecium* 133, it carried both *optr*A and *poxt*A genes, specifically, the *optr*A gene was in a Tn6261 transposon (Table 1), while the gene *poxt*A was in short contig not possible to assign to a MGE. The results obtained highlighted the ability of *E. faecium* 133 to transfer to recipient strain (*E. faecium* 64/3) LZD resistance genes, both before and after transit-triggered stress (Table 2). The transfer frequency of the post-transit strain was significantly higher than pre-transit one ($p = 7 \times 10^{-4}$, Supplementary Table 2), with values of $3.05 \pm 0.11 \times 10^{-4}$ CFU/mL and $8.87 \pm 0.40 \times 10^{-5}$ CFU/mL, respectively (Table 2). The phenotypic analysis on transconjugants strains revealed the same MIC values (*i.e.* 8 μg/mL) of donors (undigested and digested) (Table 2). The genotypic analysis showed the transfer of the two LZD resistant determinants, *optr*A and *poxt*A, in both experiments (Table 2). Because of the gene localizations it is unlikely that they are encoded in the same MGE without hypothesizing a plasmidic rearrangement. This is currently under investigation.

The strain *E. faecium* 106, on the contrary, was not able to transfer LZD resistance, both before and after the transit-triggered stress. This result is likely due to the genomic context of the *optr*A gene; although it is located on the same contig as Tn558, this element belongs to the Tn554 family of staphylococcal transposons, which are characterized as non-conjugative (Kehrenberg and Schwarz, 2005). The lack of an

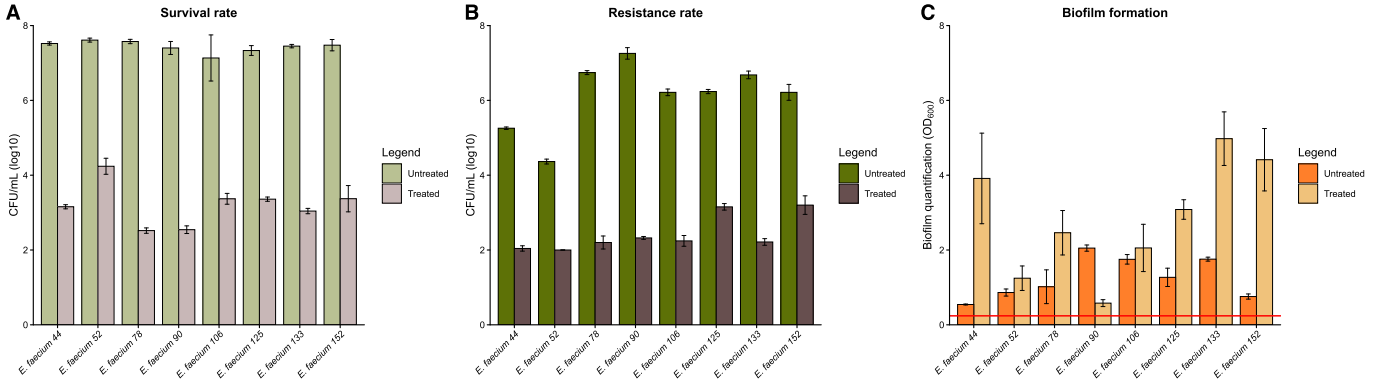

Fig. 3. Survival, resistance and biofilm formation ability of *E. faecium* strains before and after *in vitro* simulated upper gastrointestinal transit. (A) Survival rate expressed as CFU/mL (log10) of untreated and treated samples for each strain. (B) Resistance rate expressed as CFU/mL (log10) of colonies recovered after plating on antibiotic-supplemented media for untreated and treated samples. (C) Biofilm formation before and after transit, expressed as normalized optical density (OD600). Error bars indicate standard deviation. The red horizontal line in panel C represents the cut-off value (OD = 0.24) as the threshold for classification as strong biofilm producers. Strain codes are reported on the x-axis.

Table 2
Conjugation experiments and transfer of linezolid (LZD) resistance genes among *E. faecium* strains.

Isolate (recipient × donor)	Donors' LZD MIC	Donors' LZD gene	Frequency of transfer	N° of confirmed transconjugants/total putative transconjugants	Transconjugants' LZD MIC	Transconjugants' LZD gene
E. faecium 64/3 × *E. faecium* 106 ND	8 μg/mL	*optr*A	/	/	/	/
E. faecium 64/3 × *E. faecium* 106 D	8 μg/mL	*optr*A	/	/	/	/
E. faecium 64/3 × *E. faecium* 133 ND	8 μg/mL	*optr*A/*poxt*A	$8{,}8 \times 10^{-5}$	6/10	8 μg/mL	*optr*A/*poxt*A
E. faecium 64/3 × *E. faecium* 133 D	8 μg/mL	*optr*A/*poxt*A	$3{,}05 \times 10^{-4}$	9/10	8 μg/mL	*optr*A/*poxt*A

D, digested; ND, non-digested; LZD, linezolid; MIC, Minimum Inhibitory Concentration.

autonomous conjugation machinery, coupled with the probable chromosomal integration of the transposon, explains the failure to recover transconjugants in the filter mating experiments.

