



Short Communication

Characterization of antimicrobial resistant *Empedobacter* from fresh meat and meat preparations

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ABSTRACT

Empedobacter has been identified as an opportunistic pathogen that frequently exhibits resistance to multiple antibiotics, including some of those known as of last-resort. This study describes the phenotypic and genotypic characterization of carbapenem-resistant *Empedobacter* isolates obtained from retail fresh meat and meat preparations. The antimicrobial susceptibility of 62 isolates to 15 common antibiotics was assessed using the broth microdilution method. Additionally, whole genome sequencing (WGS) was performed on 24 of these isolates to determine their taxonomic classification and to identify antimicrobial resistance genes (ARGs), as well as their chromosomal or plasmid-borne location. Resistance to meropenem, ciprofloxacin, amikacin, gentamicin, chloramphenicol, tetracycline, and/or colistin was frequently detected, with 61.3 % of the *Empedobacter* strains being classified as multi-drug resistant (MDR) despite the absence of breakpoints for some of the antibiotics tested. WGS revealed the presence of *bla*_{EBR-1} genes in all *Empedobacter falsenii* isolates and the single *Empedobacter tilapia* isolate, of a chromosomal *ere*(D) gene in one *E. falsenii* isolate, and of *tet*(X2) genes in eight *E. falsenii* isolates, seven of them harboured in plasmids.

These findings underscore the need for further research to determine the role of neglected non-ESKAPE bacteria, such as *Empedobacter*, in the spread of antimicrobial resistance in meat production systems.

1. Introduction

Antimicrobial resistance (AMR) has become an important global problem with consequences for both public health and the economy (WHO, 2022). Among the various contributors to this crisis, the genus *Acinetobacter* stands out as one of the most prevalent carriers of acquired antimicrobial resistance genes (ARGs) in food products (Fernández-Trapote et al., 2024). In light of this, the authors recently conducted a study to determine the prevalence and concentration of antimicrobial resistant, including carbapenem-resistant, *Acinetobacter* spp., in pork, beef, chicken, and turkey fresh meat and meat preparations (Puente et al., 2025). *Acinetobacter* spp. was detected in 74 % of the

analysed meat samples, with concentrations reaching up to 3.13 log₁₀ CFU/g. The recovered isolates were found to carry several resistance genes, underscoring their potential role in the spread of AMR through the food chain (Puente et al., 2025). However, it is of note that, despite this, no *Acinetobacter* isolates were obtained through the use of CHROMagar™ *Acinetobacter* supplemented with CHROMagar™ MDR Selective Supplement (CHROMagar, Paris, France), a selective medium used for the detection of carbapenem-resistant *Acinetobacter* spp. This no detection of *Acinetobacter* spp. strains obtained from meat indicated they were susceptible to carbapenem antibiotics, as it was subsequently confirmed by Puente et al. (2025). Nevertheless, several carbapenem-resistant isolates recovered on this medium were later

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identified as *Empedobacter*, a genus of Gram-negative, non-spore-forming and non-motile bacteria (Vandamme et al., 1994). This genus comprises several species, including *Empedobacter brevis*, *Empedobacter collagenolyticum*, *Empedobacter haloabium*, *Empedobacter falsenii* (formerly known as *Wautersiella falsenii* (Zhang et al., 2014)), *Empedobacter stercoris*, *Empedobacter tilapiae*, and *Empedobacter sedimenti* (Parte et al., 2020) (<https://lpsn.dsmz.de/genus/empebacter>, last access February 30, 2025), which are distributed across diverse environments, ranging from animal faeces and fish intestines to lake sediments (Schauss et al., 2015; Young-Ok et al., 2019; Ruan et al., 2023). While human infections caused by *Empedobacter* are rare, some species such as *E. brevis* and *E. falsenii* have been isolated from clinical samples and are recognized as opportunistic pathogens (Vandamme et al., 1994; Kämpfer et al., 2006). It is also of note that members of this genus frequently display resistance to multiple antibiotics, including carbapenems and colistin, and may act as reservoirs for ARGs (Sharma et al., 2016; Collins et al., 2018; Zeng et al., 2020; Olowo-okere et al., 2022; Martinez et al., 2023).

The detection of carbapenem-resistant strains of *Empedobacter* in fresh meat, along with the fact that this bacterium is frequently identified as one of the most abundant genera in diverse meat types (Wang et al., 2022; Emamjomeh et al., 2023), raises concerns about the potential role of this bacterial genus in the spread of AMR in the meat production chain. Considering that there is limited research on the AMR profile of non-clinical *Empedobacter* strains, this study aimed at investigating the antibiotic susceptibility, and the occurrence of genes associated with AMR, in a collection of carbapenem-resistant *Empedobacter* isolates previously obtained in a survey of fresh pork, beef, chicken and turkey meat and meat preparations collected from supermarkets in Spain (Puente et al., 2025).

2. Materials and methods

2.1. Collection of *empedobacter* isolates

The *Empedobacter* isolates characterized in this study were obtained from different fresh meat and meat preparations (Table S1) analysed in a previous investigation (Puente et al., 2025). Meat preparations include fresh meat that has been reduced to fragments, which has had foodstuffs, seasonings or additives added to it or which has undergone processes insufficient to modify the internal muscle fibre structure of the meat, thus preserving the characteristics of fresh meat (Point 1.15. of Annex I to Regulation (EC) No 853/2004 - <https://eur-lex.europa.eu/eli/reg/2004/853/oj/eng>). In such analyses, in summary, 25 g of each meat sample were homogenized for 2 min in a Stomacher (IUL Instruments, Barcelona, Spain) with 225 mL of Buffered Peptone Water (BPW, Merck KGaA, Darmstadt, Germany) supplemented with 1 mg/L vancomycin, 1.5 mg/L cefsulodin and 5 mg/L cephadrine (Sigma-Aldrich, Saint Louis, USA). The isolates were collected after plating on CHROMagar™ *Acinetobacter* medium supplemented with CHROMagar™ MDR Selective Supplement (CHROMagar) following an incubation at 35 °C for 24 h, and were identified as members of the *Empedobacter* genus (log score value > 1.70) by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS, Microflex LT model, Bruker-Daltonics, USA). A total of 62 *Empedobacter* isolates were stored

at −80 °C for further phenotypic and genotypic characterisation.

