Pol. J. Environ. Stud. Vol. XX, No. X (XXXX), 1-13
DOI: *10.15244/pjoes/208255* ONLINE PUBLICATION DATE: 2025-12-03

Original Research

Investigation of the Virulence Factors of *Enterococcus* Strains Isolated from Seawater

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Received: 19 March 2025
Accepted: 14 July 2025

Abstract

This study aimed to investigate the antimicrobial resistance and virulence factors of *Enterococci* isolated from seawater using both phenotypic and genotypic methods. A comparison of the phenotypic virulence factors between *Enterococcus faecalis* and *Enterococcus faecium* strains revealed that gelatinase activity was 91.3% and 89.5%, respectively. Caseinase activity was observed at rates of 65.2% for *E. faecalis* and 31.5% for *E. faecium*. The capacity for strong adhesion in biofilm formation was 41.3% in *E. faecalis* and 42.1% in *E. faecium*. Serum resistance activity was noted at 54.3% for *E. faecalis* and 31.6% for *E. faecium*. Remarkably, hemagglutination activity showed a strong activity in 39.1% of *E. faecalis* strains, while *E. faecium* exhibited 0% activity. Antibacterial activity against *Staphylococcus aureus* ATCC 25923 was 32.6% for *E. faecalis* and 5.3% for *E. faecium*, and against *Escherichia coli* ATCC 25922, the activity was 23.9% and 0%, respectively. It was determined that one *E. faecalis* strain (2.17%) and one *Enterococcus gallinarum* strain (100%) contained at least four resistance genes. Additionally, one *E. faecalis* strain (2.17%) harbored up to 11 of the 12 tested antibiotic resistance genes. Notably, two *E. faecalis* strains (3.03%) exhibited the most virulent characteristics, encompassing 12 virulence gene regions. In contrast, one *E. gallinarum* strain (100%) manifested the least virulent characteristics, comprising three virulence gene regions. These findings indicate that marine *Enterococci* could pose a public health threat, necessitating ongoing surveillance.

Keywords: antibiotic resistance, *Enterococcus*, *E. faecalis*, *E. faecium*, virulence, seawater

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Introduction

Marine environments are critical ecosystems that necessitate thorough evaluation regarding public health implications. Recent studies have focused on the effects of pathogens originating from direct or indirect fecal pollution in aquatic environments [1-4]. *Enterococci*, for instance, can infiltrate individuals engaged in recreational activities in contaminated seawaters through various routes, including dermal contact, natural body orifices, oral ingestion, and the consumption of contaminated fish and shellfish [3, 5]. These bacteria can withstand environmental fluctuations, such as temperature, salinity, and pH variations, demonstrating their adaptability [5-7]. Research has detected *Enterococci* in various marine products, including fish and shellfish, underscoring the potential risk of transmission to humans.

Water bodies are impacted by diffuse sources of fecal pollution, with the presence of antibiotic-resistant *Enterococci* in coastal environments linked to human waste. The discharge of antimicrobial agents into the environment, whether from agricultural, industrial, or medical sources, can accelerate the propagation of antibiotic-resistant *Enterococci* in water, soil, food, and wastewater. This issue arises from the overuse and misuse of antibiotics in human and veterinary medicine, agriculture, and aquaculture, as well as inadequate disposal practices [3, 5, 8-13]. This problem is particularly prevalent in *Enterococcus faecalis* and *Enterococcus faecium*, species responsible for human infections. *Enterococcus* spp. has garnered significant attention due to its ability to acquire multiple antibiotic resistance factors. This acquisition can occur through various mechanisms, including the transfer of plasmids and transposons, chromosomal exchange, or mutations [3-5, 7, 8, 11-14].

Enterococci exhibit not only antibiotic resistance but also produce virulence factors that enhance their pathogenic potential [7, 12, 15]. Their ability to cause disease is enhanced by these virulence factors, alongside their inherent resistance mechanisms. These bacteria possess both chromosomal and plasmid-encoded determinants, which enable adherence to host tissues, biofilm formation, and evasion of host immune responses [7, 9, 16]. Key virulence factors include aggregation substances, extracellular surface proteins, collagen-binding adhesins, as well as enzymes like gelatinase and hyaluronidase. Additionally, *Enterococci* are capable of producing hemolysins and possess sex pheromones that facilitate conjugation and gene transfer [6, 7, 14, 15, 17-19].

The rise in enterococcal infections and their multiple antibiotic resistance characteristics necessitate a thorough investigation into the mechanisms influencing these infections. In this study, we conducted an extensive analysis of virulence factors in 66 *Enterococcus* strains previously isolated from the Marmara Sea and Black Sea regions (Kilyos, Rumelikavağı, Yeşilyurt, and Üsküdar). A comprehensive set of phenotypic and genotypic analyses was used to evaluate these strains. Specifically, we examined genes encoding virulence factors such as those responsible for binding to extracellular matrix proteins (*ace*), gelatinase activity (*gelE*), aggregation factor (*agg*), adhesion to host tissues and biofilm formation (*esp*), regulation of gelatinase and serine protease expression (*fsr*), production of cytolysin (*cylM*, *cylB*, *cylA*), and the presence of enterococcal adhesion protein (*efaAfs*, *efaAfm*). We also analyzed genes for sex pheromones (*cpd*, *cob*, *ccf*) in *Enterococci*, alongside genes associated with antibiotic resistance, including those for vancomycin (*vanA*, *vanB*), streptomycin (*ant(6)-la*), gentamicin (*acc(6')-aph(2'')*), kanamycin (*aphA-3*), rifampicin (*rpoB1*, *rpoB2*), ampicillin (*bla(Z)*), erythromycin (*ermB*), chloramphenicol (*catpIP*), and tetracycline (*tetM*, *tetL*). Current literature suggests that these virulence and resistance genes have a significant impact on the pathogenesis of enterococcal infections, posing a substantial risk to human health [3-5, 7-15]. This research aims to contribute valuable data for epidemiological studies and enhance our understanding of the infectious potential of environmental *Enterococcus* isolates, which are frequently underestimated regarding their capacity to cause epidemics.

