Seasonal prevalence and antimicrobial resistance profiles in *Enterococcus* spp. identified from mussels farmed along the coasts of the Abruzzo region

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Abstract

The present study aimed to investigate the antimicrobial resistance (AMR) circulation through the different seasons in the *Enterococcus* genus isolated from mussels (*Mytilus galloprovincialis*) for human consumption and farmed along the coasts of the central Adriatic Sea (Abruzzo region, Italy). A total of 250 mussels were collected, and 32 *Enterococci* (90.62% *Enterococcus faecium* and 9.37% *Enterococcus durans*) were identified using the VITEK 2 system (bioMérieux, France). Antibiograms included 26 molecules used for the treatment of veterinary and human infections. Biomolecular screenings involved 45 genetic determinants responsible for AMR. Results showed mainly resistance against tetracycline (44.44%), vancomycin (27.78%), quinupristin-dalfopristin (16.67%), nitrofurantoin, and linezolid (11.11%). Concerning the antibiotic resistance genes (ARGs), multiplex end-point polymerase chain reaction assays mostly amplified *tet*C (59.37%), *tet*D (50.00%), *cfr* (43.75%), *van*A and *van*D (37.50%), *vat*E (21.87%), *vat*D, *poxt*A, and *qnr*S (18.75%), and 52.67% and 35.11% in winter and spring seasons, respectively. The consistent environmental ARG circulation confirms the genetic pollution of marine environments, and the season variable (water temperatures) significantly influences their horizontal circulation and phenotypical expression. The AMR phenomenon, defined as uncontrolled, represents a crucial public health concern that needs to be monitored.

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Introduction

Among the marine environmental bacteria, the *Enterococcus* genus is also part of the natural intestinal microbiota in mammalian and fish species and humans (Abdel-Raheem *et al*., 2024). The horizontal transmission of genetic mobile forms has played a consistent role in the diffusion of the antimicrobial resistance (AMR) phenomenon involving both commensal and pathogen strains. *Enterococci* have a crucial role as reservoirs of many antibiotic resistance genes (ARGs) (Paillard *et al*., 2022). The most amplified determinants involved both veterinary-administrated antimicrobials such as β-lactams (*bla* genes), tetracyclines (TET) (*tet* genes), *etc*., and both against the so-called critically important antimicrobials (CIAs) whose usage is restricted only to the human species, *i.e*., glycopeptides (*van*A, *van*B, *van*D, *van*N, *etc*.), oxazolidinones (*cfr*, *optr*A, *poxt*A, *etc*.), streptogramin (*vat*D, *vat*E, *vga*A, *etc*.). For this reason, *Enterococcus* spp. was included among the ESKAPE pathogens that are responsible for nosocomial infection in human and veterinary medicine (De Oliveira *et al*., 2020).

Furthermore, the increased discovery of multi-drug resistant (MDR) *enterococci* against different antimicrobial classes has attracted more attention from many scientific groups. Indeed, many *Enterococcus* species have shown resistance to vancomycin (VAN) (also harboring *van* genes), determining their identification with the acronym VRE; another important concern is represented by the consistent diffusion of resistance profiles against the oxazolidinone antimicrobial family, with special regard to the linezolid one. Indeed, the increased lack of susceptibility against this molecule has been justified by the discovery of many genetic determi-

nants widely diffused among *enterococci* (isolated from different environments and media) (Sacramento *et al*., 2019). Another important variable in the AMR phenomenon has been covered by the marine water temperatures among the different seasons, combined with the different urbanization levels of many geographical areas. Winter and spring seasons are considered as referring periods when ARGs can be mostly amplified due to their consistent horizontal transmissions, suggesting that thermal variations cover the role of physical triggers, including the wide activation of bacterial metabolism and enzymes. Indeed, it can be considered as an evolutionary process aiming to guarantee their survival (Kormos *et al*., 2022). For this purpose, this study aimed to study the phenotypic and genotypic resistance profiles of 32 *enterococci* identified from 250 mussels (*Mytilus galloprovincialis*) collected in three different seasons (spring, summer, and winter) in a mariculture farm along the coasts of the central Adriatic Sea (Abruzzo region, Italy). The obtained biomolecular results were intended to provide original data concerning the AMR phenomenon and possible correlation with the environment in a low-investigated marine area.

