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Seafood as a source of antibiotic resistant *Enterococcus* spp

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ABSTRACT

Seafood is a crucial global food source, but its role as a reservoir for antibiotic-resistant pathogens is concerning. This study focuses on the prevalence of antibiotic resistance in retail seafood, using *Enterococcus* spp. as an indicator. Seven categories of raw seafood were collected from retail markets in Central Italy. Out of 422 samples, 288 tested positive for *Enterococcus* spp., resulting in a prevalence rate of 68.25% (CI95%: 63.66%-72.51%). The most common species were *E. faecalis* (48%) and *E. faecium* (17.4%). *Enterococcus* spp. was most frequently found in cephalopods, salmon, bivalves, and crustaceans than in sea bass and bream. The odds of *Enterococcus* spp. recovery were higher in frozen than in fresh samples, while there was no difference between farm-raised and wild-caught seafood. A subset of 102 isolates was selected for antibiotic resistance testing, showing the highest resistance rates to quinupristin/dalfopristin (52.94%) and tetracycline (27.45%). Over 70% of isolates were multidrug-resistant. Additionally, strains resistant to vancomycin and oxazolidinones, two last-resort antimicrobials, were detected. Vancomycin resistance was observed in *E. casseliflavus* and *E. gallinarum*, which are naturally resistant due to the *vanC* chromosomal cluster. The *optrA* gene, responsible for resistance to oxazolidinones and phenicols, was found in *E. thailandicus* and *E. faecium*, where it was located on to a transferable plasmid. In conclusion, this study confirms the widespread presence of *Enterococcus* spp. in retail seafood. The detection of multi-resistant isolates and resistance to last-resort antimicrobials highlights significant human health risks, stressing the importance of including *Enterococcus* spp. in seafood antibiotic resistance surveillance.

1. Introduction

Seafood represents an important source of proteins for humans, worldwide. Global seafood consumption has risen sharply from the pro-capita consumption of 9.9 kg in the 1960s to the 20.5 kg of 2019 (European Commission, 2019). In Europe, the average consumption of seafood is even higher, as it was estimated at 24 kg (live weight) per inhabitant/year (European Commission, 2019). To respond to the increasing demand of seafood, the rise of aquaculture has expanded rapidly, though wild-caught fishery remains significant. Most of the seafood present on the market is imported: in Europe, the second largest importer of seafood worldwide, approximately 73% of imported seafood products are from developing countries (CBI, 2021).

The presence of antibiotic-resistant organisms (AROs) in marine environments is well-documented. Marine bacteria, such as *Vibrio* spp. and *Aeromonas* spp., are often carriers of antibiotic resistant genes (ARGs) (Albini et al., 2022). In addition, AROs may derive from the terrestrial environment, *e.g.* from hospitals, cities and farms and reach the sea through rivers or sewage. The presence of antibiotics or antibiotic residues derived by human activities or from aquaculture

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determines a selective pressure in this ecological niche, further increasing the risk for AROs selection, maintenance and spread in the marine environment (Schar, Klein, Laxminarayan, Gilbert & Van Boeckel, 2020). Seafood can be contaminated by AROs and/or ARGs and, being imported, may contribute to the global spread of antibiotic resistance (Albini et al., 2022). There is a growing concern that antibiotic-resistant pathogens in seafood could reach humans through foodborne routes. Indirect transmission is also possible: ARGs can be horizontally transmitted from seafood bacteria to the commensal flora, expanding the pool of ARGs available to pathogens. The rising consumption of ready-to-eat products containing raw fish, like sushi and sashimi, in European countries could further increase AROs and ARGs transmission risk from seafood to the consumer.

Despite the increasing importance of seafood as a commodity, and the documented presence of AROs, Europe has no surveillance plan for antibiotic resistance in seafood. In fact, while antimicrobial resistance (AMR) has been monitored in production animals since 2014, no data are currently available for seafood. Several researchers emphasize the need for systematic AMR surveillance in this sector (Collineau et al., 2023; Schar et al., 2020). However, technical challenges complicate implementing such programs. Among them, the choice of indicators for AMR: *Escherichia coli*, which is widely used in terrestrial animals, is not a normal inhabitant of marine organisms and when present, typically originates from the terrestrial environment (EFSA/ECDC, 2022). Marine bacteria like *Aeromonas* or *Vibrio*, lack an effective standardization of techniques for antibiotic susceptibility testing (Tate et al., 2022) and most of these bacteria species do not survive freezing, limiting their use as AMR indicators in frozen foods. Seafood encompasses a wide range of animals, from bivalves to crustaceans and finfish, inhabiting varied marine environments with different biological traits. Thus, data on potential AMR indicator bacteria in seafood are needed to support effective AMR surveillance in this sector.

Enterococcus spp. are part of the normal gut microbiota of animals and humans and this genus often included as an indicator in surveillance plans for AMR in terrestrial animals. This bacterial genus is commonly found in a range of habitats, including marine water and beach sand, as a possible consequence of fecal contamination (Davis et al., 2022). Unlike *E. coli, Enterococcus* spp. can persist the marine environment, thanks to their ability to resist harsh conditions and high salt concentrations (Davis et al., 2022; Sánchez-Valenzuela, Benomar, Abriouel, Cañamero & Gálvez, 2010; Zaheer et al., 2020). *Enterococcus* spp. has been recovered from fish intestines, bivalves and processed seafood (Sánchez-Valenzuela et al., 2010). Though present in varied settings, some species of *Enterococcus* are also emerging as human pathogens. In 2019, antibiotic-resistant *E. faecalis* and *E. faecium* were linked to 4450 and 10,000 deaths in the EU, respectively (Mestrovic et al., 2022). Resistance to last-resort antimicrobials, such as vancomycin and oxazolidinones, are surging in these two species causing further public health concerns (Zaheer et al., 2020).

This study aimed to assess antibiotic resistance in retail seafood, using *Enterococcus* spp. as an indicator. Our approach involved several steps. First, we measured the presence of *Enterococcus* spp. in Italian retail seafood products, across different categories, geographical origins, and preservation methods. Second, we tested a representative subset of isolates for antibiotic susceptibility, focusing on antibiotics critical for human health. Finally, isolates with critical resistance were genomically characterized to assess if their resistance determinants could be horizontally transferable.