Experimental

Bacteria

In this study, 66 strains of *Enterococcus*, previously isolated from seawater samples collected from the Marmara Sea and the Black Sea (specifically from Kilyos, Rumelikavağı, Yeşilyurt, and Üsküdar) [5], were analyzed. These areas are significant for tourism, swimming, and fishing [5]. The strains were first resuscitated from stock cultures stored at -86ºC and then reconfirmed using the API®20 Strep test kit (Biomerieux, France). *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection, USA) served as a positive control for examining the phenotypic and genotypic virulence factors of *Enterococci* isolated from seawater.

Investigation of Antibiotic Susceptibility and Virulence Factors of *Enterococci* Strains Phenotypically

Antibiotic Susceptibility

The antibiotic susceptibility and resistance profiles of *Enterococci* were examined using the Kirby-Bauer disk diffusion method, following the standards established by the Clinical and Laboratory Standards Institute (CLSI) [20]. The study utilized several antibiotics, including nalidixic acid (NA 30), Streptomycin (S 10), Penicillin (P 10), Chloramphenicol (C 30), Vancomycin (VA 30), Tetracycline (TE 30), Ciprofloxacin (CIP 5),

Kanamycin (K 30), Amikacin (AM 30), Ampicillin (AM 10), Erythromycin (E 15), Gentamicin (CN 10), and Rifampicin (RIF 5).

Hemolytic Activity

Columbia Agar (Merck, Germany) medium, supplemented with 5% sheep blood, was utilized to evaluate the hemolytic activity of *Enterococci* strains. This activity was assessed by observing clear zones around the bacterial colonies following 24 to 48 h of incubation at 37ºC. As positive controls, *Streptococcus pneumoniae* ATCC 6301 was used for α-hemolytic activity, *Staphylococcus aureus* ATCC 25923 for β-hemolytic activity, and *Escherichia coli* ATCC 25922 for γ-hemolytic activity [8, 19, 21].

Gelatinase Activity

To evaluate gelatinase activity, we modified the method originally developed by Dahlén et al. [22]. We used a Brain Heart Infusion Broth (Merck, Germany) medium containing 4% gelatin. After incubating the samples for 24 h at 37ºC, we examined the tubes for liquefaction. The tubes were then stored at +4ºC for 30 min. Ongoing liquefaction in the medium indicated a positive presence of gelatinase activity.

Caseinase Activity

Mueller-Hinton Agar (Oxoid, UK) medium with 3% skim milk powder was used to assess caseinase activity. This activity was determined by observing the transparent zones around colonies on the medium after 24 h of incubation at 37ºC. *Bacillus subtilis* served as the positive control, while *Escherichia coli* was used as the negative control [8].

Biofilm Formation

To assess biofilm formation activity, we used a flat-bottomed, sterile polystyrene 96-well microplate. The results were quantitatively evaluated using spectrophotometry following staining with 1% crystal violet for 15 min [23]. *E. faecalis* ATCC 29212 served as the positive control. The evaluation criteria for the biofilm formation activity of the *Enterococci* strains were based on those defined by Stepanovic et al. [24].

Serum Sensitivity

Serum sensitivity was investigated using the microplate method. *Enterococci* were prepared at a concentration of (10^5) bacteria per mL and mixed with human serum in equal volumes in microplate wells. Samples were inoculated into Nutrient Agar (Oxoid, UK) medium at specified time intervals (0, 60, 120, and 180 min) and incubated at 37ºC for 24 h. Serum sensitivity of the *Enterococci* was evaluated according to Benge's criteria [25]. *E. faecalis* ATCC 29212 served as the positive control.

Hemagglutination Activity

To assess hemagglutination activity, we prepared a 1:1 mixture of 3% human erythrocytes and a trypsin-treated solution. The results were evaluated as follows: strong hemagglutination (++++) was indicated by precipitation within 2 min, medium strong (+++) between 2 and 10 min, weak (++) after 10 min, and very weak (+) when observed at 4x magnification after 10 min [26].

Antibacterial Activity

The Agar-Sandwich method, originally developed by Mary-Harting et al. [27], was modified for assessing antibacterial activity. *Enterococcus* strains were prepared following the McFarland No. 1 turbidity standard, along with indicator bacteria. The standard bacteria, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, served as indicators in this experiment. *Enterococci* were inoculated at three locations on M17 Agar plates. Tubes of BHI Broth containing 0.8% agar and a 1% concentration of indicator bacteria were then poured onto the M17 Agar plates with the *Enterococcus* bacteria. All Petri dishes were incubated at 37ºC for 24 h. Antibacterial activity was considered positive if the strains formed a zone with a diameter greater than 2 mm [28].

Investigation of Virulence Factors of *Enterococci* Strains Genotypically

Chromosomal and Plasmid DNA Isolation

Chromosomal DNA from *Enterococci* strains was isolated using the IDPURETM Universal Spin Column Genomic DNA Mini Kit (IDLabsTM, Canada), while plasmid DNA was isolated using the SpinKlean Plasmid DNA Miniprep Kit (Biomatik, Canada), all following the manufacturer's instructions. Chromosomal and plasmid DNA bands were visualized via electrophoresis on a 1% agarose gel at 110 V for 60 min, using a UV transilluminator (Kodak GL 1500). *E. faecalis* ATCC 29212 was used as a positive control.

Plasmid Profile Sizes

To visualize the migration of λ DNA used as a marker on a horizontal agarose gel, we utilized a UV transilluminator. We plotted curves adhering to the logarithm of their standard sizes. By calculating the correlation coefficient and the slope of the curve for each sample, we determined the sizes of plasmid DNA isolated from *Enterococci* strains [6].