Materials and Methods

Sample collection and processing

Sampling activities lasted from March 2023 till January 2024. A total of 250 bivalve lamellibranches, belonging to the *Mytilus galloprovincialis* species, were collected from a mariculture farm located along the coasts of the Abruzzo region in the central Adriatic Sea (georeferencing data: 42° 08.7810’ N 14° 46.0265’ E). Specimen collections were divided into three different months: March (C1), June 2023 (C2), and January 2024 (C3), as schematically represented in the following Table 1.

In agreement with the European Regulation No. 853/2004 and No. 627/2019, mariculture waters, belonging to the screened farm, were classified as A-category (European Parliament and Council of the European Union, 2004; European Commission, 2019). It implied their further prompt commercialization for human consumption. All tested mussels were directly conferred (under refrigerated conditions) for microbiological and biomolecular assays to the laboratory at the Department of Veterinary Medicine (University of Teramo, Italy).

First, it was performed a sterile collection of animal tissues (with special regard to the gastroenteric system), using mono-usage scalpels (Monopec Scalpels, Thermo Fisher Scientific™, Waltham, MA, USA), and pools of 5 subjects were composed. Therefore, a total of 50 pools were obtained. From each pool, 25-gram aliquots were introduced into stomacher bags (BagMixer®, Interscience, Puycapel, Cantal, France), and 225 mL of buffered peptone water (ThermoFisher Scientific™, Waltham, MA, USA) was added to perform ten-fold dilutions, as described in the ISO 6887-3:2017 (ISO, 2017). Specimens were stomached for 90 seconds and were successively incubated at 37°C for 18-24 hours.

Identification and antimicrobial susceptibility tests

The first part, belonging to the identification procedures, was designed as a culture-dependent study; indeed, a selective screening was performed in agreement with the international standard for *Enterococcus* spp. ISO 7899-2:2000 (ISO, 2000). More in detail, from each incubated homogenized broth culture, a loopful was directly plated onto selective Slanetz-Bartley agar (ThermoFisher Scientific™, Waltham, MA, USA) supplemented with TTC Solution Oxoid™ (ThermoFisher Scientific™, Waltham, MA, USA) and incubated for 18-24 hours at 37°C, as described by the manufacturer’s instructions. The morphological suspect of *Enterococcus* colonies (red-maroon-pink ones) was followed by an automated biochemical method using the VITEK® 2 System (bioMérieux, Paris, France). More specifically, pure colonies were collected and introduced into a sterile vial, which contained 0.45 NaCl sterile solution, gaining a final turbidity in the range of 0.50-0.64 McFarland. Once solutions were prepared, they were involved as cultures useful for identification with cards VITEK® ID-GP (VITEK® 2 system, bioMérieux, Paris, France). After loading, results were provided within 8 hours of starting. Multiplex end-point polymerase chain reactions (PCR) were performed to confirm *enterococci* identifications, as described by Jackson *et al*. (2004). For all above-mentioned screenings, positive controls [ATCC 35667 for *Enterococcus faecium* (Fioriti *et al*., 2021), ATCC 6056 for *Enterococcus durans* (Tarek *et al*., 2022)] and negative ones (sterile water) were included.