2.2. Antibiotic susceptibility testing

The collection of 62 *Empedobacter* isolates were tested for susceptibility to 15 antibiotics using the broth microdilution method with Sensititre™ EUVSEC3 plates (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. The antibiotics and their respective concentration ranges (in µg/mL) were: ampicillin (1–32), meropenem (0.03–16), ciprofloxacin (0.015–8), azithromycin (2–64), amikacin (4–128), gentamicin (0.5–16), tigecycline (0.25–8), ceftazidime (0.25–8), cefotaxime (0.25–4), chloramphenicol (8–64), colistin (1–16), nalidixic acid (4–9), tetracycline (2–32), trimethoprim (0.5–16), and sulfamethoxazole (8–512). The minimum inhibitory concentration (MIC) was determined for each antibiotic as the lowest concentration without visible bacterial growth in the well. Since clinical breakpoints for *Empedobacter* species are not established by either the Clinical Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), CLSI breakpoints for other non-Enterobacterales Gram-negative organisms (CLSI, 2021) were used to classify the antimicrobial susceptibility profiles. For colistin, the EUCAST breakpoint for *Acinetobacter* spp. (EUCAST, 2024) was applied, as CLSI does not provide breakpoints for non-Enterobacterales Gram-negative organisms in this case. No breakpoints were available for ampicillin, azithromycin, tigecycline, nalidixic acid, trimethoprim or sulfamethoxazole. Consequently, the MIC₉₀, defined as the MIC of an antimicrobial that inhibits the growth of 90 % of the isolates tested, was determined by calculating the 90th percentile of the MIC values of these antibiotics. Figures representing the MIC values of the different antibiotics for the *Empedobacter* isolates analysed were prepared using ggplot2 R-package.

2.3. Characterization by whole genome sequencing

A total of 24 *Empedobacter* isolates, each from a positive sample (except for 2 samples, which isolates were contaminated even after several steps of single colony isolation and sequencing), were subjected to whole genome sequencing (WGS). DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions for Gram-negative bacteria. The final elution was carried out with 100 µL of Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA) to enhance DNA concentration. The final DNA concentration was assessed using the Qubit® 3.0 Fluorometer and the dsDNA HS assay kit (Thermo Fisher Scientific).

WGS was performed using a hybrid approach: short-read sequencing was conducted on an Illumina NovaSeq 6000 Sequencing System at Macrogen (Seoul, Korea), while long-read sequencing was carried out in house on a GridION platform (Oxford Nanopore Technologies, Oxford, UK) using R10.4.1 flowcells. Illumina sequencing libraries were prepared using the Nextera XT DNA Library Preparation kit (Illumina Inc., San Diego, CA, USA) while Nanopore libraries were obtained with the Native Barcoding Kit 24 v.14. Illumina raw reads were processed by Trim Galore (v.0.6.6) (<https://github.com/FelixKrueger/TrimGalore>) to remove adapters, low quality reads (Phred score <20), short reads (<75 bp), and reads with more than two ambiguous nucleotides. Bowtie2

Table 1

Number of isolates and number of samples from which they were isolated. Meat origin of samples is indicated on rows and packaging conditions on columns. Values between brackets indicate the number of samples analysed. MAP: Modified atmosphere packaging.

	MAP		Vacuum		Unpackaged	
	Isolates	Samples	Isolates	Samples	Isolates	Samples
Beef	1	1 (7)	1	1 (5)	7	2 (8)
Chicken	10	7 (12)	–	–	2	2 (6)
Pork	4	2 (11)	–	(5)	2	1 (9)
Turkey	16	4 (13)	8	3 (6)	–	–
Meat preparations	2	1 (5)	–	–	9	2 (2)