2. Material and methods

2.1. Sampling and isolation

Seven categories of samples of raw commercial seafood were chosen basing upon the data about seafood consumption in Italy. Prevalence estimation sampling was used to identify the number of samples to collect considering a 20% expected prevalence, 95% confidence levels and 10% precision (Thrusfield, 2018), resulting in 62 samples for each category. The samples were collected from 10 different hypermarkets in Central Italy between June 2020 and May 2021. Information about the origin of the sample (Food and Agriculture Organization (FAO) fishing areas), raising claims (farm-raised *vs*. wild-caught) and sold form (fresh *vs*. frozen) were recorded for all samples. Samples were packed in sterile bags, placed on ice in a coolbox, and immediately transported to the laboratory, where they were processed within 2 h after arrival. Frozen fillets were allowed to thaw at ambient temperature in their original envelopes. The samples were processed following the UNI EN ISO 6887–3, using sterile material. Briefly, portions of fish, fillets, crustaceans (approx. 25 g each) were cut with sterile scalpels under aseptic conditions. For the bivalves, the 25 g included flesh and intravalvular liquid. Samples were placed in Stomacher bags (VWR International Srl, Milan, Italy), diluted 1:10 with buffered peptone water, homogenized with Stomacher® and incubated at 37 °C per 24 h. Then, 10 μl of the suspension was spread on Slanetz & Bartley agar. After 48 h incubation at 37 °C, typical dark red or maroon colonies were isolated on Tryptic Soy Agar and incubated at 37 °C for 24 h. Isolates were identified using a MALDI-TOF MS instrument (Bruker Daltonics, Bremen, Germany) with Microflex LT Smart Biotyper with FlexControl Biotyper 3.4 software (Bruker Daltonics, Bremen, Germany) and then stored in 20% glycerol Luria-Bertani medium at −20 °C for further analysis.

2.2. Selection of isolates

To estimate the prevalence of the antibiotic resistance of Enterococcus spp. isolates, a representative subset of isolates was selected. To ensure the inclusion of all category of seafood, a blocked randomization method was used, with seven strata each representing a category (Petrie & Watson, 2013). The selection took into account a total population of 422 samples, an expected prevalence of 50%, a confidence level of 95% and a precision of 10%, resulting in 102 enterococcal isolates (https://epitools.ausvet.com.au/).

2.3. Antibiotic susceptibility

The susceptibility of 102 enterococcal isolates was tested against a range of antibiotics, including chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), linezolid (LZD), nitrofurantoin (NIT), penicillin (PEN), quinupristin/dalfopristin (SYN), streptomycin (STR), tetracycline (TET), and vancomycin (VAN). The testing was performed using Minimal Inhibitory Concentration (MIC) commercial plates (Sensititre CMV3AGPF, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. MIC values of oxazolidinones and vancomycin were further confirmed by standard broth microdilution method (CLSI, 2020). E. faecalis ATCC 29212 (ielab Calidad, S.L., Alicante, Spain) was used as the quality control strain.

The classification of the isolates into susceptible, intermediate or resistant to chloramphenicol, ciprofloxacin, daptomycin, erythromycin, linezolid, quinupristin/dalfopristin, tetracycline, nitrofurantoin, penicillin and vancomycin was based on the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2019) clinical breakpoints. Since CLSI breakpoints were available only for streptomycin to high-level resistance testing (HLSR), interpretation of MIC values for streptomycin was based on the epidemiological cut-off values (ECOFF) defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (version v13.0, www.eucast.org): here, non-wild-type isolates were reported as 'resistant'. In the statistical analysis intermediate results were grouped with the resistant ones, according to what suggested by Magiorakos et al. (2012). Isolates that showed resistance to at least 3 antibiotics from distinct classes were classified as multidrug-resistant.

2.4. Amplification experiment

All 102 enterococcal isolates of the subset were screened by PCR for the presence of both the oxazolidinones-resistance genes (*cfr* and *cfr*-like, *optrA*, and *poxtA*) (Cinthi et al., 2022) and most common vancomycin-resistance genes (*vanA* and *vanB*) using primer pairs previously described (Fioriti et al., 2020). The *poxtA*-carrying *E. faecium* EF3 (Fioriti et al., 2021), the *cfr*-, *optrA*-carrying *E. faecium* E35048 (Morroni et al., 2018), *E. faecium* BM4147 (*vanA*) and *E. faecalis* ATCC 51299 (*vanB*) isolates (Fioriti et al., 2020) were used as positive controls in PCR experiments. The PCR products were subjected to Sanger sequencing to confirm the actual presence of the above-mentioned resistance genes.

2.5. PFGE analysis

Macrorestriction with SmaI endonuclease (New England Biolabs, Beverly, MA) and pulsed-field gel electrophoresis (PFGE) analysis, for a preliminary enterococcal typing, were performed as described elsewhere (Ripa et al., 2001). The banding pattern was interpreted according to the criteria of Tenover et al. (1995).

2.6. WGS and sequence analysis

Bacterial genomic DNA was extracted by the QIAcube automated extractor using DNeasy PowerLyzer PowerSoil Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). In order to characterize the resistome, the sequence type and the genetic element responsible for the spread of antibiotic resistance genes, extracted DNA was subjected to WGS with two approaches: Illumina MiSeq platform (Illumina®, San Diego, CA, USA) using a 2 x 150 bp paired-end technology and a long-read sequencing approach (MinION, Oxford Nanopore Technologies, Oxford, UK). Hybrid assembly was performed with Unicycler v.0.4.8 (https://github.com/rrwick/Unicycler). *In silico* identification of acquired antimicrobial resistance genes and ribosomal mutations involved in oxazolidinone resistance were carried out using dedicated tools available at the Center for Genomic Epidemiology available at http://www.genomicepidemiology.org/ (*e.g.*, MLST v.2.0, ResFinder 4.1, LRE-finder 1.0) and by the BLAST suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Easyfig tool was used to compare relevant genetic elements (https://mjsull.github.io/ Easyfig/).

2.7. Conjugation experiments

Conjugal transfer was performed on a membrane filter as described previously (Brenciani et al., 2016). In mating experiments, all isolates carrying oxazolidinone resistance genes were used as donors. The chloramphenicol-, and linezolid-susceptible *E. faecium* 64/3 strain, was used as recipient (Werner, Klare & Witte, 1997). Transconjugants were selected on Brain Heart Infusion Agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with chloramphenicol (10 mg/L), fusidic acid (25 mg/L), and rifampicin (25 mg/L) (Sigma Aldrich, St. Louis, MI). Transconjugants were tested for the presence of oxazolidinone resistance genes by PCR and for their susceptibility to chloramphenicol and linezolid. *Sma*I-PFGE was carried out and patterns analyzed to confirm the genetic background of transconjugants. Conjugation frequencies were expressed as number of transconjugants per recipient cell. The *poxtA*-carrying *E. faecium* EF3 (Fioriti et al., 2021) was used as positive control in conjugation experiments.

