Research Article

Antibiotic Resistance in *Enterococcus faecalis*: Broad-Spectrum Drug Target Identification Using Subtractive Genomics

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Abstract | Multidrug-resistant *Enterococcus faecalis* (MDR *E. faecalis*) is a growing concern worldwide, with higher resistance levels detected in humans. The increasing prevalence of MDR *E. faecalis* decreased the effective treatment options, making infections more difficult to manage. The aim of this study was to identify potential novel drug targets in MDR *E. faecalis* using a subtractive genomics approach. The *E. faecalis* core proteome was retrieved from the EDGAR 3.5 database. After filtering the paralogous sequences using Cluster Database at High Identity with Tolerance (CD-HIT), the Database of Essential Genes (DEG) was used to identify *E. faecalis's* essential proteins. DEG came up with 10 essential proteins. The Virulence Factor Database (VFDB) detected sixteen virulence factors in the core proteome. The core genome analysis showed that 4 genes of *E. faecalis* conferred antibiotic resistance *via* biofilm formation, antiphagocytosis, adherence to surfaces, and production of several enzymes such as gelatinase, hyaluronidase, and glutamyl endopeptidase. The NCBI BLAST results showed that no sequence from *E. faecalis* was homologous to the human proteome, gut microbiota, or anti-target proteins. The obtained results of the NCBI BLASTn analysis of the Microbial Nucleotide Database revealed that fourteen bacterial strains can be effectively targeted by a single broad-spectrum antibiotic aimed at the essential proteins of *E. faecalis*. The druggability results showed that 4 *E. faecalis* essential proteins had homologs in Drugbank and Therapeutic Target (TT) Databases. Three of these drug targets were enzymes, including fumarate reductase flavoprotein subunit, bacterial ribosomal ATPase RbbA (Bact rbbA), and ribosomal small subunit pseudouridine synthase A, while a single enzyme was acting against a multidrug resistance protein 1. This study, for the first time, elucidated novel drug targets against the MDR *E. faecalis*. Among them are enzymes and transporters indispensable to the MDR *E. faecalis*.

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Introduction

Antimicrobial resistance poses a significant threat to the public health, particularly with the rise of multidrug-resistant (MDR) microorganisms. Among these, *Enterococcus* species have emerged as a leading cause of nosocomial infections, garnering considerable attention. These bacteria have shown an

exceptional ability to acquire and intrinsically develop resistance genes (Wu *et al*., 2025). They can lead to severe health issues such as endocarditis, urinary tract infections (UTIs), wound infections, bacteremia, and abdominal infections. The majority of persistent *Enterococcus* infections are associated with biofilm formation on medical devices, including catheters, pacemakers, prosthetic devices, and heart valves. This is mainly attributed to the high transmission rates and antimicrobial resistance to a broad spectrum of antibiotics (Boccella *et al*., 2021; Wu *et al*., 2025). *E. faecalis* is particularly prevalent in clinical samples among *Enterococcus* spp. (Hota *et al*., 2024). Currently, the Food and Drug Administration (FDA) approved several antibiotic therapies for vancomycin-resistant *E. faecalis* (VRE), mainly quinupristin-alfopristin and linezolid, having notable limitations upon treating serious *E. faecalis* infections (Cairns *et al*., 2023). These limitations are due to their intrinsic bacteriostatic properties, adverse toxicity profiles, complications with administration, and the potential for developing further resistance (Cairns *et al*., 2023; Hota *et al*., 2025).

The rise of resistance in *E. faecalis*, particularly against glycopeptides and β-lactams, presents significant challenges in treating the infections caused by these Gram-positive bacteria (Gagetti *et al*., 2019; Sarathy *et al*., 2020). Glycopeptides inhibit bacterial cell wall peptidoglycan polymerization by binding to the D-Ala-D-Ala terminal of the peptidoglycan precursors. Meanwhile, the β-lactams act as suicide substrates for penicillin-binding proteins (PBPs), essential for cell wall synthesis. *E. faecalis* possesses at least 6 putative PBP genes, with *pbp5* being linked to the intrinsic tolerance against β-lactams, due to its low binding affinity for ampicillin and cephalosporins. Resistance to ampicillin has been associated with mutations in *pbp5*, particularly substitutions near the active site, which reduce β-lactam binding (Hunashal *et al*., 2023). Moreover, the presence of a β-lactamase gene (*blaZ*) has also been identified in *E. faecalis* and *E. faecium*, allowing for inactivation of the β-lactam antibiotics through cleavage of the β-lactam ring (Geraldes *et al*., 2022).

Enterococci are recognized for containing numerous mobile genetic elements (MGEs), such as plasmids, insertion sequences, transposons, integrons, and pathogenicity islands. Many of these genetic elements carry genes that play a role in virulence and antimicrobial resistance (Schaffer *et al*., 2023).

Daptomycin (DAP) resistance in *E. faecalis* is associated with mutations of the envelope stress response (CESR) and cell membrane phospholipid metabolism genes. Genes that regulate CESR (*liaFSR*) (Prater *et al*., 2021) and phospholipid biosynthesis (*cls*, cardiolipin synthesis) are often mutated and implicated in DAP resistance (Nguyen *et al*., 2023). *E. faecalis* vancomycin resistance is based on the presence of van operon variants such as the *VanA* and *VanB* operons. Both operons encode a ligase that modifies the vancomycin binding site, essential for the antibiotic's effectiveness (Kardos *et al*., 2024).

Several previous studies have shown that resistance to aminoglycosides is due to covalent modifications of the aminoglycosides by the enterococcal cytoplasmic enzymes and poor transport of antibiotics across the enterococcal cell membrane (Chatterjee *et al*., 2024; Galgano *et al*., 2025). These covalent modifications reduce binding of the aminoglycosides to their intended intracellular targets *via* steric hindrance. In *E. faecalis*, the enzyme 6′-acetyltransferase (AAC(6′)-Ii) is chromosomally encoded and is responsible for modifying several aminoglycosides, including tobramycin, sisomicin, kanamycin, and netilmicin (Miller *et al*., 2014).