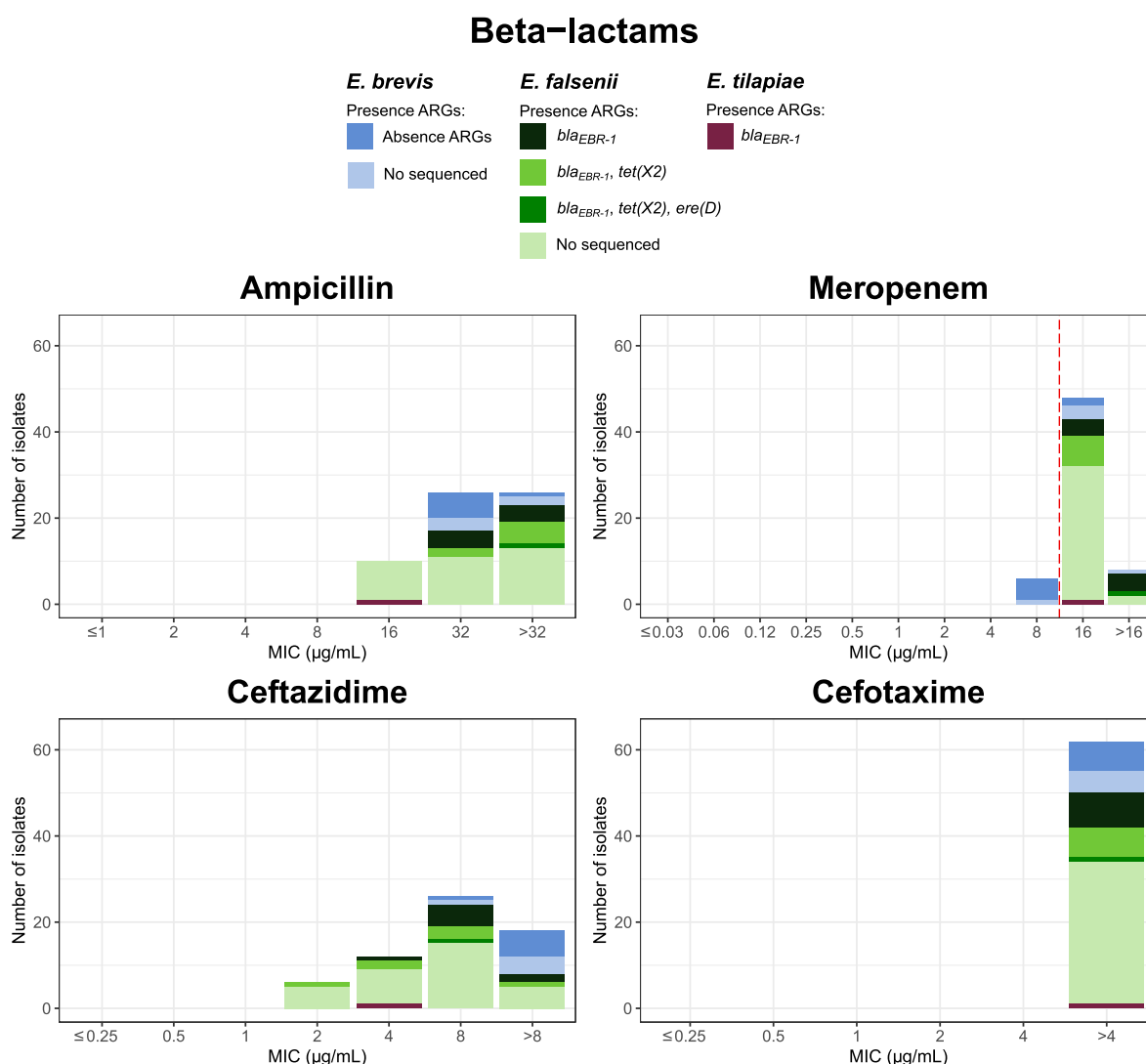


Fig. 1. Minimum inhibitory concentration (MIC) values of the beta-lactam antibiotics tested for 62 *Empedobacter* isolates obtained from fresh meat and meat preparations. Different colours indicate the *Empedobacter* species to which they belong and the presence of known ARGs according to the WGS analysis of a subset of 24 strains. Clinical breakpoints are indicated by a dotted vertical red line.

(v.2.2.9) (Langmead et al., 2019) was used to identify contaminant DNA and remove reads from the phiX174 Illumina spike-in (National Center for Biotechnology Information (NCBI) accession number NC_001422) and potential human contamination using the GRCh38.p13 human genome (NCBI accession number GCF_000001405.39). For Nanopore sequencing, adapters were removed from raw reads (obtained from the GridIon platform using MinKNOW software, in.fastq format) using Porechop (<https://github.com/rrwick/Porechop>). Quality filtering was performed with NanoFilt (<https://github.com/wdecoster/nanofilt>), selecting reads with a quality score higher than 10. Hybrid assembly, integrating both Illumina and Nanopore reads, was carried out by Unicycler (v.0.5.0) and genome quality was assessed with CheckM2 (Chklovski et al., 2023). Furthermore, taxonomic assignment was performed using GTDB-Tk (v.1.7.0) (Chaumeil et al., 2020).

To confirm the taxonomic identification, two phylogenetic trees were elaborated, one based on the core-genome and other on Average Nucleotide Identity (ANI), using reference genomes of five *Empedobacter* species validly published: *E. brevis* ATCC 43319^T (Accession nr: GCF_000382425.1), *E. falsenii* 1681-1 (GCF_013488205.1), *E. sedimenti* DT-LB-19^T (GCF_030005555.1), *E. stercoris* CIP 110833^T (GCF_965137335.1), and *E. tilapiae* MRS2^T (GCF_004785645.1); downloaded from the NCBI. Furthermore, in order to clarify the taxonomy of

three isolates initially unidentified at the species level, an additional phylogenetic tree was constructed using ANI values. This analysis included the previously identified *E. falsenii* isolates, the three unidentified isolates, all *E. falsenii* genomes available in the NCBI database, and one genome of *E. tilapiae* (ULE_I141). For the core-genome tree, Bakta (v.1.3.1) (Schwengers et al., 2021) was employed to annotate the genome sequences, whereas Panaroo (v.1.5.2) (Tonkin-Hill et al., 2020) was used to compare the core-genome genes. Finally, the core-genome genes were aligned and a Neighbor-Joining (NJ) phylogenetic tree with a 1000 bootstrap test was obtained by MAFFT (online service, <https://mafft.cbrc.jp/alignment/server/index.html>, v.7.511) (Katoh et al., 2019). Additionally, the phylogenetic relationship between the 24 *Empedobacter* sp., genomes of the present study and all reported *Empedobacter* sp. strains downloaded from the NCBI, mainly obtained from humans, animals, food and environmental samples, was analysed. To this end, a phylogenetic tree based on ANI values of these genomes was done. This ANI tree revealed that some of these genomes had been incorrectly annotated, and consequently, they were taxonomically assigned by GTDB-Tk (Chaumeil et al., 2020). For all ANI trees, dRep (v.3.5.0) (Olm et al., 2017) was used to calculate ANI distance values, while the plots were done by *ggtree* R-package using the distance matrix obtained from dRep.