2.8. Nucleotide sequence accession number

The WGS data are available under the BioProject ID PRJNA1141356 (accession numbers of the genomes: JBFTEJ000000000 and JBFTEK000000000). The pV662*optrA* plasmid of the *E. faecium* V662 and the Tn*6674*-like transposon of the *E. thailandicus* V872 nucleotide sequences have been assigned to following GenBank accession numbers: OR209137 and OR863616, respectively.

2.9. Statistical analysis

The prevalence of *Enterococcus* spp. in seafood samples was calculated as described with its 95% confidence interval (CI95%).

The proportion of phenotypically resistant isolates was calculated by dividing the number of resistant isolates by the total number of isolates tested against that antimicrobial.

The association between the recovery of an isolate of *Enterococcus* spp. and a set of variables including the area of origin of the sample (FAO zone), the sold form (fresh *vs*. frozen), the category of the sample and the sample type (wild-caught *vs.* farm-raised), were investigated using univariate analysis. The odds ratio (OR) and the 95% CI were calculated measure the strength of association of the predictor variable on the outcome. For the category of the samples, category 1 was used as baseline. The significant level was set at level $p \leq 0.05$. The same approach was applied to investigate the associations between the presence of a multidrug-resistant (MDR) isolate and the set of variables described above. The analysis was performed using Stata software version 16.1 (StataCorp LCC, College Station, TX, USA).

3. Results

3.1. Isolation, identification and distribution of the isolates

From the starting collection of 422 samples, 288 (68.25%, CI95%: 63.66%−72.51%) resulted positive for *Enterococcus* spp. (Table 1). In the seafood category sample C (cephalopods), E (salmon), F (bivalves) and G (crustaceans), more than 70% of the samples were positive (Table 1). An association was found between the isolation of *Enterococcus* spp. and seafood categories C (cephalopods), E (salmon), F (bivalves), and G (crustaceans), as compared to category A (sea bass and brim) (Table 2). Frozen samples had higher odds of being positive for *Enterococcus* spp. than fresh samples, while no association was found for farm-raised *vs* wild-caught seafood (Table 2).

A total of 282 isolates belonging to the genus *Enterococcus* were collected. Of the 15 bacterial species identified, the most prevalent was

Table 1
The prevalence rate of *Enterococcus* spp. among seafood category samples.

Seafood category samples	Negative samples		Positive samples		Total
	N	%	N	%	
A (bass)	30	46%	35	54%	65
B (anchovy)	19	50%	19	50%	38
C (cephalopods)	17	25%	51	75%	68
D (cod)	26	39%	40	61%	66
E (salmon)	14	23%	46	77%	60
F (bivalves)	14	23%	47	77%	61
G (crustaceans)	14	22%	50	78%	64
Total	134	32%	288	68%	422

Table 2
Associations between the presence of *Enterococcus* spp. and the seafood category sample, the frozen *vs* fresh status and the wild-caught *vs* farm-raised variables.

Category	Odds ratio (OR)	CI 95%	p-value
A (bass)	1	–	–
B (anchovy)	0,86	0,36–2,06	0,7061
C (cephalopods)	2,57	1,16–5,75	0,0107
D (cod)	1,32	0,62–2,80	0,4342
E (salmon)	2,82	1,22–6,62	0,0076
F (bivalves)	2,88	1,25–6,75	0,0063
G (crustaceans)	3,06	1,34–7,16	0,0036
frozen *vs* fresh	1.69	1.08–2.70	0.0156
wild-caught *vs* farm-raised	1.1	0.71–1.70	0.6540

Table 3
Number and distribution of *Enterococcus spp*. isolates from seafood samples.

Seafood category samples	Seafood samples	*Enterococcal species isolated*								
		E. aquimarinus	*E. casseliflavus*	*E. duranas*	*E. faecalis*	*E. faecium*	*E. fluvialis*	*E. gallinarum*	*E. gilvus*	*E. hirae*
A	Bass	1	2		2				1	1
(sea bass and bream)	Gilt-head bream		1		6	5				1
	Sea bass				2					
B	Anchovy		5		6	2			3	
(anchovy)										
C	Squid		1		8	1				1
(cephalopods)	Octopus				5	2		1		1
	Cuttlefish				12	2				
	Flying squid		2		1	1				
D	Cod fish		2		12	4			2	2
(cod)	Hake		2		11	1				
E	Salmon		5		30	1		1	1	
(salmon)										
F	Mussels		1		4	6				3
(bivalves)	Clams				7	19				1
G	Shrimp		2	2	16	3	1			
(crustaceans)	Prawns				9			1		
	Langoustines		1	1	6	2		1		1
	Total	1	24	3	137 (48.6)	49	1	4	7	11
	(%)	(0.4)	(8.5)	(1.1)		(17.4)	(0.4)	(1.4)	(2.5)	(3.9)

E. faecalis (137/282, 48.6%), followed by *E. faecium* (49/282, 17.4%), *E. casseliflavus* and *E. thailandicus* (24/282, 8.5%) and *E. hirae* (11/282, 3.9%). The other species were represented by few or only one isolates (Table 3). Eight isolates were not identified at the specie level and were reported as *Enterococcus* spp.

For individual species, *E. faecalis* was isolated from all sample types, while *E. faecium* was not recovered in bass, sea bass and prawn. The only isolates of *E. aquimarinus* and *E. phoenicula* were isolated from bass, whereas *E. fluvialis* and *E. saccharoliticus* were both from shrimp.

3.2. Antibiotic susceptibility testing of the Enterococcus isolates

The prevalence of the antibiotic resistance of *Enterococcus* spp. isolates was tested on a subset of 102 isolates. The distributions of the MIC values for each antibiotic are shown in Table 4. The highest percentages of resistance occurred in response to quinupristin/dalfopristin (54/102, 52.94%) and tetracycline (28/102, 27.45%). Less of the 20% of the isolates was resistant to ciprofloxacin (20/102, 19.61%), erythromycin (16/102, 15.7%), chloramphenicol (7/102, 6.86%) and penicillin (2/102, 1.96%). In addition, 27 (26.47%), 27 (26.47%), 1 (0.98%), 61 (59.80%), 41 (40.19%) and 10 (9.8%) isolates were classified as intermediate for nitrofurantoin, quinupristin/dalfopristin, tetracycline, ciprofloxacin, erythromycin and chloramphenicol, respectively (Table 4).