Antimicrobial susceptibility tests (ASTs) were performed using the VITEK® 2 system (bioMérieux, Paris, France) by loading two specific cards: AST-P658 and AST-P659, including 26 antimicrobials. More in detail, veterinary law-allowed and CIAs molecules were considered such as amoxicillin/clavulanic acid, ampicillin, ciprofloxacin, daptomycin, gentamycin, imipenem, kanamycin, levofloxacin, linezolid (LZD), nitrofurantoin (NIT), quinupristin/dalfopristin (QPD), streptomycin, teicoplanin, tigecycline, trimethoprim, trimethoprim/sulfamethoxazole, VAN, benzylpenicillin, cefoxitin, cefazoline, clindamycin, erythromycin, mupirocin, oxacillin, rifampicin, and TET. According to the manufacturer’s instructions, bacterial suspensions (containing *Enterococcus* strains) with a final turbidity of 0.5 McFarland were used as mother-solutions. From each one of them, an aliquot of 280 µL was directly added to vials with 3 mL of sterile NaCl solution (0.45%). The last step was cards loading in the systems, and the respective results were obtained after 24 hours. Antibiograms, performed with the VITEK® 2 system, also provided the minimum inhibitory concentration values that were determined in agreement with the Clinical & Laboratory Standard Institute breakpoints (CLSI, 2017). The definition of MDR bacteria was used for strains that were not susceptible to three or more antimicrobial classes, as reported by Magiorakos *et al*. (2012).

Phenotypic indexes calculation

The resulting MDR *enterococci* were involved in the AMR index determination. More in detail, the multiple antibiotic resist-

Table 1. Screened mussels involved in the present scientific investigation.

Italian region	Screened farm	N. of sampled subjects	Months
Abruzzo	Casalbordino (CH, Italy) 42° 08.7810’ N 14° 46.0265’ E	C1: 80 mussels C2: 80 mussels C3: 90 mussels	March 2023 June 2023 January 2024

C1, first collection; C2, second collection; C3, third collection.

ance index (MAR_{index}) and the resistance pattern one (ARPA), as calculated by Adesiyan *et al*. (2022). MAR_{index} was determined by the ratio of the sum of the discovered resistance profiles and the total of tested antimicrobials, and a referring threshold, about MAR_{index}, was established at the value of >0.2 (Mok *et al*., 2021); it means that if values were higher than 0.2, it could be considered a representation of high pollution caused by humans and zootechnic sector, as reported by Titilawo *et al*. (2015). The ARPA index was obtained by performing the ratio between the number of discovered resistances and the total number of tested bacteria, as described by Deng *et al*. (2020).

Biomolecular assays: virulence gene and antibiotic resistance gene detection

All identified *enterococci* were successively tested by performing uniplex or multiplex end-point PCR assays. DNA extraction was obtained using the commercial kit High Pure PCR Template Preparation Kit (Roche®, Indianapolis, IN, USA), and pellets were resuspended with 100 µL of DNAse-free water (Nuclease-Free Water, ThermoFisher Scientific™, Waltham, MA, USA). Specimen storage was at -80°C. *Agg*2, *gel*E, *cyl*A, *cyl*B, and *cyl*M were the screened genetic determinants as virulence factors. Reaction volumes were performed in 25 µL, and the thermocycler setting agreed with Çardak *et al.* (2022). ARGs detection included a total of 45 genetic determinants, as schematically represented in *Supplementary Table 1* (Celli and Trieu-Cout, 1998; Maynard *et al*., 2004; Baddour *et al*., 2007; Srinivasan *et al*., 2007; Bailey *et al*., 2010; García *et al*., 2017; Nomura *et al*., 2018; Shaw *et al*., 2018; Bender *et al*., 2019; Kishk *et al*., 2020; Ayobola *et al*., 2021; Çardak *et al*., 2022), and they were searched in all identified *enterococci* (both from phenotypically resistant and susceptible ones).

Amplicons, obtained by PCR assays, were loaded onto agarose gels at different concentrations (1.5-2.0%) depending on sequence seizure. For this purpose, specific DNA ladders (Genetics, DNA Marker, Düren, Germany) of 50 bp and 100 bp were used as referments and the running time was set at 80 Volts for 40 minutes. Purification procedures were applied for the suspected positive bands using commercial kits (QIAquick® PCR Purification Kit, Hilden, Germany). This step was followed by sequencing (Sanger one) performed by BioFab Research (Rome, Italy), and sequences were successively studied using the BALSTN system (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch).