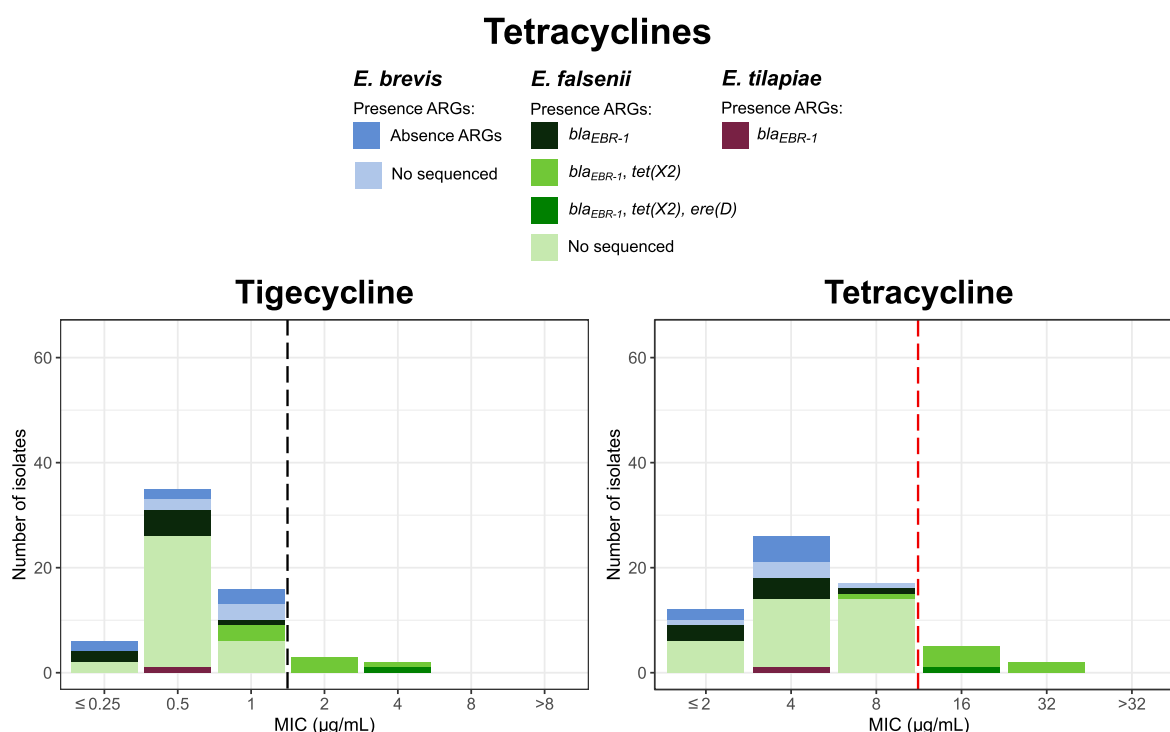


Fig. 2. Minimum inhibitory concentration (MIC) values of the tetracycline antibiotics tested for 62 *Empedobacter* isolates obtained from fresh meat and meat preparations. Different colours indicate the *Empedobacter* species to which they belong and the presence of known ARGs according to the WGS analysis of a subset of 24 strains. Clinical breakpoints are indicated by a dotted vertical red line and MIC₉₀ by a dotted vertical black line.

The presence of known ARGs in the genomes was analysed using BLASTn (Camacho et al., 2009) against the ResFinder database (downloaded on October 24, 2022) (Florensa et al., 2022), applying a cut-off of 80 % for identity and gene coverage. The detection of plasmids was performed using Platon (Schwengers et al., 2020).

2.4. Accession numbers

The 24 *Empedobacter* genome fasta files are available at the NCBI under the BioProject accession number PRJNA1210498.

3. Results

The 62 *Empedobacter* isolates characterised in this study were obtained from 26 out of 89 meat samples, with 46 isolates classified as *E. falsenii* (74.2 %), 12 as *E. brevis* (19.4 %), 1 as *E. tilapia* (1.6 %) and 3 as *Empedobacter* sp. (not assigned at species level). In particular, isolates were collected from three out of 25 samples of pork meat, four out of 20 beef meat, seven out of 19 turkey meat, nine out of 18 chicken meat samples, and three out of seven mixed meat preparations (Table 1).

3.1. Antimicrobial susceptibility

The collection of the 62 *Empedobacter* isolates showed growth at the highest antimicrobial concentrations available in the Sensititre™ panel for cefotaxime (MIC >4 µg/mL) and colistin (MIC >16 µg/mL) (Fig. 1, Fig. S1). Thus, considering the EUCAST *Acinetobacter* clinical breakpoint (>2 µg/mL), all *Empedobacter* isolates could be classified as colistin-resistant. However, it was not possible to determine whether *Empedobacter* isolates could be considered also resistant to cefotaxime, as the maximum concentration of the antibiotic in the panel was 4 µg/mL whereas the CLSI clinical breakpoint for resistance of non-Enterobacterales Gram negative bacteria is ≥ 64 µg/mL. Similarly, the resistance status to ceftazidime could not be established, since the observed MICs ranged between 2 and >8 µg/mL (Fig. 1), and the CLSI

clinical breakpoint is set for non-Enterobacterales Gram negative bacteria at ≥32 µg/mL.

Of the 62 isolates, 56 (90.3 %) were resistant to meropenem, with MICs ≥16 µg/mL (Fig. 1); 32 to gentamicin (51.6 %), with MICs ≥16 µg/mL (Fig. S2); 26 to amikacin (41.9 %), with MICs ≥64 µg/mL (Fig. S2); and four to ciprofloxacin (6.5 %), with MICs ≥4 µg/mL (Fig. S3). Additionally, resistance to chloramphenicol was observed in only two isolates, belonging to the *E. brevis* and *E. falsenii* species, both showing MICs of 64 µg/mL (Fig. S1). For tetracyclines, tigecycline MICs ranged from ≤0.25 to 4 µg/mL, with 8 % (5/62) of the isolates exhibiting a MIC value above the MIC₉₀ (≤1 µg/mL) (Fig. 2). Tetracycline MICs varied from ≤2 to 32 µg/mL, with seven isolates being classified as resistant, based on MICs exceeding the CLSI clinical breakpoint for non-Enterobacterales Gram negative bacteria of ≥16 µg/mL (Fig. 2). For the remaining antimicrobials, considerable variability in MICs was noted. Ampicillin MICs ranged from 16 to >32 µg/mL (MIC₉₀ >32 µg/mL) (Fig. 1), while for trimethoprim MICs varied from 2 to >16 µg/mL (MIC₉₀ >16 µg/mL) (Fig. S4). Isolates showed MICs spanning the entire range of the panel for both nalidixic acid (≤4 to >64 µg/mL), with only one isolate exhibiting a MIC value higher the MIC₉₀ (≤64 µg/mL), and sulfamethoxazole (≤8 to >512 µg/mL), with 8 % (5/62) of the isolates exhibiting a MIC value above the MIC₉₀ (≤256 µg/mL) (Fig. S3, Fig. S4). Regarding azithromycin, 6 out of 62 isolates (9.7 %) showed MIC values higher to the MIC₉₀ (≤2 µg/mL) (Fig. S1).