None of the isolates tested demonstrated resistance or intermediate susceptibility to daptomycin.

As regard to last-resort antimicrobials one isolate of *E. faecium* (V662), resulted resistant to linezolid and one *E. gallinarum* and 12 *E. casseliflavus* isolates were resistant to vancomycin.

For individual species, *E. faecium* and *E. thailandicus* were the enterococcal species resistant or non-susceptible to a major number of antibiotics tested ($n = 8$), followed by *E. faecalis* and *E. faecium,* which were resistant or non-susceptible to six antibiotics (Table 5).

Although the 13.72% (14/102) of the isolates were susceptible to all antibiotics or resistant to only one antibiotic, the simultaneous resistance to at least 3 antibiotics was observed in 70.59% (72/102) of the isolates, with 38 different resistance phenotypes, as reported in table 5. The major number of MDR isolates belonged to *E. faecalis* and *E. faecium* ($n = 34$ and $n = 18$, respectively) (Table 6).

No associations were found between the status or category of the sample and the recovery of a MDR isolate. Similarly, no association was found between the wild-caught/farmed raised category or the geographical origin (FAO zones) of the sample and the recovery of a MDR isolate.

3.3. Amplification experiments and genotypic characterization of isolates carrying oxazolidinones and glycopeptides determinants

Amplification experiment was performed on a subset of 102 Enterococcus isolates. PCR screening showed that 2 out of 102 isolates, the *E. faecium* V662 and the *E. thailandicus* V872, carried the *optrA* gene, whereas all isolates were negative for *vanA* and *vanB* genes.

Sanger sequencing of the *optrA* gene followed by bioinformatic analysis revealed that the amplicons of the V662 and V872 strains were 98.02% and 99.75%, identical to the WT $optrA_{E349}$ gene respectively (accession number KP399637), respectively. BLASTP analysis of the deduced $OptrA_{V662}$ and $OptrA_{V872}$ amino acid sequences revealed 97% and 99% identity with the $OptrA_{E349}$ WT protein. The OptrA variant detected in *E. faecium* V662 showed 20 amino acid substitutions (EYKWDVDASKELYNKQLEIG), whereas that from *E. thailandicus* V872 showed 4 amino acid substitutions (EDDM) compared to the $OptrA_{E349}$.

Interestingly, among the 102 isolates we detected 12 isolates of *E. casseliflavus* and one of *E. gallinarum*, which commonly carry the non-transferable *vanC* chromosomal cluster conferring intrinsic vancomycin resistance (García-Solache & Rice, 2019a). In order to demonstrate the presence of *E. casseliflavus* clonal groups a *Sma*I-PFGE experiments were performed. It showed that three of 12 *E. casseliflavus* (V826, V828 and V858) isolates exhibited the same PFGE pattern (pulsotype A), while V832 and V551 exhibited the closely related A1 and A2 pulsotypes, respectively. Moreover, two isolates showed the pulsotype B (V448) and the closely related pulsotype B1 (V457). Finally, V429 had pulsotype C, V629 pulsotpe D, V857 pulsotype E and V717 and V666 showed the same PFGE pattern (pulsotype F).

3.4. WGS analysis

Resistome analysis revealed that *E. faecium* V662 and *E. thailandicus* V872 isolates, besides *optrA*, shared two acquired antibiotic resistance genes: *erm*(B) (macrolide, lincosamide and streptogramin group B resistance) and *fexA* (phenicols resistance). Moreover, *E. faecium* V662 isolate showed: *lnu*(B) (lincosamide resistance), *lsa*(E) (lincosamide, streptogramin A and pleuromutilin resistance), *ant*(6)-Ia, *aac*(6′)-Ii and *aac*(6′)-*aph*(2″) (aminoglycoside resistance), *tet*(M) and *tet*(L)

Enterococcal species isolated								
E. mundtii	*E. phoeniculicola*	*E. pseudoavium*	*E. saccharolyticus*	*E. sulfureus*	*E. thailandicus*	*E. spp*	Total for sample (%)	Total for category (%)
	1	2			1	1	12 (4.3)	33
		2			1	1	17 (6)	(17.7)
		2					4 (1.4)	
					1		17 (6)	17 (6)
2					4		17 (6)	53
					3	1	13 (4.6)	(18.8)
					2		16 (5.7)	
		1			1	1	7 (2.5)	
				1	2	2	27 (9.6)	41
							14 (5)	(14.5)
					2	1	41 (14.5)	41 (14.5)
					3		17 (6)	48
					3	1	31 (11)	(17)
1			1				26 (9.2)	49
					1		11 (3.9)	(17.4)
							12 (4.3)	
3 (1.1)	1 (0.4)	7 (2.5)	1 (0.4)	1 (0.4)	24 (8.5)	8 (2.8)	282	

(tetracycline resistance) and *dfrG* (trimethoprim resistance) while *E. thailandicus* V872 isolate exhibited only the *erm*(A) gene (macrolide, lincosamide and streptogramin group B resistance).

Both isolates lacked the mutations in 23S rRNA and L3/L4 ribosomal proteins involved in oxazolidinone resistance and *E. faecium* V662 isolate was associated to sequence type ST8.

Further bioinformatic analysis indicated that the *optrA* gene had a plasmid and chromosomal localization in *E. faecium* V662 and *E. thailandicus* V872, respectively.

3.5. pV662optrA plasmid analysis

In the *E. faecium* V662 the 68,303-bp *optrA*-carrying plasmid (34% GC content), named pV662*optrA* (GenBank accession no. OR209137), exhibited 57 ORFs encoding proteins >50 amino acids (Fig. 1 and Table S1). BLASTN analysis revealed that pV662*optrA* was 97% identical (coverage range 62% - 71%) to four enterococcal plasmids: (i) pAT40b-a (accession no. CP097035) of *E. faecalis* AT40b-a from pet food in Switzerland; (ii) pT17-1-optrA-57k (accession no. CP109840) and p47-61 (accession no. CP091102) of two porcine *E. faecium* isolates from China; (iii) pFYY063-optrA-70 K (accession no. CP116030) of *E. casseliflavus* CQFYY22-063 from swine feces in China (Fig. 1).