Statistical analysis

The SPSS 20.0 Software (IBM, Chicago, IL, USA) was used to perform the statistical analysis. Data were first observed with descriptive analysis, with special regard to the Shapiro-Wilk test for the normality assumption parameter ($p<0.05$). The two-tailed *t*-test was used to compare ARG distribution among phenotypically resistant and susceptible *Enterococcus* strains between the different sampling steps. Finally, confidential intervals (CI) at 95% were calculated, if applicable, for all percentage values.

Results

Identification, antimicrobial susceptibility test, and antibiotic resistance gene results

A total of 32 *Enterococcus* strains were identified from 250 *Mytilus galloprovincialis* farmed in a mariculture site located along the coasts of the Central Adriatic Sea (Abruzzo region, Italy). More in detail, 29/32 or 90.62% (CI 95%: 80.52-100.00%) were *E. faecium* and 3/32 or 9.37% (CI 95%: 0.16-18.58%) were *E. durans*. Basing on the sampling design and collection days, *E. faecium* was identified as follow: 13/29 or 44.82% (CI 95%: 26.72-62.92%) from C3 (January 2024), 11/29 or 37.93% (CI 95%: 20.28-55.58%) from C1 (March 2023), and 5/29 or 17.24% (CI 95%: 0.35-30.98%) from C2 (June 2023). *E. durans* was only identified from C3 (January 2024).

Among the tested antimicrobials, resistance to the TET class was mainly observed in 8/18 or 44.44% (CI 95%: 21.49-67.39%) of resistant *enterococci*, followed by 5/18 or 27.78% (CI 95%: 7.09-48.47%) to glycopeptide (VAN), 3/18 or 16.67% (CI 95%: 0.16-33.18%) to the streptogramin (QPD), and 2/18 or 11.11% (CI 95%: 0.03-21.92%) to NIT and LZD.

Based on the collection season, resistant *enterococci* (9/18 or 50.00%; CI 95%: 26.91-73.09%) were mainly identified from C3 resulting statistically different than C1 (7/18 or 38.88%; CI 95%: 16.36-61.40%) with $p<0.02$ and C2 (2/18 or 11.11%; CI 95%: 0.04-22.18%), with a $p<0.001$.

Among the resistant *enterococci*, 7/18 or 38.88% (CI 95%: 16.36-61.40%) were classified as MDR because not susceptible to three or more antimicrobial classes. A detailed representation of all phenotypic resistance profiles is illustrated in Figure 1.

The phenotypical resistance indexes were calculated, as described in the Materials and Methods section. MAR_{index} was 0.53 from the identified enterococci. More specifically, it was 0.38 in C3, 0.34 in C1, and 0.07 in C2. The ARPA value was 0.44.

A total of 131 ARGs were amplified, and more specifically, 69/131 or 52.67% (CI 95%: 44.12-61.22%) genetic determinants were harbored by bacteria belonging to the C3, 46/131 or 35.11% (CI 95%: 26.94-43.28%) to C1, and 16/131 or 12.21% (CI 95%: 6.61-17.81%) to C2. Comparing ARG distributions among collection steps, statistical differences were observed between C3-C1 ($p<0.001$) and C3-C2 ($p<0.01$).

Among ARGs discovered from *enterococci* DNA, *tet*C and *tet*D were mainly amplified, presenting the following respective percentage values: 19/32 or 59.37% (CI 95%: 42.36-76.38%) and 16/32 or 50.00% (CI 95%: 32.68-67.32%). *Cfr* genetic determinant (resistance against oxazolidinone antimicrobial class) was discovered from 14/32 or 43.75% (CI 95%: 26.57-60.93%) strains. Concerning *van* genes, *van*A and *van*D were amplified from 12/32 or 37.50% (CI 95%: 20.73-54.27%) and *van*N was discovered in 6/32 or 18.75% (CI 95%: 5.23-32.27%) *enterococci*. Genetic determinants for streptogramin and fluroquinolones were also observed: *vat*E (7/32 or 21.87%; CI 95%: 7.55-36.19%), *vat*D and *qnr*S (6/32 or 18.75%; CI 95%: 5.23-32.27%).