It is worth highlighting that, based on CLSI clinical breakpoints for non-Enterobacterales Gram negative bacteria and the EUCAST colistin breakpoint for *Acinetobacter*, 38 out of 62 *Empedobacter* isolates (61.3 %) were classified as multidrug-resistant (MDR), exhibiting resistance to at least one agent in three or more antimicrobial categories (Table S2). However, the lack of established CLSI or EUCAST breakpoints for ampicillin, azithromycin, tigecycline, nalidixic acid, trimethoprim, and sulfamethoxazole prevented definitive susceptibility classification for these antibiotics.

3.2. Taxonomic assignment

A total of 13 *E. falsenii*, seven *E. brevis*, the unique *E. tilapia* isolate, and the three *Empedobacter* sp. isolates were whole-genome sequenced. Phylogenetic analyses based on core-genome alignment (Fig. 3) and ANI tree (Fig. 4) supported the GTDB-Tk classifications. Of note, the three unassigned isolates by GTDB-Tk (ULE_I136, ULE_I140 and ULE_I145) consistently formed a distinct cluster closely related to *E. falsenii* genomes in the core-genome and ANI tree (Fig. 3; Fig. 4). Further ANI analysis, using both all available *Empedobacter* genomes (Fig. 4), and a focused set of *E. falsenii* genomes from the NCBI database (Fig. S5), confirmed that these three isolates belong to *E. falsenii*, as they clustered above the 95 % ANI threshold for species delineation (Chun and Rainey, 2014) with all *E. falsenii* genomes.

All *Empedobacter* strains analysed, except 6 *E. brevis* isolates, were resistant to meropenem (Fig. 1), while only 5 and 7 *E. falsenii* isolates

were resistant to tetracycline (Fig. 2). Moreover, one *E. brevis* and one *E. falsenii* isolates were resistant to chloramphenicol (Fig. S1), six *E. brevis*, 19 *E. falsenii* and the *E. tilapia* isolate to amikacin (Fig. S2), eight *E. brevis*, 23 *E. falsenii* and *E. tilapia* isolates to gentamicin (Fig. S2), and three *E. falsenii* and one *E. brevis* to ciprofloxacin (Fig. S3). In addition, all isolates were resistant to colistin.

The phylogenetic tree using ANI values of the *Empedobacter* genomes characterized in this study and reference genomes from diverse origins available in the NCBI demonstrated that some of these genomes had been incorrectly annotated, while the identification provided by GTDB-Tk was more accurate (Fig. S6). For instance, some isolates classified as *Empedobacter* sp. were finally identified at the species level, and some *E. falsenii* were re-classified as *E. stercoris*, a species that was published later (Kämpfer et al., 2006; Zhang et al., 2014; Schauss et al., 2015) (Fig. S6). Finally, it was observed that the majority of the *E. brevis* genomes isolated from meat in the present study appears closely related to