Linezolid is a bacteriostatic antibiotic effective against Gram-positive bacteria, and acts by binding to the 23S rRNA component of the ribosome. This binding interferes with docking of the aminoacyl-tRNA in the ribosomal A site, inhibiting both the peptide delivery and elongation of the polypeptide chain (Krawczyk *et al*., 2024). The *cfr* gene is associated with the mobile element IS256, which plays a crucial role in the horizontal transfer of antibiotic resistance genes and may influence the expression of the resistance determinants (Torabi *et al*., 2023).

Enterococcus faecalis; a nosocomial bacterial pathogen, has developed various resistance mechanisms to combat the antibiotic therapy, making it a significant concern in healthcare settings. The main resistance mechanisms of *E. faecalis* include: Active efflux of antibiotics out of the cell, alterations in cell wall structure, reducing antibiotic binding, production of enzymes that inactivate antibiotics, changes in antibiotic target sites, and reducing the binding affinity (Hollenbeck and Rice, 2012). Furthermore, transposons located in the

plasmids confer resistance to *E. faecalis*. Therefore, elucidating a broad-spectrum novel drug target identification using subtractive genomics will enable the development of effective therapies against the MDR *E. faecalis*. The main objective of this study was to identify potential novel drug targets in *E. faecalis* using a subtractive genomic approach to overcome MDR, improving the patient's outcomes.

Materials and Methods

Retrieval of the core proteome from the EDGAR 3.5 database

The core proteome of *E. faecalis* strains was retrieved from the EDGAR Version 3.5 database (EDGAR, https://edgar3.computational.bio). The reference strain, *E. faecalis* OG1RF (Accession number: CP 002621.1) and the core proteome that showed hits against the reference strain were further investigated to predict new potential drug targets for MDR *E. faecalis*. These hits were based on the BLASTp sequence analysis. Overall, in all analyses, E-values below $1e^{-5}$ were deemed significant; a threshold of $1e^{-5}$ or less was applied (such as $1e^{-10}$ or $1e^{-50}$) for more reliable results (Choudhuri, 2014). A schematic workflow showing the process of discovering the essential druggable proteins in *E. faecalis* is depicted in Figure 1.

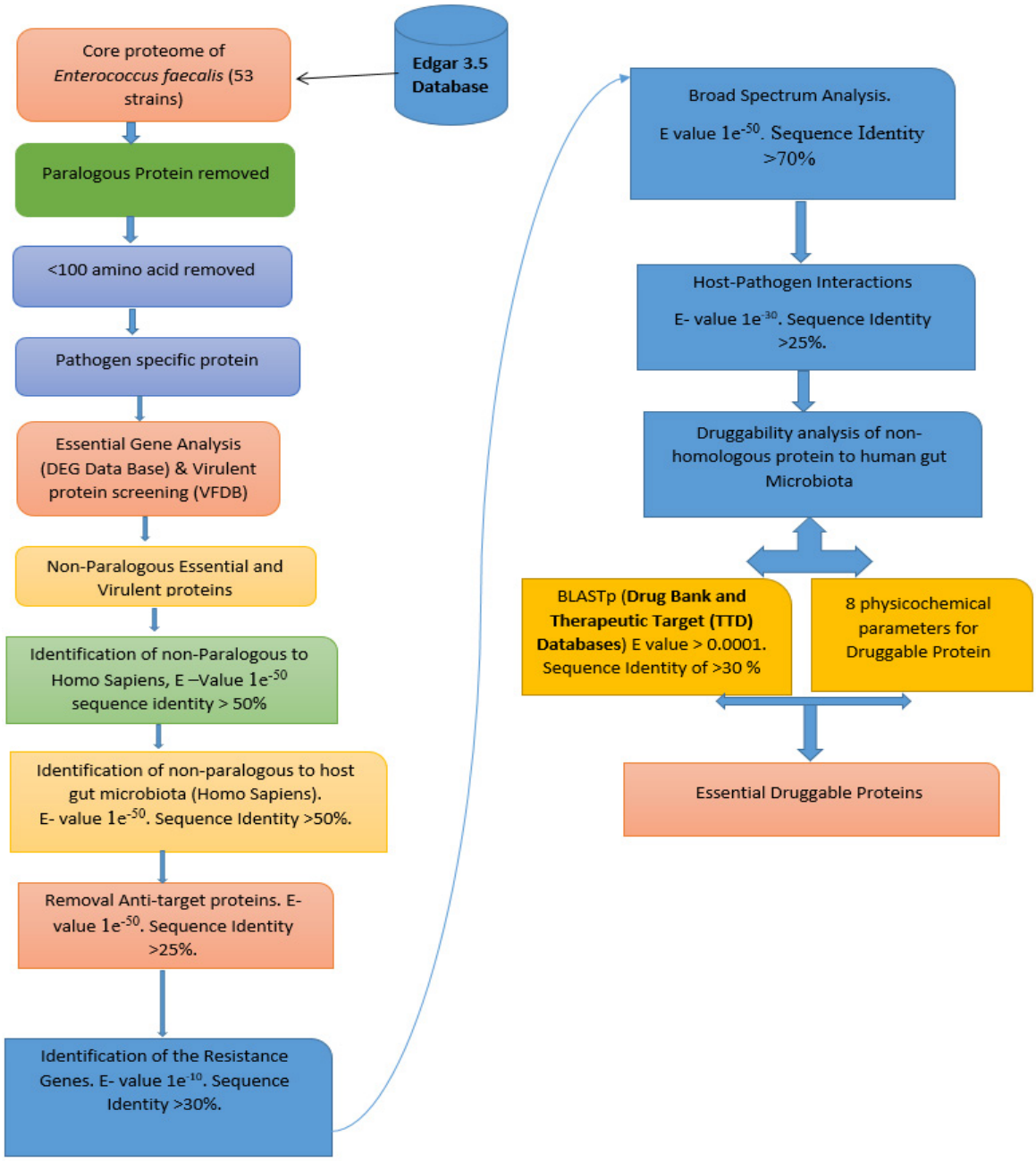

Figure 1: *A schematic workflow showing the process of discovering the essential druggable proteins in Enterococcus faecalis. Where; DEG (Database of Essential Genes) and VFDB (The Virulence Factor Database).*