Discussion

The present culture-dependent study was performed to biomolecularly screen *Enterococcus* strains identified from mussels (*Mytilus galloprovincialis*) farmed for human consumption in a mariculture site located along the coasts of the Abruzzo region in the central Adriatic Sea. More in detail, the 29 *E. faecium* and 3 *E. durans* involved presented consistent ARGs harboring, which resulted marginally phenotypically expressed, as described in the Results section. The obtained original data concerning the wide genetic determinants circulation in the marine environment enforced the scientific hypothesis of horizontal gene transmissions among human enteric microbiota with animal ones (with special regard to whether foodstuffs were raw or undercooked consumed),

as suggested by Svanevik *et al*. (2023). Bivalve lamellibranches, including the *Mytilus* genus, represent ideal bio-sentinels of the genetic marine environmental pollution among strict pathogen and commensal bacteria (including *Enterococcus* genus) (Radisic *et al*., 2024); for this scientific purpose, the present study investigated phenotypic and genotypic resistance profiles from *Enterococcus* spp. identified from mussels in three different seasons (spring, summer, and winter).

The obtained phenotypic resistance profiles were mostly highlighted against TET (44.44%; CI 95%: 21.49-67.39%), VAN (27.78%; CI 95%: 7.09-48.47%), QPD (16.67%; CI 95%: 0.16-33.18%), NIT and LZD (11.11%; CI 95%: 0.03-21.92%), as described in the Results section. The observed resistance prevalence resulted in line for TET but low for VAN if compared with the percentage values of 45.76% (CI 95%: 33.05-58.47%) TET and 37.28% (CI 95%: 24.94-49.62%) VAN discovered from *Enterococcus* spp. isolated from seafood products (ready-to-eat) by Igbinosa and Beshiru (2019). Çardak *et al*. (2022) observed similar resistance prevalence against TET (50.00% CI 95%: 40.94-59.06%), but lower for VAN (8.54%; CI 95%: 3.48-13.60%). These observed differences find their scientific explanations in the reduced administrations of VAN if compared with the TET ones as treatments for infections with special regard to the zootechnical sectors, as observed, in the mammalians, by Lengliz *et al*. (2022) and Amuasi *et al*. (2023). On the contrary, Abdel-Raheem *et al*. (2024) observed similar phenotypic resistance profiles, reported in this study, from n. 54 *enterococci* isolated from different kinds of seafood (*Oreochromis niloticus*, *Argyrosomus regius*, and shrimp): 90.7% to TET and 37.0% to VAN. Their biomolecular investigations and sequence analyses demonstrated that the increased VAN-resistance profile diffusion was caused by antimicrobial abuse in human medicine. The environmental repercussions were supported by the improper filtration methods of wastewater coming from hospitals and genotypically enforced by the horizontal transmissions among the different bacterial genera. Furthermore, the highest MAR_{index} value was 0.53, observed in this study, which was in agreement with 0.55, discovered by Igbinosa and Beshiru (2019). MAR_{index} and ARPA values of this study were interpreted as