Other antibiotic resistance genes, beside *optrA*, were located in pV662*optrA: fexA* (*orf48*), two copies of *erm*(B) (*orf42/orf50*) and *aac*(6′)-*aph*(2″) (*orf10*) (Fig. 1 and Table S1). Moreover, a complete conjugation region (from *orf26* to *orf40*), *rep* (*orf6*) (belonging to the inc18 family), *parA* (*orf7*) (responsible for plasmid replication and partitioning, respectively), a ε-ζ-ώ toxin/antitoxin (*orf51* to *orf53*) and restriction and modification systems (from *orf23* to *orf25*) (involved in the plasmid persistence in the enterococcal population), were also detected in pV662*optrA*.

3.6. Chromosomal genetic context of the optrA gene

The *E. thailandicus* V872 showed a chromosomal *optrA*-carrying region (13,671 bp in size) that exhibited a high nucleotide identity (99.78%) and coverage (86%) with the multiresistance transposon Tn*6674* (12,932 bp in size) (accession no. MK737778) (Fig. 2). Both the transposons were inserted into the *radC* gene as previously described (D. Li et al., 2019).

A more in-depth sequence analysis of the *E. thailandicus* V872 Tn*6674*-like displayed three main rearrangements compared to the wild type trasponson (Fig. 2):

(i) The Tn*6674*-like is devoid of the *ant*(9)-Ia gene responsible for spectinomycin resistance;
(ii) The Tn*6674*-like had the *araC* gene encoding a transcriptional regulator;
(iii) A different localization of the *erm*(A) and *fexA* genes. The former was located downstream of the *optrA* gene while the latter was downstream of the *tnpA/tnpB/tnpC* transposases. Notably, in the Tn*6674*-like transposon the *erm*(A) gene was truncated at the 3′-end, indeed it was devoid of 171 bp compared to wild-type gene (561/732 bp).

3.7. Transferability of the optrA gene

In vitro conjugation assays were performed to demonstrate possible transfer of the *optrA* gene. It was successfully transferred only from *E. faecium* V662 to *E. faecium* 64/3 recipient with a frequency of 3×10^{-4} per recipient. Two transconjugants exhibited resistance to chloramphenicol and reduced susceptibility to linezolid. PCR and Sanger sequencing indicated that all transconjugants acquired the *optrA* gene.

4. Discussion

The *Enterococcus* genus is considered one of the predominant bacterial group found in foods due to its ubiquity and its resistance to harsh conditions in food production and storage (Chajęcka-Wierzchowska, Zadernowska & García-Solache, 2020). Our study confirms that *Enterococcus* spp. is also common in retail seafood, with an overall prevalence of approximately 70%. This aligns with reports by Tate et al. in the United States (Tate et al., 2022) and by Boss et al. in imported seafood in Switzerland (Boss, Overesch & Baumgartner, 2016). However, the prevalence of enterococci in seafood varies widely in the literature, from 20% in seafood from Iran to over 90% in imported pangasius and shrimp in Denmark (Ellis-Iversen et al., 2020; Noroozi, Momtaz & Tajbakhsh, 2022). Factors influencing prevalence estimates include the seafood type, processing and storage conditions, and the detection methods used. In our study, *Enterococcus* spp. was more frequent in categories such as salmon, cephalopods, bivalves, and crustaceans, with recovery odds about three times higher than in sea

Table 4
Distribution of MICs among 102 *Enterococcus* spp. isolates. Gray shading indicates range of values actually tested for each antibiotic. Black vertical bars indicate threshold values for resistance, dotted lines indicate intermediate threshold, according to CLSI clinical breakpoints or EUCAST epidemiological cut-off values.

Antimicrobial agents	No. (%) isolates by MIC, (μg/ml)																	
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Chloramphenicol									12 (11.76)	73 (71.56)	10 (9.80)	7 (6.86)						
Ciprofloxacin					1 (0.98)	2 (1.96)	18 (17.64)	61 (59.80)	20 (19.61)									
Daptomycin (*E. faecium*)					1 (0.98)	2 (1.96)	9 (8.82)	7 (6.86)	1 (0.98)									
Daptomycin (non *E. faecium*)					22 (21.57)	21 (20.59)	31 (30.39)	8 (7.84)										
Erythromycin					24 (23.53)	21 (20.59)	18 (17.65)	18 (17.65)	5 (4.90)	16 (15.68)								
Linezolid						2 (1.96)	5 (4.90)	80 (78.43)	14 (13.72)	1 (0.98)								
Nitrofurantoin								5 (4.90)	3 (2.94)	53 (51.96)	12 (11.76)	2 (1.96)	27 (26.47)					
Penicillin					11 (10.78)	13 (12.74)	15 (14.70)	49 (48.04)	8 (7.84)	4 (3.92)	2 (1.96)							
Quinupristin/Dalfopristin						14 (13.72)	7 (6.86)	27 (26.47)	10 (9.80)	40 (39.21)	4 (3.92)							
Streptomycin																99 (97.05)	1 (0.98)	2 (1.96)
Tetracycline							66 (64.70)	6 (5.88)	1 (0.98)	1 (0.98)	3 (2.94)	25 (24.50)						
Vancomycin					1 (0.98)	23 (22.55)	46 (45.09)	18 (17.65)	1 (0.98)	13 (12.75)								

Table 5
Number and resistance phenotype of multidrug-resistant *Enterococcus* spp.