Identification of the non-paralogous protein sequences

The cluster database at high identity with tolerance (CD-HIT) tool filtered the paralogous sequences of the *E. faecalis* core proteome (Wei *et al.*, 2023). Non-paralogous protein sequences were identified using bio-tools such as cd-hit version 4.8.1 (Li and Godzik, 2006; Fu *et al.*, 2012; The Galaxy, 2024). The algorithm parameters were set to a sequence identity threshold of 60 %, an alignment band width of twenty amino acids, a tolerance for redundancy was set at 5, and exclusion of the sequences of <100 amino acids in length, using a global sequence identity. Sequences longer than 100 amino acids are typically regarded as essential for bacteria. However, sequences that are more than 60 % identical tend to be paralogous, thus, they were excluded (Omeershffudin and Kumar, 2023).

Identification of the essential proteins

The microorganism's essential proteins are indispensable for their survival and are naturally represent good drug targets. The Database of Essential Genes (DEG) Version 15.2 (www.essentialgene.org) includes an extensive list of all microorganisms essential genes and proteins (Luo *et al.*, 2021) and was used to identify the *E. faecalis's* essential proteins. The non-paralogous proteins were subjected to BLASTp against the bacterial essential genes deposited in DEG. The cut-off for e-value of $1e^{-50}$ and a bit score of >100 was used.

Identification of the proteins containing virulence factors

Enterococcus faecalis establishes itself and causes diseases in the human hosts with the assistance of various virulence factors (Too and Masila, 2024), which are the major determinants of pathogenicity. The Virulence Factor Database (VFDB) Version 1 (https://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) was used to identify the *E. faecalis* virulence proteins (Zhou *et al.*, 2025). The database contains 5 major virulent proteins involved in adherence, antiphagocytosis, biofilm formation, enzymes, and toxins. The core proteome was subjected to BLASTp against the core dataset of VFDB. The E-value was $1e^{-50}$ with an alignment cutoff bit score of >100.

Identification of the protein non-homologous to the human proteome

The essential proteins of *E. faecalis* were subjected to BLASTp of human proteins to identify the non-homologous proteins (*Homo sapiens* taxid: 9606) (Altschul *et al.*, 1997). Identification of the non-homologous proteins ensures that proteins important to the host are not targeted by the drugs. The E-value was $1e^{-50,}$ and the sequence identity to >50 % (Sharma and Kumar, 2016).

Identification of the genes non-homologous to human gut microbiota

To exclude genes that shared high sequence similarity to human gut microbiota (NCBI txid: 408170) (Jovel *et al.*, 2016), the essential genes of *E. faecalis* were subjected to BLASTn against the human gut metagenome 16S ribosomal RNA database (human gut metagenome NCBI txid: 408170) (Schoch *et al.*, 2020). The E-value was $1e^{-50,}$ and the sequence identity to > 50% (Sharma and Kumar, 2016). Gut microbiota is essential for the homeostasis of metabolic processes and the maintenance of immunity (Thursby and Juge, 2017).

Identification of the anti-target proteins and the 3d protein structures propensity

The binding of drugs to essential host proteins can have devastating consequences to the host metabolic pathways. Thus, it is very important to make sure that the new drugs do not bind to the host proteins. The host proteins whose functions are adversely affected by the drugs are called anti-target proteins. Such anti-target proteins include P-glycoprotein (P-gly), adrenergic receptor, dopaminergic receptor, and ether-a-go-go-related protein. The *E. faecalis* essential proteins were subjected to NCBI BLASTp against known 210 anti-target proteins (Fatoba *et al.*, 2021). The E-value was $1e^{-50,}$ and the sequence identity to >25 %.

Rational drug design requires an advanced understanding of the molecular structures; therefore, *E. faecalis* essential proteins were further screened against solved protein structures in the Protein Data Bank (PDB) using NCBI PSI-BLAST, with the E-value set at $1e^{-50}$ and the sequence identity of >40 %. At 40 % or above sequence identity, during homology modeling, similar and excellent models can be computed (Lihan *et al.*, 2023). The protein sequences were aligned using Clustal Omega (Sievers *et al.*, 2011).

Identification of the resistance genes in E. faecalis

The *E. faecalis* core genome was subjected to BLASTn to identify the resistance genes using the Resistance

Gene Identifier (RGI) of the Comprehensive Antibiotic Resistance Database (CARD), CARD 2023 (https://card.mcmaster.ca/) (Alcock *et al.*, 2023). The E-value was $1e^{-10,}$ and the sequence identity to >30 %.

Broad-spectrum antibiotic drug targets

It is generally beneficial that the antimicrobial drug targets several bacterial spp. To elucidate broad-spectrum antibiotic targets in various bacteria, the *E. faecalis* essential genes were subjected to BLASTn against the NCBI Microbial Nucleotide Database. The E-value was $1e^{-50,}$ and the sequence identity to >70 %.