4. Discussion

The prevalence of *E. faecalis* and *E. faecium* over the non-*faecalis* non-*faecium Enterococcus* species such as *E. hirae*, *E. casseliflavus*, and *E. durans* among the isolates from RTE meat samples, as well as the isolation rate of *E. faecium*, close to 30%, are in line with the results reported in previous studies on RTE meat samples (Chajęcka-Wierzchowska et al., 2016).

Interestingly, the isolation rate of *E. faecium* observed in this and previous studies mirrors that reported for the enterococcal community of the human gut (Almeida-Santos et al., 2025) and is also comparable to rates detected in the environment (Vignaroli et al., 2013) with frequencies ranging between 26% in environmental samples and 47% in the human gut. This finding highlights the high genomic plasticity and adaptability of *E. faecium* (Zhong et al., 2019) underscoring the need for monitoring to limit its persistence and dissemination.

This need is further stressed by the fact that 36% (8 out of 22) of the genetically distinct strains (as resulted by PFGE analysis) was resistant to at least one of the tested antibiotics. Notably, two strains showed resistance against LZD and were positive for the *optr*A gene (strains *E. faecium* 106 and *E. faecium* 133). The strain *E. faecium* 133 carried also another LZD resistance gene (*poxt*A). The gene *poxt*A is frequently associated with the genes *fex*B, *tet*M and *tet*L (Dejoies et al., 2021), also detected in strain *E. faecium* 133. However, those genes were identified in short contigs that were not possible to assemble or assign to a MGE. Dejoies et al., 2021 described that the two genes (*poxt*A and *optr*A) were carried by different plasmids, in clinical isolates. LZD remains a first line treatment for vancomycin resistant *E. faecium* infections, and the increasing detection of LZD resistant strains in hospital settings highlights the need for enhanced surveillance and WGS based characterization of resistance genotypes (Misiakou et al., 2023). The MDR strain *E. faecium* 133 was assigned to the infrequently detected sequence type (ST) 142 (Kawalec et al., 2007; Bezdicek et al., 2023), which belongs to the clonal complex 9, previously associated with a poultry source (Freitas et al., 2009). The other LZD resistant strain, *E. faecium* 106, showed a novel sequence type related to ST1818 (characterized by a new allele of the *gdh* gene), which has previously been reported in antibiotic resistant strains isolated from both clinical settings (Li et al., 2024) and farms (Lei et al., 2021).

These findings suggest a broad distribution of LZD resistance, detected even in genetically rare or previously unassigned strains from RTE foods. Notably, in the two strains, *E. faecium* 106 and *E. faecium* 133, the *optr*A gene was associated with Tn558 and Tn6261, respectively, both of which are well known to mediate the horizontal transfer of LZD resistance genes (Hou et al., 2024; Zhang et al., 2021). Furthermore, the *optr*A gene was mobilized *via* conjugation, highlighting the potential for the strain *E. faecium* 133 to disseminate LZD resistance.

Beyond their antibiotic resistance, the eight sequenced genomes of the selected *E. faecium* strains suggested the potential to produce biofilm and carried key virulence factors (*i.e*, *acm* and *efa*A_{fm}). The gene *acm* encodes for the collagen binding protein (Nallapareddy et al., 2003) required for surface adhesion to the extracellular matrix (Roer et al., 2024), while the gene *efa*A_{fm} encodes an endocarditis antigen protein, which plays a crucial role in the colonization and persistence of enterococcal infections (Koleini et al., 2025). Since both are involved in endocarditis, these findings underscore the high pathogenic potential of the isolated strains. Notably, the examined strains withstood the simulated upper gastrointestinal transit while retaining their antibiotic resistance phenotype. Furthermore, the tested strain, *E. faecium* 133, while showing a significantly lower transfer frequency, retained the ability to horizontally mobilize ARGs after simulated upper gastrointestinal digestion, in line with previous studies on antibiotic resistant enterococci (Citterio et al., 2020). This suggests that the gastrointestinal environment may serve as a highly selective environment where host induced stresses, such as acid and bile exposure, trigger bacterial survival mechanisms. Although not experimentally investigated in the present study, we can hypothesize that the induction of the SOS response, a global DNA damage response that induces the expression of genes involved in DNA repair, replication, and cell division, often associated with environmental stress, might play a role by potentially activating the machinery of MGEs (Beaber et al., 2004). Future molecular analyses will be required to confirm whether such a mechanism might explain the maintained or even enhanced gene transfer frequency observed after the digestive process, highlighting how the host environment can hypothetically accelerate the spread of resistance. However, these statistically significant *in vitro* variations in transfer frequency should be interpreted cautiously regarding their biological relevance in complex gastrointestinal environments. Most importantly, and never observed before to the authors' knowledge, most of the strains that survived the digestive process significantly increased their ability to produce biofilm. The evaluation of biofilm formation ability, before and after simulated upper gastrointestinal transit, provides insights into the adaptive behaviour of *E. faecium* under environmental stress. We hypothesize that the exposure to gastrointestinal-like conditions could impose stress stimuli, acting as an environmental trigger that upregulates genes involved in surface attachment and extracellular matrix production, thereby promoting a biofilm-mediated stress response linked to adhesion-related genes (*e.g. acm* and *efa*A_{fm}). This enhanced biofilm formation may represent a specialized, stress induced survival strategy aimed at increasing persistence within the host environment and protecting the bacterial community from fluctuating physiological conditions (Yu et al., 2020). From a biological perspective, enhanced biofilm formation following digestive stress may increase the ability of surviving cells to colonize the intestinal mucosa. The resulting protected, high density sessile community could also favor horizontal gene transfer by promoting cell proximity and plasmid-mediated conjugation. However, as these findings are based on *in vitro* models with technical replicates, further validation is required to determine whether these changes confer a true adaptive advantage during host colonization. An exception was noted for *E. faecium* 90, which showed a significant