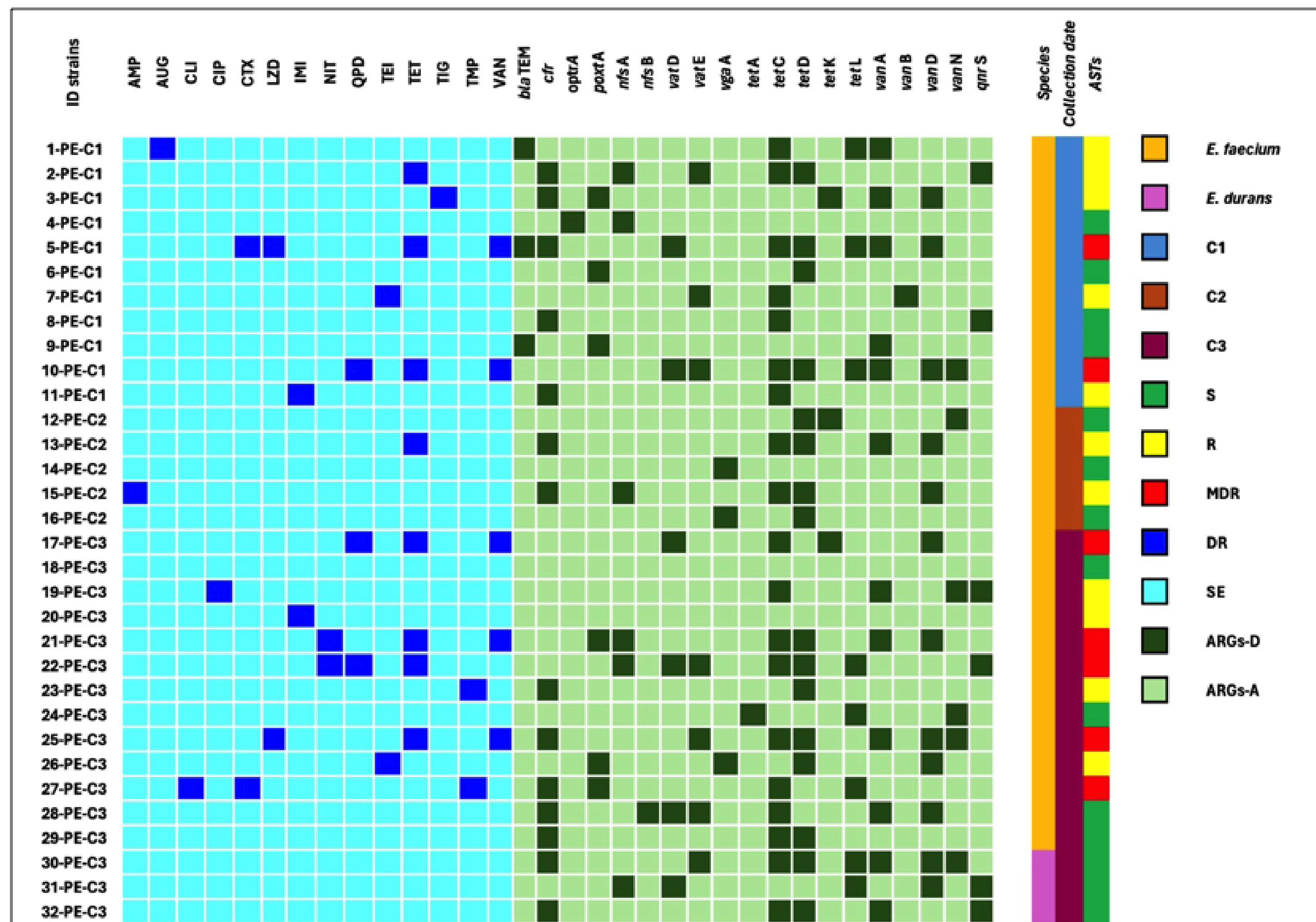

Figure 1. Heatmap of antimicrobial susceptibility tests and antibiotic resistance gene distribution among the different identified enterococci in the investigated mariculture farm. C1, collection 1 (March 2023); C2, collection2 (June 2023); C3, collection 3 (January 2024); DR, discovered resistant strains; SE, susceptible enterococci; ARGs, antibiotic resistance genes; ARGs-D, ARG detection; ARGs-A, ARGs absence; AMP, ampicillin; AUG, amoxicillin/clavulanic acid; CLI, clindamycin; CIP, ciprofloxacin; CTX, cefotaxime; LZD, linezolid; IMI, imipenem; NIT, nitrofurantoin; QPD, quinupristin-dalfopristin; TEI, teicoplanin; TET, tetracycline; TIG, tigecycline; TMP, trimethoprim; VAN, vancomycin.

described by Adesiyan *et al*. (2022) (values reported in the Results section), indicating a consistent antimicrobial pollution of the screened marine area. This trend finds its scientific foundation in the consistent anthropic pollution of marine ecosystems and with special regard to the improper wastewater management (coming from hospitals, industries, *etc*.) (Abdel-Raheem *et al*., 2024; Radisic *et al*., 2024).