Host-pathogen interactions

The proteins not found in the host but existing only in the *E. faecalis* were computed with BLASTp against the Pathogen-Host Interactions Database (PHI-base version 4.17) (www.phi-base.org) (Urban *et al.*, 2020). This was used to identify the proteins and their phenotype that are involved in the interactions with the host and have known pathogenic activities that were elucidated through rigorous laboratory experiments. The E-value was e^{-30}, and the percentage sequence identity was >25 %.

Druggability analysis of the identified non-homologous protein sequence of Enterococcus faecalis

For a protein to be considered a viable drug target, it must possess druggable properties. The Drugbank offers extensive information on drugs, including molecular details of thousands of drugs approved by the Food and Drug Administration (FDA), as well as nutraceutical and experimental drugs. The analysis of druggability of *E. faecalis* essential proteins involved screening proteins using BLASTp with an E-value threshold of < 0.0001 and sequence identity of>30 %, using Pipeline Builder for Identification of Targets version 3 (PBITV3) in Drugbank and Therapeutic Target (TTD) Databases (Wishart *et al.*, 2018; Zhou *et al.*, 2022). Proteins that demonstrated significant sequence identity compared to the core dataset of the Drugbank and Therapeutic Target databases were recognized as druggable targets.

All the essential proteins were further screened against the 8 well-established physicochemical parameters of the known druggable proteins. The criteria were hydrophobicity of -0.150 to -0.350, where in this range the protein is hydrophilic, protein's amino acids content of between 400 and 600, a mean pI of < 7.2, and molecular weight > 100 kDa (Wilkins *et al.*, 1999), the presence of a signal motif (Teufel *et al.*, 2022), absence of a PEST motif (Qile *et al.*, 2019), more than 2 N-glycosylated amino acids, and no more than a single O-glycosylated serine (Chauhan *et al.*, 2012). Proteins exhibiting relatively high target-like characteristics were selected for the druggability analysis.

Results

Sequence retrieval

The 53 complete core proteome sequences of *E. faecalis* strains were retrieved from the EDGAR 3.5 database (Supplementary File S1). These were further reduced to 6 based on BLASTp sequence alignments (Table 1), which showed hits to the reference sequence, OG1RF (Accession number: CP 002621.1), with 99 % sequence identity.

Enterococcus faecalis's non-paralogous protein sequences and essential genes

Based on the CD-HIT report on *E. faecalis*, 2218 non-paralogous protein sequences were identified from the core proteome. These 2218 non-paralogous protein sequences were further analyzed on DEG, which predicted 10 sequences as essential proteins (Table 2). The sequences of the essential proteins are shown in the Supplementary File S2.

Enterococcus faecalis's proteins containing virulence factors

Enterococcus faecalis's proteins containing virulence factors were identified using the Virulence Factor Database (VFDB). Sixteen protein sequences contained virulence factors with various functions (Table 3).

Enterococcus faecalis's sequences non-homologous to the human proteome and gut microbiota

Enterococcus faecalis essential proteins and genes were subjected to BLASTp and BLASTn, respectively, in the National Centre for Biotechnology Information (NCBI), to identify the non-homologous protein or the nucleotide sequences to the human proteome or the human gut microbiota. The BLASTp or BLASTn results showed that no sequence from *E. faecalis* OG1RF NZ CP025020 was homologous to the human proteome or gut microbiota.

Table 1: *Characteristics of Enterococcus faecalis strains that showed hits against the reference strain, OG1RF.*

Strain name	Description	Notable mobile genetic elements	Genome sequence information	Reference
ATCC 47077/ OG1RF	Human oral isolate. Resistance to rifampicin, fusidic acid, and streptomycin	chromosomal *Tn916* homologue	NCBI Bio Project PRJNA20843, Accession number: CP 002621.1	Bourgogne *et al.* (2008)
FDA-ARGOS 338 chromosome	Isolated from urine catheter. Resistance to tetracycline	Unknown	NCBI Bio Project: PRJNA231221, Accession number: CP022059.2	Sichtig *et al.* (2019)
DENG 1	Tracheal secretion. Resistance to linezolid, streptomycin, and macrolides	Pathogenicity island (PAI) *optrA* and *cfrA*	Gen Bank project 187445, Accession number: P004081.1	Yu *et al.* (2014); Wang *et al.* (2015)
ATCC29212	Isolated from a human urine sample. Resistance to clindamycin, cephalosporin, aminoglycosides	Plasmids accession numbers: CP008814 CP008815	NCBI Bio Project: PRJNA244550, Accession number: CP008816.1	Minogue *et al.* (2014)
OG1RF	Laboratory isolate at Rockefeller University. β-Lactam resistance	Uknown	Bio Project: PRJNA419201, Accession number: CP025020	Kim *et al.* (2019); Lazzaro *et al.* (2022)
C54 chromosome	Isolated from a urine sample and is resistance to linezolid and tedizolid	pC54 (64.5 kb) Accession number: CP030046	Bio Project: PRJNA476189, Accession number: CP 030045.1	Kang *et al.* (2019); Brenciani *et al.* (2022); Roy *et al.* (2020)
CVM-N48037F	Human conjunctival isolates. Resistance to linezolid, ciprofloxacin, moxifloxacin, and levofloxacin	pN48037F-1, pN48037F-2, pN48037F-3,	Bioproject: PRJNA434149 Accession number: CP028720.1	Tyson *et al.* (2018); Lee *et al.* (2017)

Table 2: *Enterococcus faecalis* essential proteins that are also potential drug targets.