Resistance phenotype*	*E. casseliflavus*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. hirae*	*E. thailandicus*	Total
number of resistances ($n = 8$)			1			1	**2**
CHL, CIP, ERY, LZD, NIT, PEN, SYN, TET						1	**1**
CHL, CIP, ERY, LZD, NIT, STR, SYN, TET			1				**1**
number of resistances ($n = 6$)		1	2				**3**
CHL, CIP, ERY, LZD, NIT, SYN			1				**1**
CHL, CIP, ERY, LZD, SYN, TET		1					**1**
CHL, CIP, ERY, NIT, SYN, TET			1				**1**
number of resistances ($n = 5$)	3	5	1		1	3	**13**
CHL, CIP, ERY, LZD, SYN		1					**1**
CHL, CIP, ERY, SYN, TET		4					**4**
CIP, ERY, LZD, NIT, SYN						1	**1**
CIP, ERY, LZD, NIT, TET			1				**1**
CIP, ERY, LZD, SYN, VAN	2						**2**
CIP, ERY, NIT, SYN, TET						2	**2**
CIP, ERY, PEN, SYN, VAN	1						**1**
ERY, NIT, STR, SYN, TET					1		**1**
number of resistances ($n = 4$)	6	7	6	1			**20**
CHL, CIP, ERY, SYN		1					**1**
CHL, CIP, LZD, SYN		1					**1**
CHL, CIP, SYN, TET		1					**1**
CHL, ERY, SYN, TET		1					**1**
CIP, ERY, LZD, SYN		2					**2**
CIP, ERY, NIT, SYN			3				**3**
CIP, ERY, NIT, TET			2				**2**
CIP, ERY, STR, TET			1				**1**
CIP, ERY, SYN, TET		1					**1**
CIP, ERY, SYN, VAN	6						**6**
CIP, SYN, TET, VAN				1			**1**
number of resistances ($n = 3$)	2	21	8		3		**34**
CHL, CIP, SYN		2					**2**
CHL, CIP, TET		1					**1**
CIP, ERY, NIT			4				**4**
CIP, ERY, SYN		11	1				**12**
CIP, ERY, TET		2					**2**
CIP, LZD, NIT			1		1		**2**
CIP, LZD, SYN		1					**1**
CIP, NIT, SYN					1		**1**
CIP, NIT, TET			1				**1**
CIP, SYN, TET		4					**4**
CIP, SYN, VAN	1						**1**
ERY, NIT, SYN			1				**1**
ERY, NIT, TET					1		**1**
ERY, SYN, VAN	1						**1**
Total	**11**	**34**	**18**	**1**	**4**	**4**	**72**

*Abbreviation: chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), linezolid (LZD), nitrofurantoin (NIT), penicillin (PEN), streptomycin (STR), quinupristin/dalfopristin (SYN), tetracycline (TET), vancomycin (VAN).

Table 6
Number of antibiotic -resistant or -intermediate *Enterococcus* spp. isolates. Percentages are shown in brackets.

		E. faecalis	*E. faecium*	*E. casseliflavus*	*E. hirae*	*E. thailandicus*	Others*	Total
Chloramphenicol	R	5 (8.77)	1 (5)	0	0	1 (20)	0	7 (6.86)
	I	8 (14.03)	2 (10)	0	0	0	0	10 (9.80)
Ciprofloxacin	R	4 (7.01)	11 (55)	3 (25)	1 (25)	1 (20)	0	20 (19.60)
	I	42 (41.17)	7 (35)	7 (58.33)	1 (25)	3 (60)	1 (25)	61 (59.80)
Erythromycin	R	9 (15.79)	3 (15)	0	2 (50)	2 (40)	0	16 (15.68)
	I	16 (28.07)	13 (65)	10 (83.33)	0	2 (40)	0	41 (40.19)
Linezolid	R	0	1 (5)	0	0	0	0	1 (0.98)
	I	6 (10.52)	3 (15)	2 (16.66)	1 (25)	2 (40)	0	14 (13.72)
Nitrofurantoin	R	0	0	0	0	0	0	0
	I	0	17 (85)	0	4 (100)	5 (100)	1 (25)	27 (26.47)
Penicillin	R	0	0	1 (8.33)	0	1 (20)	0	2 (1.96)
Quinupristin/Dalfopristin	R	49 (85.96)	2 (10)	3 (25)	0	0	0	54 (52.94)
	I	3 (5.26)	6 (30)	9 (75)	2 (50)	5 (100)	2 (50)	27 (26.47)
Streptomycin	R	0	2 (10)	0	1 (25)	0	0	3 (2.94)
Tetracycline	R	14 (24.56)	7 (35)	0	2 (50)	3 (60)	2 (50)	28 (27.45)
	I	1 (1.75)	0	0	0	0	0	1 (0.98)
Vancomycin	R	0	0	12	0	0	1	13 (12.75)
Multidrug-resistant		34 (59.65)	18 (90)	11 (91.67)	4 (100)	4 (80)	1 (25)	72 (70.59)
Full susceptible		1 (1.75)	1 (10)	0	0	0	1 (25)	3 (2.94)
Total		57	20	12	4	5	4	102

*Others: grouped species represented by one isolate only: *E. aquimarinus, E. durans, E. gallinarum, E. mundtii*.

