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Genetic and phenotypic characterization of ampicillin susceptible Enterococcus faecium clinical isolates from bloodstream infections

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DOCTORAL THESIS

Genetic and phenotypic characterization of ampicillin susceptible *Enterococcus faecium* clinical isolates from bloodstream infections

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“Faràs tot el que et proposis”

Mireia Català

Agraïments

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Paula Bierge Cabrera
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List of abbreviations

Abbreviation	Definition
AMP-R	Ampicillin resistant
AMP-S	Ampicillin susceptible
AMR	Antimicrobial resistance
ANI	Average Nucleotide Identity
aPBP	Type A Penicillin-Binding Proteins
AST	Antibiotic susceptibility test
bPBP	Type B Penicillin-Binding Proteins
BSIs	Bloodstream infections
BT	Bithionol
CA	Community-acquired
CBs	Cationic biocides
CC	Clonal complex
CFU	Colony-forming unit
CGE	Center for Genomic Epidemiology
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Antibiotic peak serum concentration
COG	Clusters of Orthologous Genes
CTns	Conjugative transposons
DCs	Dendritic cells
DNA	Deoxyribonucleic Acid
EEA	European Economic Area
Efm	*Enterococcus faecium*
EPA	Environmental Protection Agency

ESBL	Extended-spectrum β-lactamase
EU	European Union
EUCAST	Committee on Antimicrobial Susceptibility Testing
FC	Fold change
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
HA	Hospital-associated
HAMAP	High-quality Automated and Manual Annotation of Proteins
HGT	Horizontal gene transfer
HK	Housekeeping
HLRA	High-level resistance to aminoglycosides
ICEs	Integrative conjugative elements
IE	Infectious endocarditis
IS	Insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase producing
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Mbp	Megabase Pair
MDK	Minimum Duration for Killing
MDR	Multidrug-resistant
MGEs	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MLNs	Mesenteric lymph nodes
MLS	Macrolide-lincosamide-streptogramins
MLST	Multi-locus sequence typing

MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
nPB	non-Penicillin Binding
N1	N-terminal domain 1
N2	N-terminal domain 2
PASTA	Penicillin-Binding Protein and Serine/Threonine kinase Associated domain
PBPs	Penicillin-Binding Proteins
QACs	Quaternary Ammonium Compounds
RBS	Ribosome binding site
RIP	Replication initiator protein
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
SEM	Scanning Electron Microscopy
Ser/Thr	Serine/Threonine
sp.	Species
spp.	Species plural
STs	Sequence types
TA	Toxin-antitoxin system
TCS	Two-component-system
TEM	Transmission Electron Microscopy
TKCs	Time-kill curves
VBNC	Viable but non-culturable
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococcus*

VREfm	Vancomycin-resistant *Enterococcus faecium*
WGS	Whole-genome sequencing
WHO	World Health Organization

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Introduction

Chapter I: Evaluation of the efficacy of ampicillin and cephalosporins for ampicillin-susceptible *Enterococcus faecium*

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Abstract

Enterococcus faecium is a critical nosocomial pathogen, known for its high resistance to multiple antibiotics, including β-lactams, which complicates treatment strategies. This thesis investigates the genetic and molecular mechanisms driving *E. faecium*'s resistance to β-lactam antibiotics. All isolates studied were ampicillin-susceptible (AMP-S) *E. faecium* clinical strains derived from bacteremic patients at the Parc Taulí University Hospital. The research explores the efficacy of various antibiotic combinations, including ampicillin with ceftriaxone or ceftaroline, in overcoming resistance in AMP-S *E. faecium* strains. The findings suggest that while certain combinations, such as ampicillin-ceftaroline, exhibit synergy in some clinical isolates the response to combination therapy is strain-dependent, underscoring the complexity of treating infections caused by this pathogen. A key focus of the thesis is the role of genetic redundancy, particularly the presence of multiple copies of the *pbp5* gene, which encodes a Penicillin-Binding Protein (PBP) involved in β-lactam resistance in *E. faecium*. This redundancy, often carried on mobile genetic elements like plasmids, enhances the pathogen's ability to survive under selective antibiotic pressure. The study highlights how these redundant genes, along with other resistance determinants, contribute to the spread of multidrug resistance and facilitate the co-selection of resistance to different antibiotic classes. Additionally, the thesis investigates the molecular mechanisms underlying the natural resistance of *E. faecium* to cephalosporins, a phenomenon that had not been fully understood until now. We describe for the first time a clinical isolate with low MICs to cephalosporins that provided a unique opportunity for a detailed investigation of the molecular mechanisms underlying *E. faecium*'s resistance to cephalosporins, enabling the identification of specific mutations and transcriptional changes that provide deeper insights into the genetic adaptations of *E. faecium*. This thesis provides valuable insights into the complex mechanisms underlying *E. faecium* resistance to β-lactam antibiotics. The findings enhance our understanding of the adaptive strategies of this pathogen, highlighting the need for improved therapeutic strategies to address the increasing challenge of antibiotic resistance.