***Enterococcus faecalis* OG1RF NZ CP025020 protein sequence** ID	E Value	Bit score	Name of the essential protein
CVT43_RS04985	4.73E-141	405	THUMP domain-containing class I SAM-dependent RNA methyltransferase
CVT43_RS06400	1.65E-133	388	NAD(P)/FAD-dependent oxidoreductase
CVT43_RS12820	2.22E-128	364	TIGR00282 family metallophosphoesterase
CVT43_RS03355	3.25E-110	325	Phosphomevalonate kinase
CVT43_RS11635	7.80E-78	244	ABC transporter, ATP-binding protein
CVT43_RS11155	3.91E-78	241	DNA polymerase III subunit delta'
CVT43_RS02970	8.97E-76	229	pseudouridine synthase
CVT43_RS00985	5.78E-54	193	Amino acid ABC superfamily ATP binding cassette transporter, membrane protein
CVT43_RS09850	8.55E-56	184	Inorganic phosphate transporter
CVT43_RS11640	6.90E-52	173	glycosyltransferase family 2 protein

Anti-target proteins in Home sapiens and 3D protein structure propensity

Based on the NCBI BLASTp results, the predicted 10 essential proteins having huge potential to be drug targets, showed no sequence or structural similarities to the 210 human anti-target proteins. Of the 10 essential proteins, 5 displayed high sequence identity to the known 3D protein structures in the Protein Data Bank (PDB) (Table 4). Chain A, Crystal structure of SMU.472; a putative methyltransferase complexed with SAH (*Streptococcus mutans* UA159: Accession number 3LDG_A) at 62 % sequence identity, and Chain A, Putative methylase (*Clostridioides difficile* 630: Accession number 3LDU_A) at 46 % sequence identity are presented in Figure 2, aligned to *E. faecalis* OG1RF NZ CP025020 (CVT43_RS04985).

Table 3: *Enterococcus faecalis's proteins containing virulence factors.*

Enterococcus faecalis OG1RF NZ CP025020 protein sequence ID	Virulence genes	Virulence factors	Major virulence factors' mechanism
CVT43_RS04720	ace	Ace	Adherence
CVT43_RS04670	ebpA	Ebp pili	
CVT43_RS04675	ebpB		
CVT43_RS04680	ebpC		
CVT43_RS04685	srtC		
CVT43_RS08830	efaA	EfaA	
CVT43_RS10065	cpsA/uppS	Capsule	Antiphagocytosis
CVT43_RS10060	cpsB/cdsA		
CVT43_RS03620	bopD	BopD	Biofilm Formation and Quorum Sensing
CVT43_RS08035	fsrA	Fsr locus	
CVT43_RS08030	fsrB		
CVT43_RS08025	fsrC		
CVT43_RS08020	gelE	Gelatinase	Enzymes
CVT43_RS02950	hylA	Hyaluronidase	
CVT43_RS12120	hylB		
CVT43_RS08015	sprE	sprE	

Table 4: *Enterobacter faecalis essential proteins that have sequence similarity to proteins in the protein data bank.*

Enterococcus faecalis OG1RF NZ CP025020 Protein sequence ID	E-value	% Sequence identity	Protein data bank protein structure identity
CVT43_RS04985	4e-179	61.88	Chain A, Crystal structure of MU.472, a putative methyltransferase complexed with SAH. Accession no.3LDG_A
	4e-113	45.69	Chain A, Putative methylase. Accession no. 3LDU_A
CVT43_RS06400	4e-148	50.59	Chain A, NAD (FAD)-utilizing dehydrogenases. Accession no. 2I0Z_A
CVT43_RS12820	1e-125	64.91	Chain A, YMDB Phosphodiesterase Accession no. 4B2O_A
CVT43_RS03355	2e-75	40.86	Chain A, Lin0012 protein. Accession no. 3K17_A
CVT43_RS11635	3e-76	50.00	Chain A, sugar-binding transport ATP-binding protein. Accession no. 2YYZ_A

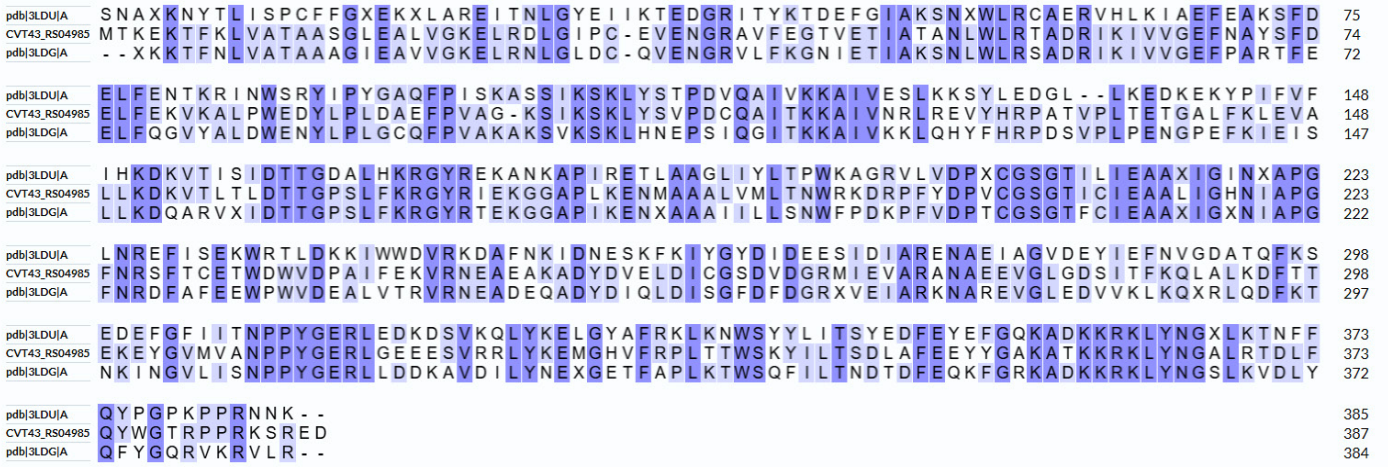

Figure 2: *Sequence alignment of Enterococcus faecalis OG1RF NZ CP025020 (CVT43_RS04985), Chain A, Crystal structure of SMU.472, a putative methyltransferase complexed with SAH (Streptococcus mutans UA159: Accession number 3LDG_A), and Chain A, Putative methylase (Clostridioides difficile 630: Accession number 3LDU_A). The 3D protein structures of 3LDG-A and 3LDU-A are available on the Protein Data Bank (PDB). The alignment shows a region of high sequence similarity. Protein sequences were aligned using Clustal Omega (Sievers et al., 2011).*