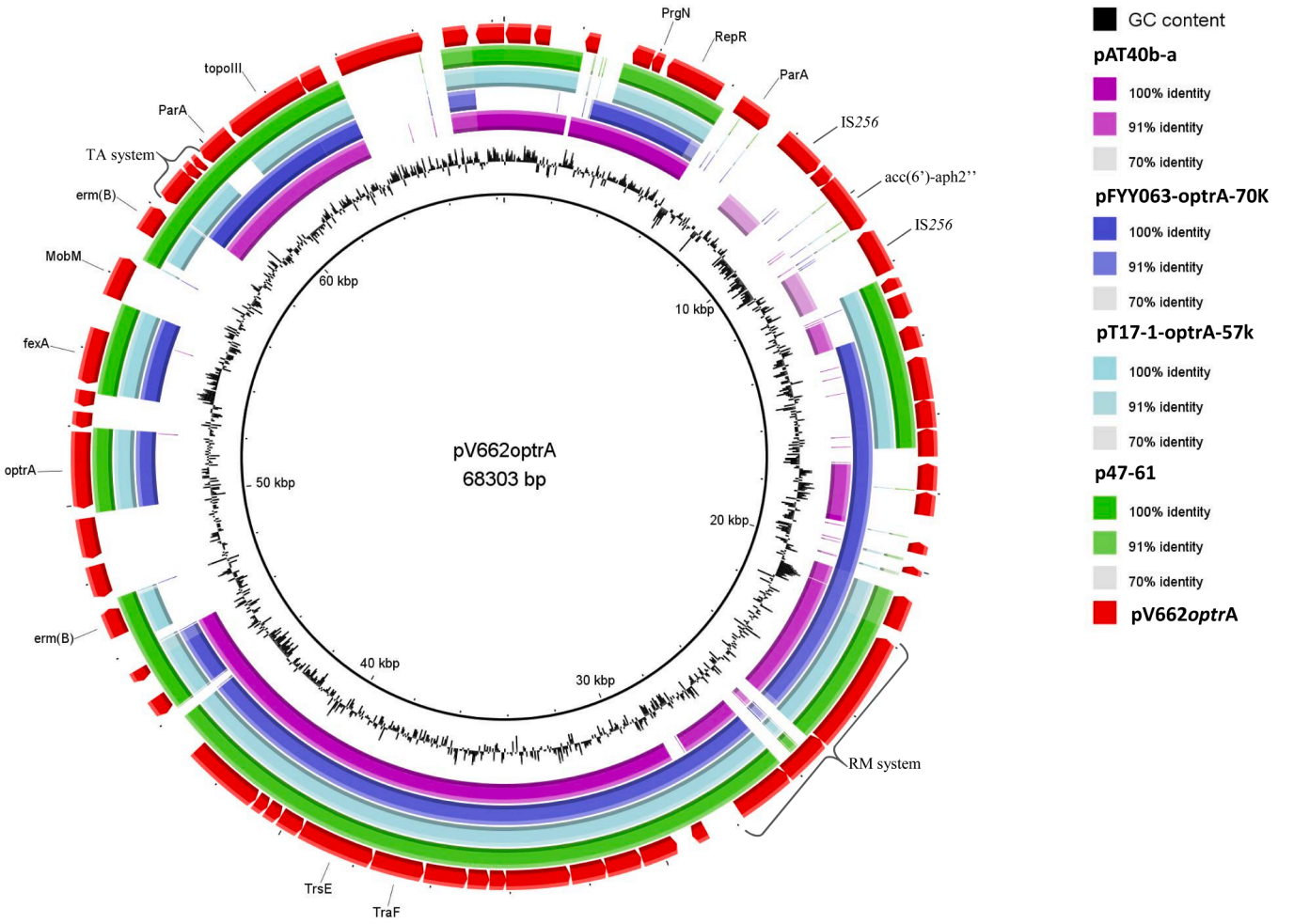

Fig. 1. Circular map of the pV662optrA plasmid of 68,303 bp, depicted in red, in comparison with other plasmids using BRIG software. Plasmids included in the analysis were as follows (inner to outer circles): pAT40b-a of *E. faecalis* AT40b-a (accession no. CP097035) indicated in purple; pFYY063-optrA-70 K of *E. casseliflavus* CQFYY22–063 (accession n. CP116030) showed in blue; pT17–1-optrA-57k of *E. faecium* (accession no. CP109840) and p47–61 of *E. faecium* (accession no. CP091102) indicated in turquoise and green, respectively. Red arrows indicate the positions and orientations of relevant genes coding: antibiotic resistance determinants, mobile/conjugation functions, plasmid replication and plasmid partition proteins all described in this study.

bass. High contamination levels in shrimp (Boss et al., 2016; Tate et al., 2022) and salmon (Boss et al., 2016) are well-documented. *Enterococcus* spp. contamination can originate from fecal pollution in the aquatic environment, explaining the high prevalence observed in crustaceans and cephalopods, which are in contact with sand and sediments, while bivalves concentrate bacteria dispersed in the water (Albini et al., 2022). Another source is cross-contamination during processing, such as evisceration and counter sales (Li et al., 2024), which can explain the high prevalence found in filleted salmon.

Regardless of the contamination sources of in seafood, the percentage of samples positive for *Enterococcus* spp. was consistently above 50% across all categories. This finding supports that this bacterial genus is ubiquitous in seafood and may serve as a source of AMR or antibiotic-resistant determinants for consumers. *E. faecalis* was the most frequently isolated species, followed by *E. faecium*, together accounting for about two thirds of the isolates.

Antibiotic resistance was common, with only around 3% of the isolates were fully susceptible to all tested antimicrobials. Resistance to quinupristin/dalfopristin, tetracyclines, fluoroquinolones and macrolides was particularly frequent, as already reported (Hirshfeld et al., 2023).

Resistance to streptogramins (quinipristin/dalfopristin) was especially prevalent in *E. faecalis*, which is naturally resistant to this antibiotic class, but it was also found in other species, including *E. faecium* (Hirshfeld et al., 2023; Tyson, Sabo, Rice-Trujillo, Hernandez & McDermott, 2018). Quinipristin/dalfopristin is a clinically significant as it treats vancomycin-resistant *E. faecium* infections (Tyson et al., 2018). Approximately one-third of isolates in our collection was resistant to tetracyclines, a similar proportion similar to what Tate et al. (2022) reported. First discovered in 1945, tetracyclines have been extensively used in human and veterinary medicine worldwide, due to their wide spectrum of actions, low toxicity and affordability (Michalova, Novotna & Schlegelova, 2004). They are among the few antibiotics approved for aquaculture use (Tate et al., 2022) and persistent in the environment, in marine biofilms (Balcázar, Subirats & Borrego, 2015). This widespread use and persistence likely contribute to resistance in *Enterococcus* spp. from various sources (Abdel-Raheem et al., 2024; Chajęcka-Wierzchowska, Zadernowska & Łaniewska-Trokenheim, 2016; Mwikuma et al., 2023; Templer & Baumgartner, 2007). In this genus, tetracyclines resistance is generally linked to mobile genetic elements and often co-occurs with resistance to other antibiotics (Igbinosa & Beshiru, 2019).

Members of the *Enterococcus* genus display low-level resistance to aminoglycosides (Zaheer et al., 2020), but high-level resistance to this antibiotic class can hinder therapeutic use in combination with beta lactams (Chow, 2000). High-level resistance to streptomycin (>1024 mcg/ml) was recorded in two isolates in our collection.