decrease in biofilm production post digestion, although it remained a strong producer. These findings suggest that while gastrointestinal stress generally promotes biofilm as a survival strategy, this adaptive mechanism remains a strain specific response. This finding further highlights the risk for RTE meat products to carry potentially pathogenic *E. faecium* strains capable of enhanced persistence upon ingestion.

To further explore the potential origins of the eight resistant *E. faecium* isolates, a comparative analysis was performed using the Patric (https://www.bv-brc.org) and PubMLST databases (https://pubmlst.org). The STs identified in this study are globally distributed and predominantly associated with livestock and farm environments. Specifically, strains belonging to ST140 (*E. faecium* 52) and ST2165 (*E. faecium* 125 and *E. faecium* 152) have been frequently isolated from pigs and cattle in Belgium, the United Kingdom, and China. The ST236 (*E. faecium* 78) displayed a strong association with poultry, having been previously detected in chicken carcasses, breast meat, and cloacal swabs across the USA, Canada, and New Zealand. Although most lineages are animal associated, ST901 (*E. faecium* 44) and ST1864 (*E. faecium* 90) have also been identified in non hospitalized individuals and marine environments, such as sea urchins, indicating their capacity to persist in diverse ecological niches outside the clinical setting. These findings support the conclusion that the resistant *E. faecium* strains contaminating RTE meat products (ciauscolo, salami, and sausages) likely follow a 'farm-to-fork' contamination route. The high genomic similarity to strains from livestock suggests that these lineages are well adapted to the selective pressures of the agricultural environment, including antibiotic exposure, and are capable of persisting throughout the food production chain. While further epidemiological data are needed to precisely quantify the transmission dynamics to the human gut, these findings highlight the role of artisanal RTE products as potential vectors of multidrug-resistant *E. faecium*, emphasizing the importance of continued food-chain surveillance.

5. Conclusions

E. faecium strains isolated from RTE meat products represent a possible source for the diffusion of clinically important ARGs; this survival capacity suggests a potential public health risk, in terms of contributing to the dissemination of resistance determinants. However, further epidemiological studies comparing consumers of RTE foods with appropriate control cohorts are needed to better elucidate the implications of consuming these products, particularly regarding the risk of bacterial transmission.

Furthermore, these findings underscore the value of WGS approaches to obtain a broad overview of antibiotic resistance and its potential mobility, as well as associated virulence. This approach once integrated in routine analyses, can represent an important improvement on monitoring and characterization of *E. faecium*, particularly supporting preventing measures essential to reduce the clinical impact of this opportunistic pathogen.

CRediT authorship contribution statement

Giorgia Piccioni: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Gabucci:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Andrea Di Cesare:** Writing – review & editing, Writing – original draft, Data curation. **Raffaella Sabatino:** Formal analysis, Data curation. **Tomasa Sbaffi:** Formal analysis, Data curation. **Gianmarco Mangiaterra:** Writing – review & editing, Formal analysis. **Maria Assunta Meli:** Methodology. **Carla Roselli:** Methodology. **Célia M. Manaia:** Writing – review & editing, Formal analysis, Data curation. **Ivone Vaz-Moreira:** Writing – review & editing, Formal analysis, Data curation. **David Savelli:** Writing – review & editing, Formal analysis, Conceptualization. **Cinzia Lorenzetti:** Investigation, Formal analysis. **Stefania Di Lullo:** Investigation, Formal analysis. **Sara Primavilla:** Investigation, Formal analysis. **Francesca Romana Massacci:** Investigation, Formal analysis. **Eleonora Scoccia:** Formal analysis. **Elena Lavinia Diaconu:** Writing – review & editing. **Alessia Franco:** Writing – review & editing. **Giuliana Blasi:** Writing – review & editing. **Barbara Citterio:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Annalisa Petruzzelli:** Writing – review & editing, Resources, Funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 from OpenAI in order to review the language (English). After using this tool/service, the authors reviewed and edited the content as needed, taking full responsibility for the content of the publication.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2026.111983.

Data availability

The raw whole genome sequences generated in this study have been deposited in NCBI under the BioProject accession number PRJNA1426155.

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