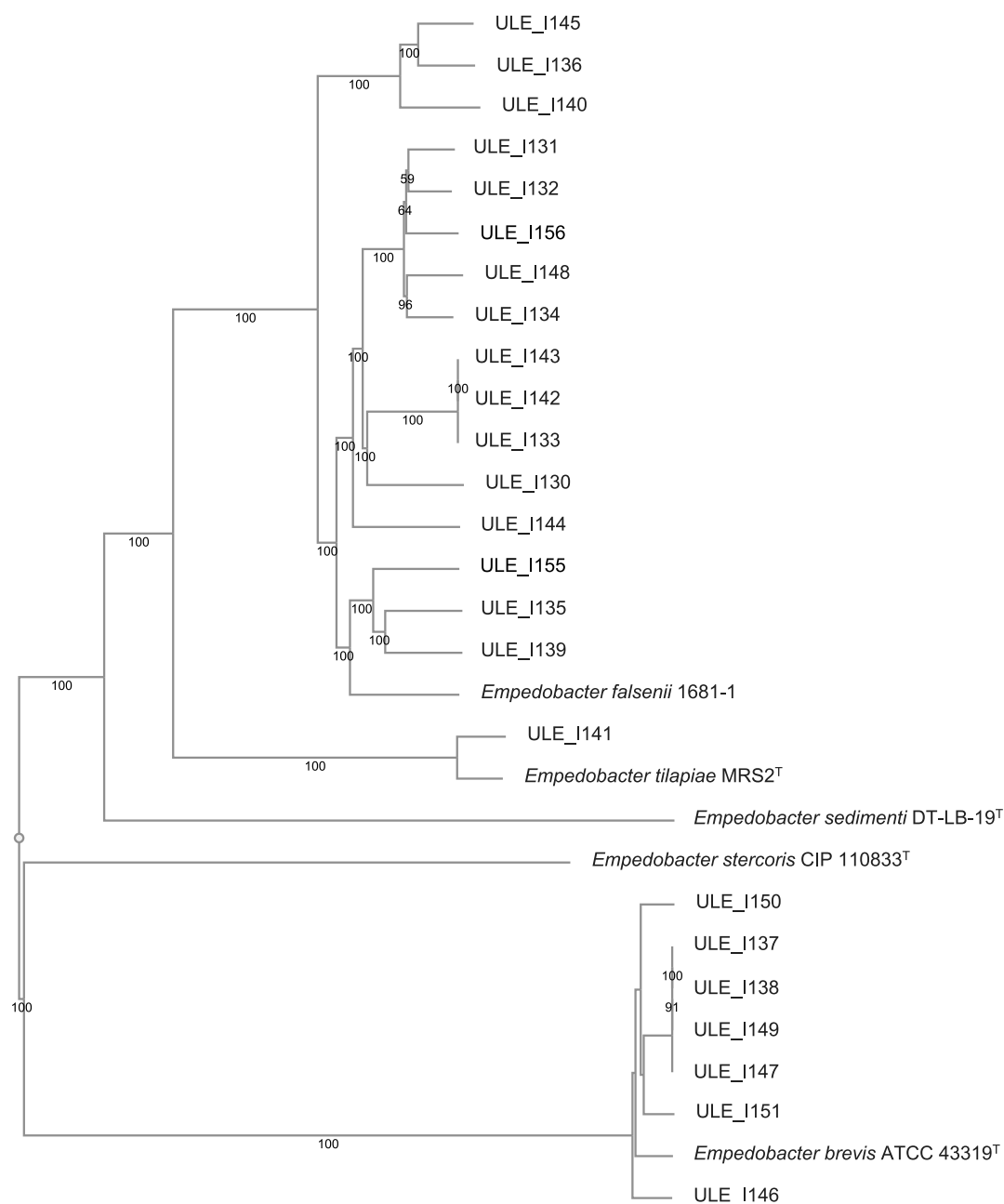


Fig. 3. Core-genome phylogenetic tree of the 24 *Empedobacter* strains isolated from fresh meat and meat preparations, and reference genomes of *Empedobacter* species.

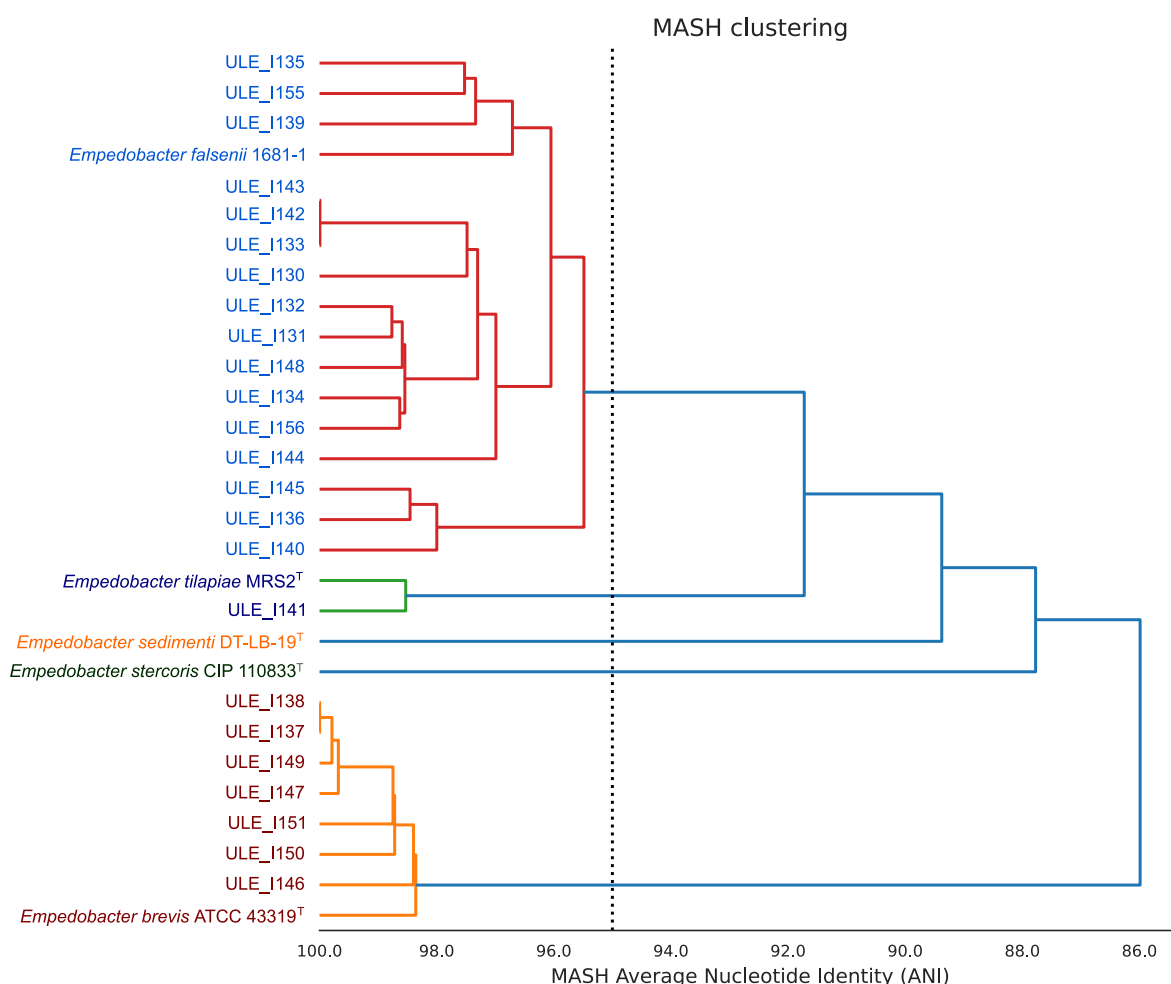


Fig. 4. ANI-based phylogenetic tree of reference genomes of *Empedobacter* and 24 isolates collected from fresh meat and meat preparations, constructed by MASH clustering. The dashed line delimits the 95 % cut-off for species definition.

other *E. brevis* from the NCBI isolated from food (Fig. S6), which was the unique *Empedobacter* isolate previously obtained from food samples together to the one not classified at species level.