Table 5: *Enterococcus faecalis OG1RF NZ CP025020 antimicrobial resistance gene and their mechanism.*

Enterococcus faecalis OG1RF NZ CP025020 protein sequence ID	*CARD short name	Antimicrobial resistance (AMR) gene family	Name of the (AMR) gene	Resistance mechanism	Drug class	Detection method
CVT43_RS03105	*vanT* gene in *vanG* cluster	glycopeptide resistance gene cluster, *vanT*	*vanT* gene conferring high-level of resistance vancomycin	Antibiotic target *al*teration, vanC-type vancomycin resistance mechanism	Glycopeptide antibiotic	Protein homolog model
CVT43_RS11365	*vanY* gene in *vanB* cluster	*vanY*, glycopeptide resistance gene cluster	*vanY* gene encodes a DD-carboxypeptidase that aids in vancomycin resistance	Antibiotic target *al*teration	Glycopeptide antibiotic	Protein homolog model
CVT43_RS06870	dfrE	trimethoprim resistant dihydrofolate reductase (dfr)	*dfrE*, dihydrofolate reductase	Antibiotic target *al*teration	Diaminopyrimidine antibiotic	Protein homolog model
CVT43_RS11705	efrA	ATP-binding cassette (ABC) antibiotic efflux pump	*efrA* is a part of the *EfrAB* efflux pump, conferring drug resistance.	Antibiotic efflux	Macrolide, fluoroquinolone, rifamycin antibiotics	Protein homolog model

*Where *CARD: The comprehensive antibiotic resistance database*

Table 6: *Bacterial strains that can be targeted by a broad-spectrum antibiotic against Enterococcus faecalis OG1RF NZ CP025020.*

Bacterial strains	E Value	% Identity	*NCBI Accession number
Candidatus Enterococcus courvalinii strain MSG2901	2e-179	77%	NZ_JAFLWI010000011.1
Enterococcus dongliensis strain K4 NODE	6e-175	77%	NZ_JARPZC010000004.1
Enterococcus thailandicus strain a523	8e-174	77%	NZ_CP023074.1
Enterococcus sulfureus ATCC 49903	1e-171	77%	NZ_ASWO01000005.1
Enterococcus aquimarinus strain DSM 17690	5e-171	76%	NZ_JXKD01000001.1
Enterococcus raffinosus strain F162_2	1e-156	77%	NZ_CP072888.1
Isobaculum melis strain DSM 13760	5e-156	76%	NZ_FOHA01000005.1
Vagococcus coleopterorum strain HDW17A	2e-155	76%	NZ_CP049886.1
Vagococcus penaei strain LMG 24833 3	1e-146	75%	NZ_NGJV01000003.1
Tetragenococcus koreensis strain KCTC 3924	5e-116	74%	NZ_CP027786.1
Vagococcus martis strain D7T301	3e-113	74%	NZ_MVAB01000001.1
Tetragenococcus muriaticus DSM 15685	2e-105	74%	NZ_AUIQ01000025.1
Pisciglobus halotolerans strain DSM 27630	1e-102	73%	NZ_FOQE01000005.1
Listeria ivanovii subsp. *ivanovii* strain WSLC 3010	2e-55	76%	NZ_CP009577.1

*Where *NCBI: National Center of Biotechnology Information.*

Enterococcus faecalis antimicrobial resistance genes and their mechanism

Enterococcus faecalis core genome was subjected to BLASTn to identify the resistance genes *via* Resistance Gene Identifier (RGI) of the Comprehensive Antibiotic Resistance Database (CARD). The core genome analysis showed that 4 genes of *E. faecalis* can confer antibiotic resistance through various mechanisms (Table 5). CVT43_RS06870 (*dfrE*, dihydrofolate reductase) showed 100 % sequence identity to the diaminopyrimidine resistance proteins, and CVT43_RS11705 (*efrA* is a part of the *EfrAB* efflux pump, conferring drug resistance) showed 100% sequence identity to macrolide, fluoroquinolone, and rifamycin antibiotic resistance proteins (Table 5). The main observed mechanisms of antibiotic resistance involved antibiotic target *al*teration and antibiotic efflux.

Broad-spectrum antibiotic drug targets

Broad-spectrum antibiotic target that was performed with NCBI BLASTn against Microbial Nucleotide Database showed that fourteen bacterial strains can be targeted by a common antibiotic, targeting essential proteins of *E. faecalis* OG1RF NZ CP025020 (Table 6).