Investigation of Antibiotic Resistance Genes and Virulence Genes

Table 1 and Table 2 display the primers and PCR conditions employed to investigate the antibiotic resistance and virulence genes of *Enterococcus* strains isolated from seawater using the PCR method. In order to detect these genes in *Enterococci* bacteria, the PCR mixture included 25 µL of Taq PCR Master Mix (2x, Biomatik), 5 µL of template DNA, 1 µL each of forward and reverse primers, and 18 µL of sterile distilled water. This mixture was processed in a Thermal Cycler (Biorad T100). PCR-amplified DNA fragments were then visualized using electrophoresis in 1% agarose gel, conducted at 110 V for 60 min, and analyzed using a UV transilluminator (Kodak GL 1500).

Table 1. Primers and PCR conditions used to detect antibiotic resistance genes in enterococci strains.

Gene	Antibiotic	Amplicon Size (bp)	"Primer Sequence (5'–3')	PCR Conditions	Cycles	Reference
vanA, *vanB*	Vancomycin	732 635	**(F)** 5' GGGAAAACGACAATTGC 3' **(R)** 3' GTACAATGCGGCCGTTA 5' **(F)** 5' ATGGGAAGCCGATAGTC 3' **(R)** 3' GATTTCGTTCCTCGACC 5'	94ºC for 5 minutes 94ºC for a minute Tm ºC (54) for a minute 72ºC for a minute 72ºC for 10 minutes	30	[29, 30]
ant(6)-la	Streptomycin	577	**(F)** 5' ACTGGCTTAATCAATTTGGG 3' **(R)** 3' GCCTTTCCGCCACCTCACCG 5'	95ºC for 10 minutes 94ºC for 30 seconds Tm ºC (54) for 30 seconds 72ºC for 30 seconds 72ºC for 10 minutes	30	[29]
acc(6') *aph(2")*	Gentamicin	675	**(F)** 5' CCAAGAGCAATAAGGGCATACC 3' **(R)** 3' ACCCTCAAAAACTGTTGTTGC 5'	94ºC for 5 minutes 94ºC for a minute Tm ºC (58) for a minute 72ºC for 2 minutes 72ºC for 10 minutes	30	[31]
aphA-3	Kanamycin	292	**(F)** 5' GCCGATGTGGATTGCGAAAA 3' **(R)** 3' GCTTGATCCCCAGTAAGTCA 5'	94ºC for 5 minutes 94ºC for 30 seconds Tm ºC (54.5) for 30 seconds 72ºC for 2 minutes 72ºC for 10 minutes	32	[32]
rpoB1, *rpoB2*	Rifampicin	1000 1000	**(F)** 5' GTCCGTTTCGGCTTTAATATA 3' **(R)** 3' AAGAAACGAGCATTCAGCAA 5' **(F)** 5' CGCAAGCGACTCAAGAACAG 3' **(R)** 3' GAGCAAATGTTCCATCTTCA 5'	95ºC for 5 minutes 95ºC for a minute Tm ºC (57) for a minute 72ºC for a minute 72ºC for 10 minutes	35	[33]
bla(Z)	Ampicillin	778	**(F)** 5' TACAACTGTAATATCGGAGGG 3' **(R)** 3' CAATAGGTTCAGATTGGCCC 5'	94ºC for 5 minutes 94ºC for a minute Tm ºC (58) for 30 seconds 72ºC for 2 minutes 72ºC for 10 minutes	35	[34]
ermB	Erythromycin	422	**(F)** 5' CATTTAACGACGAAACTGGC 3' **(R)** 3' GGAACATCTGTGGTATGGCG 5'	93ºC for 5 minutes 93ºC for a minute Tm ºC (56) for a minute 72ºC for a minute 72ºC for 10 minutes	35	[35]
catpIP	Chloramphenicol	486	**(F)** 5' GGATATGAAATTTATCCCTC 3' **(R)** 3' CAATCATCTACCCTATGAAT 5'	94ºC for 5 minutes 94ºC a minute Tm ºC (50) for a minute 72ºC for 2.5 minutes 72ºC for 7 minutes	30	[35]
tetM *tetL*	Tetracycline	657 475	**(F)** 5' GTTAAATAGTGTTCTTGGAG 3' **(R)** 3' CTAAGATATGGCTCTAACAA 5' **(F)** 5' CATTTGGTCTTATTGGATCG 3' **(R)** 3' ATTACACTTCCGATTTCGG 5'	94ºC for 5 minutes 94ºC for a minute Tm ºC (53.5) for 30 seconds 72ºC for 2 minutes 72ºC for10 minutes	30	[35]

Table 2. Primers and PCR conditions used to detect virulence genes in enterococci strains.