More than two-third of the isolates of our collection were classified

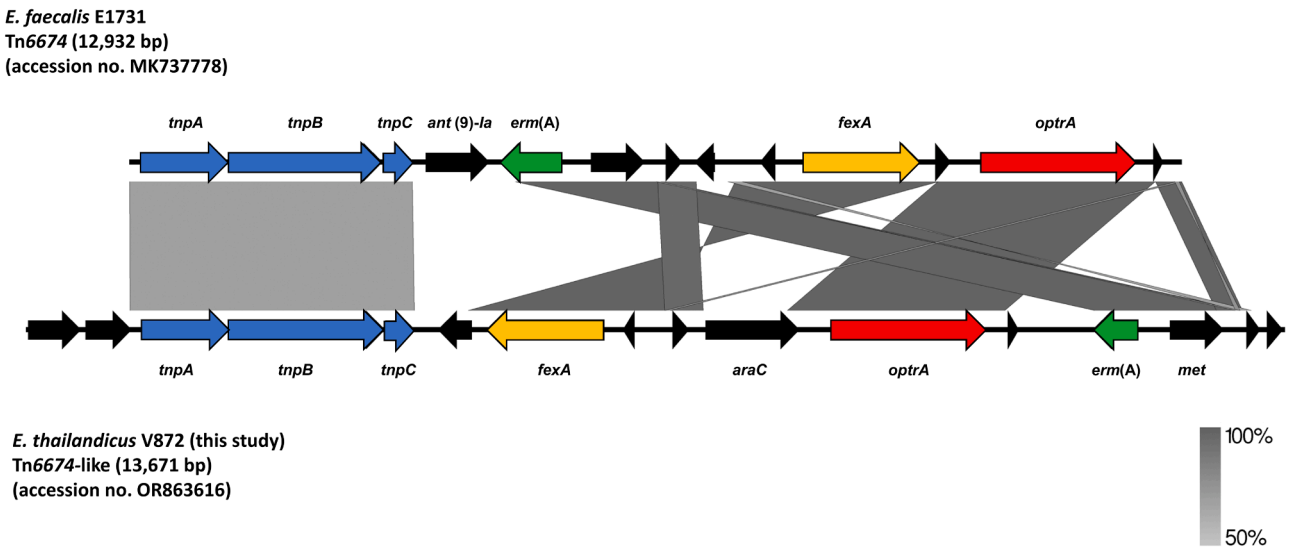

Fig. 2. Linear map of the Tn*6674*-like from *E. thailandicus* V872 in comparison with Tn*6674* wild-type from *E. faecalis* E1731 using Easyfig tool (https://mjsull.github.io/Easyfig/). The positions and transcriptional direction of the ORFs are represented with arrows. Some antibiotic resistance determinants and relevant genes described in this study are shown. The transposase genes are depicted in blue; the *optrA* gene is indicated in red, the *fexA* gene is showed in orange, whereas the *erm* (A) and ant (9)-Ia genes are shown in green and black, respectively. The identities are also shown following a gray scale between 50% and 100%.

as multidrug-resistant. In addition, we recovered *Enterococcus* species harboring resistance to last-resort antimicrobials from seafood products.

Vancomycin resistance was observed in *E. casseliflavus* and *E. gallinarum* isolates, opportunistic human pathogens, which are naturally resistant to this antibiotic due to the presence of the non-transferable *vanC* chromosomal cluster. Although rare, severe human infections caused by these enterococcal species, have recently increased (García-Solache & Rice, 2019b; Monticelli, Knezevich, Luzzati & Di Bella, 2018). The *optrA* gene, encoding an ABC-F protein that confers resistance to oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol), was found in two isolates: *E. thailandicus* V872 and *E. faecium* V662. In *E. thailandicus*, the *optrA* gene was located on the chromosome, carried by a Tn*6674*-like transposon, first described in a porcine strain of *E. faecalis* isolated in China (Li et al., 2019). To our knowledge, this is the first detection of a Tn*6674*-like transposon in *E. thailandicus* isolates.

In *E. faecium* V662, the *optrA* gene was recovered on a new transferable plasmid here named pV662*optrA*. This condition is cause of concern because plasmids are significant vehicles for spreading oxazolidinones resistance not only among bacteria of the same species, but also across different species and genera (Brenciani, Morroni, Schwarz & Giovanetti, 2022).

Our data are also confirm by the recent study of Abdel-Raheem and colleagues, regarding the emergence of linezolid-resistant -vancomycin-resistant enterococci recovered from Egyptian fish and shrimp (Abdel-Raheem et al., 2024).

The *Enterococcus* spp. exhibits genome plasticity, and can rapidly acquire DNA from other microbial community members (Davis et al., 2022). After ingestion, enterococci can transmit ARGs to other commensal flora members , including pathogens (EFSA/ECDC, 2022; Hirshfeld et al., 2023). Thus, the presence of *optrA* in a conjugative plasmid in *E. faecium* from seafood might constitute a threat for consumers.

Our findings underscore the need for a surveillance plan for antibiotic resistance in seafood and suggest including *Enterococcus* spp. among the target species. Indeed, in 2019 the United States National Antimicrobial Resistance Monitoring System (NARMS) carried out a pilot study to assess AMR in seafood. The pilot study found that *Enterococcus* spp. had a high prevalence of recovery from retail food and exhibited the highest prevalence of resistance among Gram-positive bacteria. Based on this, NARMS included *Enterococcus* spp. in the national monitoring of resistant bacteria in retail seafood starting in 2020 (Hirshfeld et al., 2023; Tate et al., 2022). Recently, the European Safety Authority recommended a baseline survey (BLSs) to assess the prevalence of AMR in seafood in the EU, complementing the current monitoring of bovine, pigs and poultry sectors (Commission Implementing Decision (EU) 2020/1729). In the BLS specifications, EFSA suggested the inclusion of *Enterococcus* spp, and particularly *E. faecium* and *E. faecalis*, which are relevant from a public health perspective, for AMR surveillance of mussels (Aerts et al., 2024).

5. Conclusion

Our study demonstrated that *Enterococcus* species are commonly present on retail seafood, though their abundance varies by seafood category. Regarding the risk of seafood as a potential source of antimicrobial-resistant organisms and antimicrobial resistance genes, we found a high prevalence of multidrug-resistant *Enterococcus* isolates. We also detected isolates resistant to last-resort antimicrobials, such as glycopeptides and oxazolidinones, which are of serious concern for human health. These findings highlight the importance of including *Enterococcus* spp. in the surveillance of antibiotic resistance in seafood.

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Ethical statement - studies in humans and animals

The authors declare that the work did not involve the use of human and animal subjects.

CRediT authorship contribution statement

Elisa Albini: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Francesca Leoni:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Francesca Romana Massacci: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Pieralisi:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Serenella Orsini:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Francesca Blasi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Alessandra Di Gregorio:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sonia Nina Coccitto:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Marzia Cinthi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Gloria D'Achille:** Writing – review & editing, Methodology, Investigation. **Gianluca Morroni:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Carmen Maresca:** Writing – review & editing, Validation, Supervision, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Eleonora Scoccia:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Du Xiang-Dang:** Writing – review & editing, Methodology, Investigation. **Eleonora Giovanetti:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Andrea Brenciani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Chiara Francesca Magistrali:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2024.100604.

Data availability

Data will be made available on request.

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