Table 7: *Phenotypic characteristics of host-pathogen interactions of Enterococcus faecalis.*

Enterococcus faecalis OG1RF NZ CP025020 protein sequence ID	*PHI-Base ID	Gene	E-Value	% Identities	Pathogen species	Phenotype
CVT43_RS11635	6319	PotA	4.21e-67	41.24	*Streptococcus pneumoniae*	Reduced_virulence
	8968	ProV	4.31e-40	33.93	*Klebsiella pneumoniae*	Reduced_virulence
	6579	VraD	2.95e-39	37.32	*Staphylococcus aureus*	Reduced_virulence
	8623	Cab_ (NMB0789)	1.45e-38	36.40	*Neisseria meningitidis*	Unaffected_ pathogenicity
	11542	B0446	4.62e-35	35.58	*Brucella melitensis*	Unaffected_ pathogenicity
CVT43_RS09850	9451	PitA	6.74e-68	37.89	*Staphylococcus aureus*	Reduced_virulence
CVT43_RS11640	6142	GlcV	1.30e-49	32.81	*Listeria monocytogenes*	Reduced_virulence
	2656	PmrF	1.24e-39	27.45	*Salmonella enterica*	Reduced_virulence
	7950	ArnC	1.02e-36	27.50	*Klebsiella pneumoniae*	Unaffected_pathogenicity

*Where *PHI: Pathogen-host interaction.*

Table 8: *Comparison of Enterococcus faecalis essential proteins against known druggable proteins.*

Enterococcus faecalis OG1RF NZ CP025020 protein sequence ID	Number of homologous alignment(s)	*PBITV3 database identifiers	Protein homologs (Identity %)	Drug names
CVT43_RS06400	1	DB03147	Fumarate reductase flavoprotein subunit (26%)	Flavin adenine dinucleotide
CVT43_RS11635	2	DB00778	Multidrug resistance protein 1 (33 %)	Roxithromycin
		T62977|RBBA_ECOLI	Bacterial Ribosomal ATPase RbbA (Bact rbbA) (33 %)	Hygromycin B
CVT43_RS11155	1	DB02930	DNA polymerase III subunit tau (34 %)	Adenosine 5′-(γ-thiotriphosphate)
CVT43_RS02970	1	DB03419	Ribosomal small subunit pseudouridine synthase A (36 %)	Uracil

*Where *PBITV3: pipeline builder for identification of targets version 3.*

Host-pathogen interactions

Analysis of host-pathogen interactions revealed that 3 non-homologous *E. faecalis* proteins had various pathogenic impacts on the host. These were CVT43_RS11635 and CVT43_RS11640; both had reduced virulence and unaffected pathogenicity, while CVT43_RS09850 displayed reduced virulence (Table 7).

Druggability of the non-homologous protein sequence of Enterococcus faecalis

Analysis of the druggability of *E. faecalis* essential proteins using various proteomics tools showed that 7 proteins were hydrophilic and 3 were hydrophobic. Only CVT43_RS00985 had both the Signal and PEST motifs. CVT43_RS11155 had no N-glycosylation site. CVT43_RS11155, CVT43_RS02970, and CVT43_RS09850 had isoelectric points (pI) above 7. Only CVT43_RS06400 had the amino acid sequence of above 400 amino acids (Supplementary File S3). The lowest number of O-glycosylation sites was predicted in the CVT43_RS12820 and CVT43_RS02970, where both had 7 O-glycosylation sites.

Further analysis of the druggability of the essential proteins was performed through comparing them to the druggable proteins in the Drugbank and Therapeutic Target (TT) Databases, using Pipeline Builder for Identification of Targets version 3 (PBITV3). The obtained results of these analyses showed that 4 *E. faecalis* essential proteins had homologs in Drugbank and Therapeutic Target (TT) Databases (Table 8). Three of these drug targets were enzymes, including Fumarate reductase flavoprotein subunit, Bacterial Ribosomal ATPase RbbA (Bact rbbA), and Ribosomal small subunit pseudouridine synthase A.

Discussion

Multidrug-resistant *E. faecalis* is one of the most predominant isolates in hospital environments. It poses a serious challenge to the enterococcal infection treatment; hence there is an urgent need to elucidate novel drug targets. Indeed, *E. faecalis* is exceptionally good at rapidly developing resistance to any antimicrobial agent (Esmail *et al.*, 2019; Guan *et al.*, 2024). This bacterium has already been ahead of antimicrobial drugs because of its intrinsic and acquired resistance. Intrinsic resistance originated from the core genome, while acquired resistance was gained through the horizontal exchange of mobile genetic elements. *Enterococci* present a significant difficulty in clinical practice due to their genomes' malleability, inherent resistance to several antimicrobial drugs, and capacity to attract and spread antibiotic resistance determinants (Guan *et al.*, 2024). One of the biggest issues that physicians deal with when treating enterococcal disease is the emergence of resistance during treatment. This could ultimately result in treatment failures and, in many cases, raise the mortality rate of those patients infected with *E. faecalis* (Guan *et al.*, 2024). Instead of acquiring new resistance determinants, this problem is especially pertinent when the mechanism of resistance entails mutations in already-existing genes. Thus, to overcome MDR *E. faecalis*, it is important to target its essential proteins because they are crucial to its survival and are rarely mutated.

Of the 53 *E. faecalis* strains retrieved from the EDGAR 3.5 database, 6 exhibited sequence similarity of up to 99 % with the reference strain. This indicates that the essential proteins identified in *E. faecalis* OG1RF (NZ CP025020) are also relevant to these strains for the development of novel broad-spectrum antibiotics. All 6 strains have been experimentally confirmed to be resistant to various antibiotics. After removing paralogous protein sequences, the non-paralogous proteins were further analyzed, resulting in the identification of 10 essential proteins. These proteins are critical for the survival of *E. faecalis*, making them excellent targets for drug development. The majority of these proteins are either enzymes or transporters, which means they possess naturally occurring ligands that can be manipulated during rational drug design.

Sixteen proteins contained virulence factors that could assist *E. faecalis* in biofilm formation, avoid phagocytosis, adhere to surfaces and enzymes, including gelatinase that aid the bacteria to degrade gelatin through hydrolysis, hyaluronidase, an enzyme that breaks down hyaluronic acid, and *sprE* gene that encodes a glutamyl endopeptidase, an enzyme that breaks down proteins. Similar virulence factors have been previously experimentally observed in sixty *E. faecalis* urinary isolates (Hashem *et al.*, 2021), although in *E. faecalis* OG1RF NZ CP025020, no cytolysin (*Cyl*) virulence factors were observed. Furthermore, no homologous *E. faecalis* sequences were observed in the core proteome of the human and gut microbiota, and the anti-target proteins, making these essential proteins excellent drug targets.