Gene	Function	Amplicon Size (bp)	Primer Sequence (5'–3')	PCR Conditions	Cycles	Reference
ace	Binding to extracellular matrix proteins	320	**(F)** 5' AAAGTAGAATTAGATCCACAC 3' **(R)** 3' TCTATCACATTCGGTTGCG 5'	94 ºC for 5 minutes 94 ºC for a minute Tm ºC for a minute 72 ºC for a minute 72 ºC for 10 minutes	30	[36]
gelE	Gelatinase	402	**(F)** 5' AGTTCATGTCTATTTTCTTCAC 3' **(R)** 3' CTTCATTATTTACACGTTTG 5'			
efaAfm	Enterococcal adhesion protein	735	**(F)** 5' AACAGATCCGCATGAATA 3' **(R)** 3' CATTTCATCATCTGATAGTA 5'			
efaAfs	*E. faecalis* adhesion protein	705	**(F)** 5' GACAGACCCTCACGAATA 3' **(R)** 3' AGTTCATCATGCTGTAGTA 5'			
agg	Aggregation factor	1553	**(F)** 5' AAGAAAAAGAAGTAGACCAAC 3' **(R)** 3' AAACGGCAAGACAAGTAAATA5'	94 ºC for 5 minutes 94 ºC for2 minutes Tm ºC for 2 minutes 72 ºC for 2 minutes	1	
cylA	Cytolysin	517	**(F)** 5' TGGATGATAGTGATAGGAAGT 3' **(R)** 3' TCTACAGTAAATCTTTCGTCA 5'	92 ºC for 15 seconds Tm ºC for 15 seconds 72 ºC for 15 seconds	30	
cylM		742	**(F)** 5' CTGATGGAAAGAAGATAGTAT 3' **(R)** 3' TGAGTTGGTCTGATTACATTT 5'			
cylB		843	**(F)** 5' ATTCCTACCTATGTTCTGTTA 3' **(R)** 3' AATAAACTCTTCTTTTCCAAC 5'	72 ºC for 10 minutes		[37]
cpd		782	**(F)** 5' TGTTGGGTTATTTTTCAATTC 3' **(R)** 3' TACGGCTCTGGCTTACTA 5'			
ccf	Sex pheromone	543	**(F)** 5' GGGAATTGAGTAGTGAAGAAG 3' **(R)** 3' AGCCGCTAAAATCGGTAAAAT 5'			
cob		1405	**(F)** 5' AACATTCAGCAAACAAAGC 3' **(R)** 3' TTGTCATAAAGAGTGGTCAT 5'			
fsr	Gelatinase and serine protease regulation	3268	**(F)** 5' AACCAGAATCGACCAATGAAT 3' **(R)** 3' GCCCCTCATAACTCAATACC 5'	95 ºC for 5 minutes 94 ºC for 30 seconds Tm ºC (55) for 30 seconds 72 ºC for 2.5 minutes 72 ºC for 10 minutes	30	[38]

bp: Base pairs, **(F):** Forward primer, **(R):** Reverse primer. **Tm:** Binding temperature, **Tm(*ace*):** 53 ºC; **Tm(*gelE*):** 50 ºC; **Tm(*efaAfm*):** 49 ºC; **Tm(*efaAfs*), Tm(*cob*), Tm(*cylB*):** 50 ºC; **Tm(*agg*), Tm(*cylA*), Tm(*cylM*), Tm(*cpd*):** 52 ºC; **Tm(*ccf*):**54 ºC

Statistical Analysis

The data analysis was conducted using the SPSS 22.0 statistical program. The normal distribution of the two independent groups of variables was assessed with the Kolmogorov-Smirnov test. For variables that did not exhibit a normal distribution, the Mann-Whitney U test was applied. Comparisons of phenotypic and genotypic virulence factors were performed using the chi-squared test and Fisher's exact test. The data analysis employed a 95% confidence interval, with a p-value of less than 0.05 indicating a statistically significant difference.

Results

Bacteria

In this study, 66 *Enterococcus* bacteria isolated from seawater in a prior investigation [5] were identified using the API®20 Strep test kit (Biomerieux, France). Among these, 46 strains (69.7%) were identified as *E. faecalis*, 19 (28.79%) as *E. faecium*, and one (1.51%) as *E. gallinarum*.

Investigation of Antibiotic Susceptibility and Virulence Factors of *Enterococci* Strains Phenotypically

Antibiotic Susceptibility

Following analysis, all strains demonstrated resistance to NA, amikacin (AK), and rifampicin (RIF) antibiotics, while remaining susceptible to streptomycin. This indicates a differential response to various antibiotic classes (Table 3). Examination of the multiple antibiotic resistance profiles revealed that three (6.5%) *E. faecalis* strains were resistant to at least five different antibiotics (NA, K, AK, CN, RIF). Additionally, two (10.5%) *E. faecalis* and two (10.5%) *E. faecium* strains showed resistance to up to 11 different antibiotics, including NA, P, C, VA, TE, K, AK, AM, E, CN,

and RIF. Two (10.5%) other *E. faecium* strains were resistant to NA, P, VA, TE, CIP, K, AK, AM, E, CN, and RIF, while one (5.3%) strain of *E. faecium* demonstrated resistance to NA, P, C, VA, TE, CIP, K, AK, E, CN, and RIF. Overall, the study identified 37 distinct multi-antibiotic resistance profiles, with *E. faecalis* exhibiting a higher resistance profile compared to *E. faecium*. No significant difference was found between *E. faecalis* and *E. faecium* strains in resistance to chloramphenicol, vancomycin, kanamycin, tetracycline, ampicillin, and gentamicin ($p>0.05$). However, there was a significant difference in resistance to ciprofloxacin, penicillin, and erythromycin ($p<0.05$).

Virulence Factors

The study found that none of the bacterial strains analyzed exhibited α- or γ-hemolysis activity; however, all strains demonstrated β-hemolytic activity. Among the 66 strains examined, 60 (90.9%) showed gelatinase activity, while four (8.7%) *E. faecalis* and two (10.5%) *E. faecium* strains did not produce gelatinase. Caseinase activity was detected in 37 (56%) *Enterococcus* strains, comprising 30 (65.2%) *E. faecalis*, 6 (31.5%) *E. faecium*, and one (100%) *E. gallinarum*.

Biofilm formation, evaluated according to Stepanovic et al. [24], revealed that 27 (40.9%) strains exhibited strong adhesion, including 19 *E. faecalis* and eight *E. faecium*. Additionally, 37 (56.1%) strains were found to have medium-strength adhesion, consisting of 25 *E. faecalis*, eight *E. faecium*, and one *E. gallinarum*. Only two (3%) strains displayed weak adhesion properties.

Furthermore, 31 (46.9%) of the 66 *Enterococcus* strains showed serum resistance, 25 (54.3%) strains were serum-sensitive, and eight (12.1%) demonstrated moderate sensitivity. The rates of serum resistance, moderate sensitivity, and susceptibility were 54.3%, 6.5%, and 39.1% for *E. faecalis*, and 31.7%, 26.3%, and 42.1% for *E. faecium*, respectively. The *E. gallinarum* strain was sensitive to human serum.