Another important observed pattern was the parallel resistance against TET and VAN in many *enterococci,* representing 5/18 or 27.78% (CI 95%: 7.09-48.47%). The scientific explanation of these results can be explained by the wide environmental diffusion of VRE, and more in detail, it is genetically associated with the harboring of *van*A-*type* (Sacramento *et al*., 2019). Indeed, in the present investigation, all phenotypically VAN-resistant (including VRE strains) and susceptible ones widely harbored the *van*A and *van*D genes (12/32 or 37.50%; CI 95%: 20.73-54.27%).

Finally, resistance against any antimicrobials classified as CIAs, such as LZD (11.11% (CI 95%: 0.03-21.92%) and QPD (16.67%; CI 95%: 0.16-33.18%) was also observed. A similar pattern, 20.40% (CI 95%: 8.34-32.46%) was also discovered by Abdel-Raheem *et al*. (2024) from *E. faecium* identified from *Argyrosomus regius* fished in Egypt. The increasing resistance against LZD represents a crucial alert because this antimicrobial is largely administered to threaten VRE infections in the human species (Yang *et al*., 2023).

Concerning ARGs, *tet* genes were widely amplified (as described in the Results section): more specifically *tet*C (19/32 or 59.37%; CI 95%: 42.36-76.38%) and *tet*D (16/32 or 50.00%; CI 95%: 32.68-67.32%), while *tet*K was marginally amplified (3/32 or 9.37%; CI 95%: 0.16-18.58%). These results were in contrast with previous studies that mainly discovered *tet* genes from *Enterococcus* spp. (identified from finfish and bivalves), with the following prevalence percentages: *tet*K 75.90% (CI 95%: 53.19-98.61%) reported by Abdel-Raheem *et al.* (2024); *tet*A 51.90% (CI 95%: 32.57-71.23%); and *tet*M 55.60% (CI 95%: 27.71-79.79%) by Igbinosa and Beshiru (2019).

Oxazolidinone ARGs were also widely amplified from the identified *enterococci,* and with special regard, the *cfr* one was discovered from the extracted DNA of 14/32 or 43.75% (CI 95%: 26.57-60.93%) bacteria. *Poxt*A gene, also responsible for LZD resistance, was harbored both in resistant and susceptible strains (6/32 or 18.75%; CI 95%: 5.23-32.27%). Abdel-Raheem *et al*. (2024) found mainly amplified the *optr*A gene (20.40%; CI 95%: CI 95%: 8.34-32.46%) (genetic mobile vector of resistance against LZD), which was discovered in the present biomolecular investigation from 1 *E. faecium*. These differences are explained by the consistent plasticity of these nucleotide sequences, as suggested by Echeverria-Esnal *et al.* (2023).

Another important antimicrobial class, involved in the observed resistance profiles, is represented by the glycopeptide class (VAN). Indeed, *van*A and *van*D (37.50%; CI 95%: 20.73-54.27%) were widely harbored in all *enterococci*. A similar prevalence 37.00% (CI 95%: 15.65-58.35%) for *van*A was discovered from VRE by Abdel-Raheem *et al*. (2024), but lower than 63.60% (39.01-88.19%) observed by Igbinosa and Beshiru (2019). The VRE environmental diffusion (aquatic ecosystems, soil, nosocomial ones, *etc*.) represents a crucial public health concern, and the *van* genes have presented numerous modifications to the oligo sequences over the years, as suggested by O'Toole *et al*. (2023).