Five essential protein sequences displayed high sequence similarities to known 3D structures in the protein data bank. These proteins can be used for homology modelling in rational drug design. None of the essential proteins had a known 3D structure in the protein data bank. Thus, to overcome antimicrobial resistance in *E. faecalis*, the atomic resolution structures of some of these proteins must be determined.

Analysis of the *E. faecalis* core genome for resistance genes demonstrated that it had 4 genes related to antimicrobial resistance. These genes were detected by homology modelling. The genes showed that antimicrobial resistance in *E. faecalis* involved antibiotic target alteration and efflux pump. Rana *et al.* (2023) discovered up to thirty-nine antibiotic resistance genes in *E. faecalis*. Broad-spectrum antibiotic analysis against the essential proteins demonstrated that fourteen bacterial strains were prone to any antimicrobial that can be used to target *E. faecalis* OG1RF NZ CP025020. Both intrinsic and acquired antimicrobial resistance mechanisms in *E. faecalis* OG1RF NZ CP025020 need further research studies.

Host-pathogen interaction (PHI) analysis of the essential proteins of *E. faecalis* OG1RF NZ CP025020 came up with 3 proteins whose interaction with the host could lead to reduced virulence or unaffected pathogenicity, based on sequence similarity to the other pathogenic bacteria. Analysis of the entire core proteome may be important to elucidate more novel proteins that are involved in host-pathogen interaction. Nevertheless, the essential proteins involved in host-pathogen interaction are excellent

drug targets. Recent PHI studies have shown that a diverse array of *E. faecalis* proteins are involved in PHI, including Gelatinase E (*gelE*), Serine protease (*SprE*), Enterococcal surface protein (*Esp*), Aggregation substance (AS), Adhesion of collagen (*Ace*), Lipoteichoic acid (LTA), Endocarditis antigen A (EfaA), Hyaluronidase (HYL), Cytolysin (Cyl), Endocarditis- and biofilm-associated pili (*Ebp*), and Enterococcal polysaccharide antigen (*EPA*) (Gök *et al*., 2020; Geraldes *et al*., 2022; Madani *et al*., 2024; Too and Masila, 2024).

The final analysis involved the druggability of the essential proteins. The physicochemical analysis demonstrated that none of the proteins met all the parameters to be considered druggable. For example, most of them had more than 2 N-glycosylated amino acids, and this is mainly attributed to the fact that not all predicted N- or O-glycosylation sites can be glycosylated (Schäffer and Messner, 2017). Only one protein had both the Signal and PEST motifs. While the absence of the PEST motifs is good, because PEST motifs serve as proteolytic recognition signals during protein degradation (Jiang *et al*., 2025), however, the lack of Signal motifs complicates the matters because a Signal motif is very important in the localization of proteins inside the cell (Sidorczuk *et al*., 2023). Further analysis on the Drugbank and Therapeutic Target (TT) Databases revealed that 4 essential proteins were druggable, mainly CVT43_RS06400 (NAD(P)/FAD-dependent oxidoreductase), CVT43_RS11635 (ABC transporter, ATP-binding protein), CVT43_RS11155 (DNA polymerase III subunit delta'), and CVT43_RS02970 (pseudouridine synthase). Although in this study, druggability was determined by analysis of physicochemical parameters and sequence comparison methods, but there are other methods that should be considered such as structure, ligand, and precedence-based methods (Agoni *et al*., 2020).

Conclusions and Recommendations

The prevalence of MDR *E. faecalis* is of a great public health concern. There is an urgent need to elucidate new drug targets to overcome microbial drug resistance. The *E. faecalis* essential proteins are the best drug targets because the bacteria cannot live without them, and are rarely mutated. Analysis of these essential proteins showed that they are not homologous to the human and gut microbiota proteome. Furthermore, any drug that can be developed to target them is also capable of tackling several other bacteria based on the broad-spectrum antibiotic analysis, such as *Enterococcus raffinosus*; a multidrug resistant bacterium, associated with endocarditis, osteomyelitis, bloodstream, hematoma, urinary tract, and intra-abdominal infections. Four of the *E. faecalis* essential proteins proved that they are druggable, providing new novel drug targets against MDR *E. faecalis*. Future researches are highly recommended to focus on *in vitro* studies on drug resistance mechanisms, elucidation of virulence factors, and design of novel drugs based on homology and molecular modeling studies.

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Novelty Statement

Using subtractive genomics, this study elucidated novel drug targets against multidrug-resistant (MDR) *Enterococcus faecalis*. Among them are essential enzymes and transporters that the bacteria can't live without. Drugs that can be designed to target these essential proteins are also capable of tackling other MDR bacteria, making them broad-spectrum antibiotics. This could lead to better patient's outcomes from the MDR bacteria. Future laboratory experiments on these proteins are needed to confirm their druggability *in vivo* or *in vitro*.

Author's Contribution

RSK: Conceptualization. RSK, JK: Formal analysis. RSK, JK: Investigation. RSK, JK, NAD: Methodology. RSK: Supervision. RSK, YAA: Validation. RSK, JK, NAD, YAA: Writing original draft. RSK, JK, NAD, YAA: Writing review and editing. RSK: Project administration.

Supplementary materials

The following supplementary materials are available for downloading: *E. faecalis* strains retrieved from the EDGAR 3.5 database (Supplementary File S1), *E. faecalis* OG1RF NZ CP025020 essential protein and DNA sequences (Supplementary File S2), and Physicochemical Druggability Properties of the Essential

Proteins (Supplementary File S3). https://dx.doi.org/10.17582/journal.NRMJ/2025/9.5.349.364

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