Regarding hemagglutination activity, *E. faecalis* strains exhibited 39.1% very strong, 36.9% strong, 10.9% weak, and 3% very weak hemagglutination. *E. faecium* strains showed 0% very strong, 5.3% strong, 47.4% weak, and 0% very weak hemagglutination. The *E. gallinarum* strain displayed no hemagglutination activity.

The study also found that 16 (24.2%) strains, including 15 *E. faecalis* and one *E. faecium*, demonstrated antimicrobial activity against *Staphylococcus aureus* ATCC 25923. Additionally, 11 (16.7%) *E. faecalis* strains showed activity against *Escherichia coli* ATCC 25922. The rates of antimicrobial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were 32.6% and 23.9% for *E. faecalis*, and 5.3% and 0% for *E. faecium*, respectively ($p<0.05$). Notably, 12 strains demonstrated antimicrobial activity against both indicator bacteria, while the *E. gallinarum* strain did not produce antimicrobial agents (Table 3).

All 66 *Enterococcus* strains (100%) analyzed in the study exhibited β-hemolysis activity. The *E. gallinarum* strain showed only β-hemolysis and gelatinase activities among 21 different virulence factors and was resistant to NA, amikacin, ampicillin, erythromycin, gentamicin, and rifampicin antibiotics (Table 3).

Investigation of Virulence Factors of *Enterococci* Strains Genotypically

Plasmid Profile

A total of 16 (24.24%) strains, comprising 13 *E. faecalis* and three *E. faecium*, were found to contain plasmids. The study determined that each strain harbors a maximum of three plasmids and collectively presents plasmids of 26 different molecular sizes. As shown in Table 4, these plasmid sizes range from 340 bp to 32,600 bp. Additionally, two *E. faecalis* strains exhibited an identical plasmid profile with a molecular size of 2,120 bp.

Antibiotic Resistance Genes

The study identified that 66 *Enterococcus* strains isolated from seawater possessed between four and 11 of 12 different antibiotic resistance genes. Specifically, two strains – one *E. faecium* and one *E. gallinarum* – carried at least four antibiotic resistance gene profiles, while one *E. faecalis* strain harbored up to 11 such profiles. Among the 66 *Enterococci* strains, 21 (31.8%) contained the *vanA* gene, 16 (24.2%) carried the *vanB* gene, 64 (96.9%) had the *acc(6')-aph(2'')* gene, 62 (93.9%) contained the *aphA-3* gene, all 66 (100%) had the *rpoB1* gene, 65 (98.5%) had the *rpoB2* gene, 60 (90.9%) had the *bla(Z)* gene, 40 (60.6%) contained the *ermB* gene, 45 (68.2%) had the *catpIP* gene, 41 (62.1%) contained the *tetM* gene, and 27 (40.9%) had the *tetL* gene. Notably, all *E. gallinarum* strains possessed the *rpoB1* and *rpoB2* genes encoding rifampicin resistance, the *ermB* gene encoding erythromycin resistance, and the *tetM* gene encoding tetracycline resistance ($p<0.05$). In contrast, all *E. faecalis* strains carried the *acc(6')-aph(2'')* gene encoding gentamicin resistance and the *rpoB1* gene encoding rifampicin resistance. Additionally, no *Enterococci* strains contained the *ant(6)-la* gene, which encodes streptomycin resistance (Table 5).

Virulence Genes

The study determined that the *E. gallinarum* strain harbored at least three virulence genes: *cylM*, *cylB*, and *cylA*. Two *E. faecalis* strains exhibited up to 12 virulence genes. Among the 66 *Enterococci* strains analyzed, 40 strains (60.6%) carried the *ace* gene, which facilitates binding to extracellular matrix

Table 3. Frequency of phenotypic distribution of antibiotic resistance and virulence factors in enterococci strain.

Antibiotic resistance and virulence factors		Frequency (%)			
		E. faecalis n (%)	*E. faecium* n (%)	*E. gallinarum* n (%)	Total n (%)
Virulence factors	H_{β}	46 (100)	19 (100)	1 (100)	66 (100)
	G	42 (91.3)	17 (89.5)	1 (100)	60 (90.9)
	C	30 (65.2)	6 (31.5)	1 (100)	37 (56)
	B_S	19 (41.3)	8 (42.1)	0 (0)	27 (40.9)
	S.R.	25 (54.3)	6 (31.6)	0 (0)	31 (46.9)
	H_S	18 (39.1)	0 (0)	0 (0)	18 (27.3)
	A_A	15 (32.6)	1 (5.3)	0 (0)	16 (24.2)
	A_B	11 (23.9)	0 (0)	0 (0)	11 (16.7)
Antibiotic resistance	NA	46 (100)	19 (100)	1 (100)	66 (100)
	S	0 (0)	0 (0)	0 (0)	0 (0)
	P	28 (60.8)	16 (84.2)	0 (0)	44 (66.7)
	C	5 (10.86)	6 (31.57)	0 (0)	11 (16.7)
	VA	19 (41.3)	11 (57.9)	0 (0)	30 (45.4)
	TE	24 (52.7)	12 (63.1)	0 (0)	36 (54.5)
	CIP	8 (17.4)	7 (36.8)	0 (0)	15 (22.7)
	K	45 (97.8)	18 (94.7)	0 (0)	63 (95.4)
	AK	46 (100)	19 (100)	1 (100)	66 (100)
	AM	18 (39.1)	11 (57.9)	1 (100)	30 (45.4)
	E	23 (50)	16 (84.2)	1 (100)	40 (60.6)
	CN	46 (100)	18 (94.7)	1 (100)	65 (98.5)
	RIF	46 (100)	19 (100)	1 (100)	66 (100)