*Vat*D (18.75%; CI 95%: 5.23-32.27%) and *vat*E (21.87%; CI 95%: 7.55-36.19%), responsible for the acetyltransferase gene expressions and for resistance against streptogramin A compounds, were also discovered from both susceptible and resistant *enterococci*. The harboring of streptogramin ARGs (also including for glycopeptides and oxazolidinones), that was defined as rare for *Enterococcus* spp. by Xuan *et al.* (2021), have been observed by recent studies as shrimps (78.18%; CI 95%: 53.46-100.00%) from *E. faecium* identified in Northern California by Hirshfeld *et al.* (2023) and in human patients as nosocomial profiles observed in *enterococci* (6.7%; CI 95%: 0.37-13.03%) identified from in human patients (Li *et al.*, 2022).

Concerning *qnr* genes, they were also amplified both from resistant and susceptible strains, and *qnr*S, among the considered ARGs, was the only one discovered in 18.75% (CI 95%: 5.23-32.27%) of them. These genetic determinants have resulted marginally amplified from *Enterococcus* spp. in mussel species; indeed, it was generally amplified from marine waters, as reported by Cho *et al*. (2020). The environmental source, as a consequence of the anthropic activities (supported by phylogenetic analyses), can be considered the scientific explanation of the observed results, in agreement with Miranda *et al*. (2022).

One more last consideration is based on the ARGs distributions among the three different collection steps. Statistical differences among C3-C1 ($p<0.001$) and C3-C2 ($p<0.01$) were observed, as described in the Results section. First of all, from the visual analysis of Figure 1, the largest ARG amounts were reported during the winter (C3) and spring (C1) seasons, also supported by a decreasing prevalence of identified *enterococci*. This environmental behavior finds its scientific explanation as a consequence of evolutionary survival mechanisms adopted by bacterial species. Indeed, it has been demonstrated that cold waters (including marine environments) act as negative stimuli to the increase of ARG horizontal transmission among bacteria genera that populate the marine microbiome, determining a wide environmental diffusion of antibiotic-resistant bacteria, as suggested by Bai *et al*. (2022). The highest distribution obtained in the present scientific investigation, during the winter season, can also be justified by the evidence reported by Kormos *et al*. (2022), who highlighted high ARG environmental distributions between December and February. This phenomenon was caused by the atmospheric abundance of rain (depending on the atmospheric peculiarities of continents) and the relative physical carrying of bacteria from the terrestrial to the marine environment in the so-defined suburban areas (Kormos *et al*., 2022). The complexity of the AMR phenomenon led to not excluding the influence of the ecosystem variable and the strong connection between bacteria and the environment. For this purpose, this study wanted to contribute to providing original data about ARG circulation in *Enterococcus* spp. differently harbored or phenotypically expressed among seasons.

Conclusions

The consistent environmental diffusion of the so-called AMR phenomenon has been enforced by the dissemination of ARGs harboring bacterial strains. Among them, the *Enterococcus* genus plays a crucial role (O'Toole *et al*., 2023). In the present scientific investigation, the discovered genetic determinants (AMR against veterinary law-allowed and CIAs ones) such as *tet*C, *tet*D, *cfr*, *van*A, *van*D, *van*N, *vat*D, *vat*E, and *qnr*S were amplified both from phenotypically resistant and susceptible *enterococci*.

From a seasonal perspective, winter and spring are characterized by a consistent amount of ARGs compared to summer. The screened area, along the coasts of the Abruzzo region in the central Adriatic Sea, can be considered a suburban one, as described by

Kormos *et al.* (2022). The observed results can be scientifically justified by the consistent impact of anthropic activities (*i.e.*, hospital wastewaters, agricultural manures, *etc.*) (Miranda *et al.*, 2022).

The improper antimicrobial administrations, both in human and veterinary medicine, are responsible for the current environmental pollution.

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Online supplementary material

Supplementary Table 1. Considered antibiotic resistance genes involved in the present biomolecular investigation.