3.3. Detection of known antimicrobial resistance genes (ARGs)

The genomes were subsequently analysed to identify known ARGs and to determine their chromosomal or plasmidic location. All *E. falsenii* isolates, as well as the single *E. tilapiae* isolate, harboured a chromosomal *bla_{EBR-1}* (encoding a subclass B1 metallo- β -lactamase) gene (Fig. 5). Isolates harbouring this ARG showed the highest MICs of the concentration range of the beta-lactams tested, and in particular were resistant to meropenem (Fig. 1). The *tet(X2)* (encoding a tetracycline-inactivating monooxygenase) was detected in eight *E. falsenii* isolates (Fig. 5). Of these eight isolates, seven carried *tet(X2)* on plasmids (Fig. 5). All tetracycline-resistant isolates harboured this gene, as well as those with MIC values above MIC₉₀ of tigecycline. Additionally, *ere(D)* (encoding an erythromycin esterase) was detected in a single *E. falsenii* isolate, which was the only strain carrying all three identified ARGs, but it was not resistant to azithromycin, the unique macrolide antibiotics employed in present study. On the other hand, none of the *E. brevis* isolates harboured known ARGs (Fig. 5), while all of them were resistant to colistin.

4. Discussion

The genus *Empedobacter*, specifically *E. falsenii* and *E. brevis*, has been

linked occasionally to human infections, including bacteraemia and urinary tract infections (Bokhari et al., 2015; Zaman et al., 2017). Moreover, some reports exist on the isolation of *Empedobacter* clinical strains namely *E. brevis* and *E. falsenii*, harbouring different ARGs and displaying multidrug resistance phenotypes (Sharma et al., 2016; Martínez et al., 2023). To the best of our knowledge, this is the first study to conduct both phenotypic and genotypic characterization of AMR in *Empedobacter* strains isolated from fresh meat and meat preparations. As the original sampling campaign was designed to investigate *Acinetobacter* spp. (Puente et al., 2025), it was not possible to accurately assess the prevalence or bacterial load of *Empedobacter* in the collected samples. Nevertheless, the detection of antimicrobial-resistant *Empedobacter* in a substantial proportion of samples, suggests that its presence in meat products may be relatively common. Previous studies have reported the presence of *E. falsenii* and *E. brevis* in retail turkey meat in Spain (Martínez-Laorden et al., 2023) and in beef and pork sausages in the United States (Ballash et al., 2021). Importantly, our findings also represent the first documented isolation of *E. tilapiae* from fresh meat, specifically from a turkey breast fillet.

All *Empedobacter* strains in this study were isolated using a selective medium commercialized for the detection of carbapenem-resistant *Acinetobacter*, which agrees with the high MIC values obtained for meropenem, the only carbapenem included in the panels for antimicrobial susceptibility testing. According to the CLSI clinical breakpoint for non-Enterobacterales Gram negative bacteria (>8 μ g/mL), 90.3 % of the isolates were resistant to meropenem, while the remaining strains exhibited a relatively high MIC of 8 μ g/mL. Remarkably, various isolates

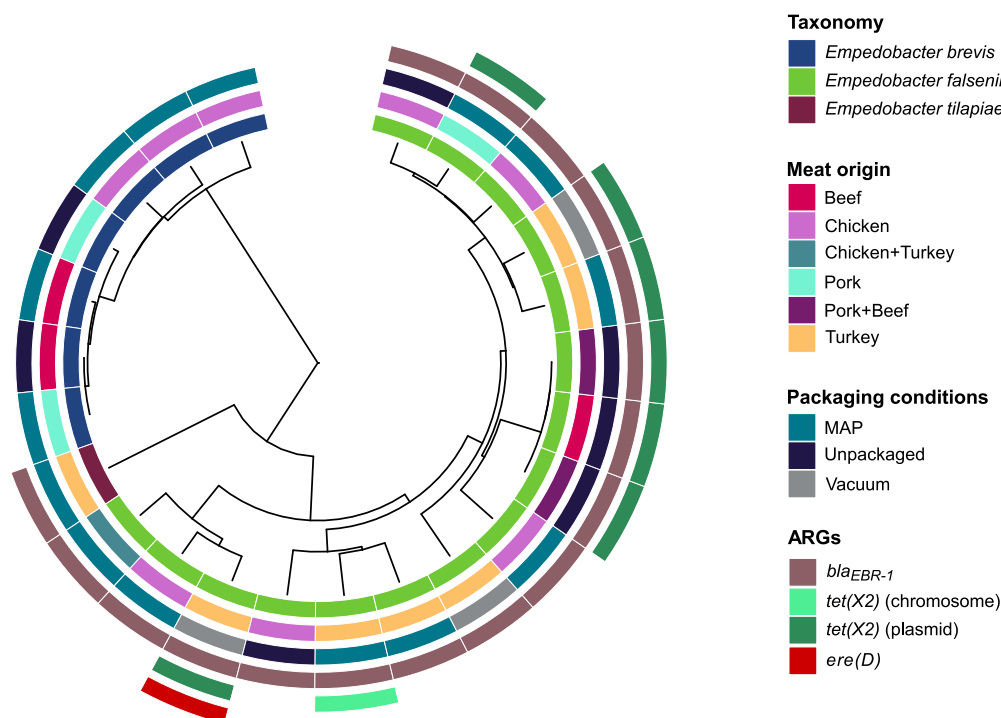


Fig. 5. ANI-based dendrogram representing from inside out the taxonomy according to the phylogenomic analyses, the origin and packaging conditions of the meat samples, and detected ARGs.

showed also resistance to fluoroquinolones (ciprofloxacin), aminoglycosides (amikacin, gentamicin), amphenicols (chloramphenicol), tetracyclines (tetracycline), and/or polymyxins (colistin), with 61.3 % of them being classified as MDR. This resistance profile is consistent with previous studies on clinical *Empedobacter* strains isolated from human infections, which showed resistance to various antibiotics, including last-resort options such as carbapenems and colistin (Sharma et al., 2016; Cheng et al., 2020; Li et al., 2023; Martinez et al., 2023; Chen et al., 2024). It is important to note that all isolates exhibited resistance to colistin despite the absence of known colistin resistance genes. Strains of *Empedobacter* resistant to colistin have been previously reported and it is speculated that *Empedobacter* species are intrinsically resistant to this antibiotic (Sharma et al., 2016; Cheng et al., 2020; Zeng et al., 2020; Olowo-okere et al., 2022; Chen et al., 2024). Some strains analysed in this study exhibited resistance to multiple antibiotic classes, including amphenicols, aminoglycosides and quinolones, despite the absence of identifiable ARGs in their genomes. This discrepancy suggests the involvement of intrinsic or alternative resistance mechanisms. Olowo-okere et al. (2022) proposed that reduced membrane permeability might explain such resistance in *E. falsenii*, based on observed resistance to fosfomycin, nitrofurantoin and trimethoprim/sulfamethoxazole. Further studies are needed to determine whether *Empedobacter* exhibit intrinsic resistance beyond colistin.