H_{β}: β-hemolysis, G: Gelatinase, C: Caseinase, B_S: Strong biofilm activity, S.R.: Serum resistance, H_S: Very strong hemagglutination activity, A_A: Antimicrobial activity against *Staphylococcus aureus* ATCC A_B: Antimicrobial activity against *Escherichia coli* ATCC 25922, NA: Nalidixic acid, S: Streptomycin, P: Penicillin, C: Chloramphenicol, VA: Vancomycin, TE: Tetracycline, CIP: Ciprofloxacin, K: Kanamycin, AK: Amikacin, AM: Ampicillin, E: Erythromycin, CN: Gentamicin, RIF: Rifampicin, n: Count of bacteria.

proteins; 59 strains (89.4%) possessed the *gelE* gene encoding the gelatinase enzyme; all 66 strains (100%) contained the *cylM*, *cylB*, and *cylA* genes responsible for cytolysis; 50 strains (75.7%) had the *agg* gene encoding the aggregation factor; and 40 strains (60.6%) exhibited the *fsr* gene, which regulates the expression of gelatinase and serine protease. Additionally, 44 strains (66.7%) and 39 strains (59.1%) contained the *efaAfs* and *efaAfm* genes, respectively, responsible for enterococcal surface proteins. The presence of pheromone-encoding genes *cpd*, *ccf*, and *cob* was noted in 24 strains (36.4%), 26 strains (39.4%), and 38 strains (57.6%), respectively.

For the *E. faecalis* and *E. faecium* strains, the prevalence of virulence genes was as follows: *ace* was found in 86.9% of *E. faecalis* and 0% of *E. faecium* ($p<0.05$), *gelE* in 91.3% and 89.4% ($p>0.05$), *efaAfs* in 95.6% and 0% ($p<0.05$), *efaAfm* in 58.7% and 63.1% ($p>0.05$), *cpd* in 41.3% and 26.3% ($p>0.05$), *ccf* in 30.4% and 63.1% ($p<0.05$), *cob* in 56.5% and 63.1% ($p>0.05$), *agg* in 84.7% and 57.9% ($p<0.05$), and the *fsr* gene in 60.8% and 63.1% ($p>0.05$).

As detailed in Table 5, the *E. gallinarum* strain used in this study possessed the *cylM*, *cylB*, and *cylA* genes among its 12 identified virulence factors. Notably, 2 (3.03%) *E. faecalis* strains exhibited the most extensive virulence profile with 12 gene regions, whereas 1 (1.51%) *E. gallinarum* strain demonstrated the least virulence, marked by the presence of three gene regions (*cylM*, *cylB*, and *cylA*).

Discussion

Enterococcus species are significant due to their prevalence in natural environments and their capacity to cause diseases. In marine environments, these bacteria are particularly concerning because

Table 4. Plasmid profile sizes.

Sample	Molecular Size (bp)
λ DNA Marker	23130, 9416, 6557, 4361, 2322, 2027, 564, 125
Bacteria No.	Plasmid Profile Sizes (bp)
AE5	2160
AE16	3210 3180
AE18	9560 8570 7240
AE20	2140 2020 340
AE21	6460 4080 3260
AE23	2120
AE24	2120
AE25	1580 1560
AE28	1920
AE31	752
AE36	2360
AE38	3960 3920
AE43	6400
AE50	32600
AE54	9320 7560 4020
AE63	1640
SB	6520 4280 4090

SB: Standard bacteria.

they exhibit high antibiotic resistance and possess virulence factors, posing a potential hazard to public health [10].

Numerous studies on *Enterococcus* species identification have shown that *E. faecalis* predominates, largely due to its abundance in the microbiota of humans and animals [39, 40]. In the present study, 46 strains (69.7%) were identified as *E. faecalis*, 19 (28.79%) as *E. faecium*, and one (1.51%) as *E. gallinarum*. This finding aligns with the results reported by Ben Said et al. [9], who also found a predominance of *E. faecalis* and *E. faecium* in seafood samples. Furthermore, Santiago et al. [4] identified these species as major enterococcal contaminants in the coastal waters of Brazil, underscoring their widespread presence in marine environments.

Matyar and Dincer [40] reported antibiotic resistance rates among *E. faecalis* and *E. faecium* isolates as follows: erythromycin (32.9% and 64.9%), ampicillin (83.6% and 92.2%), and tetracycline (54.8% and 68.8%), respectively. Our study observed a similar trend, with resistance rates for erythromycin at 50% and 84.2%, for ampicillin at 39.13% and 57.9%, and for tetracycline at 52.7% and 63.15%. Senturk [41], however, identified even higher resistance levels, including 100% resistance to NA and rifampicin, indicating an alarming rise in antibiotic resistance. These findings are consistent with those of Çardak et al. [3] and Karaman Baş et al. [42], who also documented high resistance levels in *Enterococcus* strains from seafood and coastal waters, respectively. Santiago et al. [4] further demonstrated that out of 130 *Enterococcus* isolates, 118 exhibited resistance to multiple antibiotics, underscoring the role of marine environments as reservoirs for antibiotic-resistant bacteria.

The present study found that the rates of antibiotic resistance genes in *E. faecalis* and *E. faecium* were 56.5% and 21.2% for the *tetM* gene, 43.5% and 36.8% for the *tetL* gene, 32.6% and 31.6% for the *vanA* gene, and 21.7% and 31.6% for the *vanB* gene, respectively. Additionally, it was determined that 96.96% of the strains, comprising 100% of *E. faecalis* and 94.73% of *E. faecium*, carried the *acc(6')-aph(2'')* gene. Similarly, 93.9% of the strains, including 97.8% of *E. faecalis* and 89.5% of *E. faecium*, possessed the *aphA3* gene. The study results indicate a high prevalence of the high-level aminoglycoside resistance genes *aac(6')-aph(2'')* and *aphA3*. Previous studies have shown that these genes cause high-level aminoglycoside and multi-antibiotic resistance [6, 31, 35, 40, 43, 44].

Both *rpoB1* and *rpoB2* genes are linked to rifampicin resistance. Our findings revealed that all tested strains exhibited rifampicin resistance and possessed the *rpoB1* gene, with only one strain lacking the *rpoB2* gene. This scenario may be due to 'silent genes' in the absence of mobile genes. Factors contributing to the 'silent' state of genes include mutations, normal gene regulation anomalies, laboratory condition simplifications, or errors in bacterial processing [45].