To complement phenotypic findings and better understand species-level diversity, genomic analyses were employed. The integration of core-genome and ANI based phylogenetic approaches, as recommended by Chan et al. (2012), enabled more precise taxonomic classification. This was particularly evident in three isolates with ambiguous assignments by GTDB-Tk, which were finally identified as *E. falsenii* through combined phylogenetic methods.

Among the resistance genes identified, *tet(X2)*, a variant of the *tet(X)* gene that encodes a flavin-dependent monooxygenase conferring resistance to tetracyclines (Moore et al., 2005), was detected in several *E. falsenii* isolates. Although *tet(X2)* has been associated with tetracycline resistance, it is not known to confer high-level resistance to

tigecycline. Variants of *tet(X)* have previously been detected in tetracycline- and tigecycline-resistant *Empedobacter* strains, including *E. stercoris* and *E. falsenii* (Cheng et al., 2020; Zeng et al., 2020; Chen et al., 2024), as well as in an *E. brevis* strain isolated from shrimp (Li et al., 2019). In the present study, *tet(X2)*-positive isolates were resistant to tetracycline and exhibited moderately elevated tigecycline MICs (1–4 µg/mL), exceeding those of *tet(X2)*-negative isolates. Notably, in seven *E. falsenii* isolates, *tet(X2)* was located on plasmids. The plasmid-borne nature of *tet(X2)* raises concerns, as it may facilitate horizontal gene transfer and promote the dissemination of tetracycline resistance, even if it does not result in clinically significant tigecycline resistance (Huddleston, 2014). The chromosomal metallo-beta-lactamase *bla_{EBR-1}* gene, which confers resistance to carbapenems, cephalosporins, and penicillins (Collins et al., 2018), was detected in all *E. falsenii* isolates and in the single *E. tilapiae* isolate. Different variants of *bla_{EBR}* have been previously reported in *E. brevis* (*bla_{EBR-1}*), *E. falsenii* (*bla_{EBR-2}*, *bla_{EBR-3}*, *bla_{EBR-4}*), and *E. stercoris* (*bla_{EBR-5}*) most of them isolated from clinical cases (Bellais et al., 2002; Collins et al., 2018; Olowo-okere et al., 2022; Li et al., 2023). However, to the best of our knowledge, this is the first report of a *bla_{EBR-1}* gene in *E. tilapiae*. All strains harbouring this gene were resistant to meropenem and showed MIC values exceeding 4 µg/mL for cefotaxime, which was the highest concentration tested in the antimicrobial susceptibility panel. However, it was not possible to determine their resistance status, as the clinical breakpoint for this antibiotic is higher than the concentration available in the panel (≥64 µg/mL). A similar limitation was observed for ceftazidime, where the maximum tested concentration was 8 µg/mL, but the clinical breakpoint is ≥32 µg/mL. The *ere(D)* gene, associated with erythromycin resistance, was identified in a single *E. falsenii* isolate. Although not unprecedented, Chen et al. (2024) previously detected its presence in *Empedobacter* strains from chicken farms and surrounding environments, its detection in a food-derived isolate further underscores the potential role of meat as a reservoir for resistance genes.

5. Conclusions

The findings reveal that foodborne *Empedobacter* isolates share resistance profiles comparable to those reported in clinical strains, with a high prevalence of multi-drug resistance, including to last-resort antibiotics. It is of note the presence of clinically relevant ARGs such as *bla_{EBR-1}*, *ere(D)*, and *tet(X2)*, the latter located on plasmids, raising concerns about its potential for horizontal gene transfer. Collectively, these findings suggest that *Empedobacter* has the potential to be a reservoir of ARGs in the food chain. These results highlight the need for further research to assess the role of neglected non-ESKAPE bacteria, like *Empedobacter*, in the ecology and dissemination of AMR.

CRedit authorship contribution statement

Alba Puente: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. José F. Cobo-Díaz: Data curation, Formal analysis, Writing – review & editing. Márcia Oliveira: Methodology, Writing – review & editing. Coral Barcenilla: Data curation. Elena Fernández-Trapote: Data curation. Rebeca Cordero-García: Investigation. Francesco Asnicar: Data curation. Nicola Segata: Data curation, Writing – review & editing. Mercedes López: Supervision, Writing – review & editing. Miguel Prieto: Funding acquisition, Supervision, Writing – review & editing. Filipa Grosso: Writing – review & editing. Luisa Peixe: Writing – review & editing. Avelino Álvarez-Ordóñez: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

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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.

Glossary

AMR: Antimicrobial resistance; ANI: Average Nucleotide Identity; ARG: Antimicrobial resistance gene; ARGs: Antimicrobial resistance genes; BPW: Buffered Peptone Water; CFU: Colony-forming unit; CLSI: Clinical and Laboratory Standards Institute; ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.; EUCAST: European Committee on Antimicrobial Susceptibility Testing; GTDB-Tk: Genome Taxonomy Database toolkit; MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry; MAP: Modified atmosphere packaging; MIC: Minimum inhibitory concentration; MDR: multi-drug resistant; NCBI: National Center for Biotechnology Information; WHO: World Health Organization; WGS: Whole-genome sequencing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2025.101212>.

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