Furthermore, we observed that 49.1% of the samples in the study showed resistance to at least three antibiotics. It was documented that 57% of *E. faecalis* and 46% of *E. faecium* exhibited multi-antibiotic resistance, with vancomycin resistance potentially associated with this multi-resistance. Both species are significant reservoirs of acquired glycopeptide and multidrug resistance, often carrying the most reported van genes. The expression of vancomycin-related resistance and virulence factors is characteristic of vancomycin-resistant Enterococcus phenotypes. The *vanA* phenotype is notably more virulent [46]. Globally, the *vanA* genotype is the most prevalent among human and animal isolates of *E. faecium* and *E. faecalis*, as confirmed in this study, though it is less common in other enterococcal species [47, 48].

This study also determined that *Enterococcus* strains with a plasmid profile were identical to vancomycin-resistant strains, suggesting plasmid-mediated gene transfer for vancomycin resistance [47, 48]. Moreover, *E. faecalis*, *E. faecium*, and *E. gallinarum* strains resistant to vancomycin also developed resistance to between six and 11 out of 13 different antibiotics. In this study, 98.5% of the strains were resistant to gentamicin and 95.4% to kanamycin, raising significant concerns

Table 5. Frequency of antibiotic resistance and virulence factors genes in enterococci strains.

Antibiotic resistance and virulence factors		Gene	Frequency (%)			
			E. faecalis n (%)	*E. faecium* n (%)	*E. gallinarum* n (%)	Total n (%)
Antibiotic resistance	Vancomycin	*vanA*	15 (32.6)	6 (31.6)	0 (0)	21 (31.8)
		vanB	10 (21.7)	6 (31.6)	0 (0)	16 (24.2)
	Streptomycin	*ant(6)-la*	0 (0)	0 (0)	0 (0)	0 (0)
	Gentamycin	*acc(6')-aph(2")*	46 (100)	18 (94.7)	0 (0)	64 (96.9)
	Kanamycin	*aphA-3*	45 (97.8)	17 (89.5)	0 (0)	62 (93.9)
	Rifampicin	*rpoB1*	46 (100)	19 (100)	1 (100)	66 (100)
		rpoB2	45 (97.8)	19 (100)	1 (100)	65 (98.5)
	Ampicillin	*bla(Z)*	43 (93.5)	17 (89.5)	0 (0)	60 (90.9)
	Erythromycin	*ermB*	23 (50)	16 (84.2)	1 (100)	40 (60.6)
	Chloramphenicol	*catpIP*	33 (71.7)	12 (63.1)	0 (0)	45 (68.2)
	Tetracycline	*tetM*	26 (56.5)	14 (21.2)	1 (100)	41 (62.1)
		tetL	20 (43.5)	7 (36.8)	0 (0)	27 (40.9)
Virulence factors	Binding to extracellular matrix proteins	*ace*	40 (86.9)	0 (0)	0 (0)	40 (60.6)
	Gelatinase	*gelE*	42 (91.3)	17 (89.4)	0 (0)	59 (89.4)
	Cytolysin	*cylM*	46 (100)	19 (100)	1 (100)	66 (100)
		cylB	46 (100)	19 (100)	1 (100)	66 (100)
		cylA	46 (100)	19 (100)	1 (100)	66 (100)
	Adhesion	*efaAfs*	44 (95.6)	0 (0)	0 (0)	44 (66.7)
		efaAfm	27 (58.7)	12 (63.1)	0 (0)	39 (59.1)
	Sex pheromones	*cpd*	19 (41.3)	5 (26.3)	0 (0)	24 (36.4)
		ccf	14 (30.4)	12 (63.1)	0 (0)	26 (39.4)
		cob	26 (56.5)	12 (63.1)	0 (0)	38 (57.6)
	Aggregation factor	*agg*	39 (84.7)	11 (57.9)	0 (0)	50 (75.7)
	Gelatinase and serine protease expression	*fsr*	28 (60.8)	12 (63.1)	0 (0)	40 (60.6)

n: Count of bacteria.

for treating severe enterococcal infections, as these antibiotics are now ineffective against these pathogens. The escalation of high-level aminoglycoside resistance emphasizes the necessity of developing new treatment strategies and investigating alternative antibiotics [49]. Interestingly, despite high resistance levels to gentamicin and kanamycin, no phenotypic or genotypic resistance to streptomycin was detected, an unexpected result of our study.

These findings align with prior research suggesting that *Enterococci* strains carrying these genes exhibit extensive antibiotic resistance profiles. Studies by Hashem et al. [19] and Molale-Tom and Bezuidenhout [16] highlight the crucial role of resistance genes in clinical and environmental *Enterococcus* isolates. Reports indicate that gene transfer has increased significantly due to the transfer of resistance and virulence genes between *Enterococci* and other Gram-positive bacteria, aided by factors such as peptide hormone secretion during conjugation, presence of sex pheromones, surface adhesion proteins, and the release of aggregation factors [41, 49, 50].

Studies have also identified that bacteria with sex pheromones exhibit β-hemolysis properties [41]. In our research, we found that all strains demonstrated hemolysis activity and exhibited a β-hemolysis reaction. Among these, 16 isolates with hemolytic activity were found to contain plasmids. Similarly, consistent with other studies, our findings revealed that 24 (36.4%) *Enterococci* strains with hemolytic activity possessed

the *cpd* sex pheromone, 26 (39.4%) had *ccf*, and 38 (57.6%) contained *cob*.

Recent studies have underscored the increasing significance of biofilm formation in the virulence of *Enterococci* [7, 50, 51]. Biofilm formation facilitates bacterial colonization, host tissue damage through gelatinase secretion, and enhanced hemolytic activity [7, 50, 52]. In the present study, 28 out of 66 *Enterococcus* isolates showed strong biofilm-forming capacity, with 25 of these also demonstrating gelatinase and hemolytic activity. Igbinosa and Beshiru [7] similarly highlighted the connection between biofilm formation and antimicrobial resistance in *Enterococcus* isolates from ready-to-eat seafood, noting that biofilm-associated bacteria exhibit increased survival and resistance to conventional treatments.

This study phenotypically investigated gelatinase, caseinase, biofilm-forming capacity, serum, and hemagglutination activity in 66 *Enterococci* strains. The findings showed that 42 (91.3%) and 17 (89.5%) strains of *E. faecalis* and *E. faecium*, respectively, possessed these properties. Additionally, 30 (65.2%) and 6 (31.5%) strains demonstrated caseinase activity; 19 (41.3%) and 8 (42.1%) strains exhibited biofilm-forming capacity; 25 (54.3%) and 6 (31.6%) strains showed serum activity; while 18 (39.1%) and 0 (0%) strains showed hemagglutination activity. These results align with previous research indicating that *E. faecalis* strains express higher levels of virulence factors than *E. faecium* [22, 51-53]. Overall, our study supports the conclusion that *E. faecalis* strains have a greater virulence potential compared to *E. faecium* [22, 51-53].

Antibacterial substances produced by *Enterococci* exhibit activity against both some Gram-negative bacteria and specific Gram-positive bacteria, including *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium perfringens* [54]. In the present study, 15 (32.6%) *E. faecalis* and one (5.3%) *E. faecium* strains demonstrated antibacterial activity against *Staphylococcus aureus* ATCC 25923, while 11 (23.9%) *E. faecalis* strains displayed activity against *Escherichia coli* ATCC 25922. These results align with previous research that has documented the inhibitory effects of *Enterococci* in isolates from animal-derived foods [21], seafood [3, 7, 9], wastewater [9], coastal environments [4], and clinical samples [19]. The ability of *Enterococci* to produce bacteriocins and compete with bacterial pathogens underscores their potential applications in food safety and probiotic development. These findings contribute to the expanding body of evidence emphasizing the significance of *Enterococci* in microbial control and public health.

Gelatinase, an enzyme contributing to virulence, cleaves host substrates [51]. Studies have demonstrated that bacteria harboring the *gelE* gene often exhibit resistance to vancomycin and multiple antibiotics [55]. In our study, gelatinase activity was detected in 60 strains, comprising 42 *E. faecalis*, 17 *E. faecium*, and one *E. gallinarum*. Our analysis showed that 59 strains contained the *gelE* gene, including 42 *E. faecalis* strains (91.3%) and 17 *E. faecium* strains (89.4%). Moreover, strains with the *gelE* gene also displayed resistance to vancomycin.

The *esp* and *agg* genes are virulence factors crucial for evading the host immune system and forming biofilms. Studies, including ours, have shown that *Enterococcus* strains possessing the *esp* and *agg* genes also exhibit resistance to vancomycin and multiple other antibiotics. Additionally, these strains contain sex pheromone genes (*cpd*, *ccf*, and *cob*), which are responsible for plasmid-mediated resistance transmission. Recent research has identified the *cyl*, *esp*, and *agg* genes as key elements in producing cytolysis, biofilm formation, and aggregation factors, respectively. These genes are essential for attachment to host tissue and subsequent biofilm formation. Furthermore, the co-existence of these genes has been linked to increased enterococcal virulence, as well as heightened resistance to erythromycin, high-level aminoglycosides, and notably, vancomycin [6, 55, 56].

The *efaAfs* and *efaAfm* genes encode the *Enterococcus* surface antigen A protein, a cell surface adhesion protein crucial for *Enterococci*'s attachment to host tissues. In a study examining the *efaAfm* gene [57], the prevalence of *efaAfs* and *efaAfm* was found to be 95.6% (44 strains) and 58.7% (27 strains) in *E. faecalis*, and 0% (0 strains) and 63.1% (12 strains) in *E. faecium*, respectively. Overall, 66.7% of the strains contained the *efaAfs* gene, while 59.1% contained the *efaAfm* gene.

Consequently, our study determined that the examined strains developed resistance to up to 11 out of 13 antibiotics and possessed a maximum of 12 out of 15 different virulence genes. These results align with other studies that identified high levels of antibiotic resistance and virulence genes in *Enterococcus* strains from seafood and marine ecosystems [3,13,57]. This finding further supports the hypothesis that marine environments act as reservoirs for antibiotic resistance and virulence genes.

In research investigating the plasmid content of *Enterococci* strains, Rosvoll et al. [58] identified between one and seven plasmids in 88 isolates, while Coleri et al. [59] reported one to 11 plasmids with molecular sizes ranging from 2.08 to 56.15 kb. Yuksel [6] found one to seven plasmids with sizes between 35.8 and 2.4 kb in 88 isolates. In the present study, plasmid sizes ranged across 26 different molecular sizes, with a maximum of three plasmids per strain. These plasmids varied from 340 to 32,600 base pairs (bp). Notably, two *E. faecalis* strains were observed to share the same plasmid profile with a molecular size of 2,120 bp. It is hypothesized that plasmids facilitating the transfer of antibiotic resistance and virulence genes significantly contribute to the high prevalence of virulence factors [60].

Conclusions

In conclusion, the rising prevalence of antibiotic-resistant *Enterococcus* strains exhibiting multiple virulence factors presents a substantial threat to public health. The detection of these strains in environmental samples underscores the necessity for ongoing monitoring and the establishment of effective antimicrobial stewardship programs. This study's findings add to the expanding body of knowledge on the epidemiology of *Enterococcus* species and highlight the pressing need for intervention strategies to mitigate their impact on public health.

Acknowledgements

This study was funded by Scientific Research Projects Coordination Unit of Istanbul University. Project number: 25252.

Conflict of Interest

The authors declare no conflict of interest.

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