Resum

Enterococcus faecium és un patogen nosocomial crític, caracteritzat per una elevada resistència a múltiples antibiòtics, inclosos els β-lactàmics, fet que dificulta les opcions terapèutiques disponibles. Aquesta tesi analitza els mecanismes genètics i moleculars que sustenten la resistència d'*E. faecium* als antibiòtics β-lactàmics. Els aïllats estudiats corresponen a soques clíniques d'*E. faecium* susceptibles a l'ampicil·lina (AMP-S), obtingudes de pacients amb bacterièmia a l'Hospital Universitari Parc Taulí. La recerca avalua l'eficàcia de diverses combinacions d'antibiòtics, incloent-hi l'ampicil·lina amb ceftriaxona o ceftarolina, per superar la resistència en soques AMP-S. Els resultats mostren que certes combinacions, com ampicil·lina-ceftarolina, poden generar sinèrgia en alguns aïllats clínics. No obstant això, la resposta varia segons la soca, destacant la complexitat associada al tractament d'infeccions causades per aquest patogen. Un dels aspectes clau d'aquesta investigació és l'estudi de la redundància genètica, especialment la presència de múltiples còpies del gen *pbp5*, que codifica una proteïna d'unió a la penicil·lina (PBP) implicada en la resistència als β-lactàmics. Aquesta redundància, sovint associada a elements genètics mòbils com els plasmidis, confereix a *E. faecium* una elevada capacitat d'adaptació sota pressió selectiva, contribuint així a la disseminació de resistències a múltiples fàrmacs i a la co-selecció de resistència a diferents classes d'antibiòtics. A més, s'han investigat els mecanismes moleculars que expliquen la resistència natural d'*E. faecium* a les cefalosporines, un fenomen que fins ara encara no s'ha caracteritzat plenament. Per primera vegada, s'ha identificat un aïllat clínic amb concentracions mínimes inhibitòries (MICs) baixes a les cefalosporines, proporcionant una base única per a l'anàlisi exhaustiva d'aquests mecanismes. Això ha permès identificar mutacions específiques i canvis transcripcionals que ofereixen nous coneixements sobre les adaptacions genètiques d'*E. faecium*. Aquesta tesi proporciona una contribució significativa a la comprensió dels mecanismes complexos que sustenten la resistència d'*E. faecium* als antibiòtics β-lactàmics. Els resultats obtinguts suggereixen la necessitat de desenvolupar estratègies terapèutiques més precises i efectives per fer front al creixent repte de la resistència als antibiòtics.

1. Introduction

1.1. Overview of the genus *Enterococcus*

1.1.1. Overview of enterococci and their clinical importance

Enterococci are a group of facultative anaerobic Gram-positive cocci that are classified within the phylum Bacillota and Lactobacillales order **[Figure 1] (1)**.They belong to the *Enterococcaceae* family, which encompasses a variety of species commonly found in the gastrointestinal tract (GIT) of humans, animals, and insects, where they persist as a minor component (less than 1.0%) of the human microbiota **(2)** and play a role in maintaining gut microbiota homeostasis **(3)**. Currently, over 60 species of *Enterococcus* have been identified, with *Enterococcus faecalis* and *Enterococcus faecium* being the most clinically significant**.** Other species, such as *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus hirae*, and *Enterococcus raffinosus*, are categorized as non-*faecium* and non-*faecalis* enterococci. Altogether, these microorganisms are increasingly recognized as the causal agents of bloodstream and endovascular infections **(4,5).**

During the 1960s and 1970s, studies provided evidence that *Enterococcus* species were already expanding across various environments, including mammals, birds, insects, and other animal-associated habitats **(3).** However, the molecular techniques of that time were not sufficiently advanced, and it was only in 1984 that *Enterococcus* was officially separated from *Streptococcus*, leading to the reclassification of *Streptococcus faecalis* and *Streptococcus faecium* as *E. faecalis* and *E. faecium*, respectively **(6)**. Despite recognizing the diversity within *Enterococcus*, the limited molecular tools available made it challenging to accurately classify distinct species. Today, genomics offers high-resolution capabilities, enabling precise detection and classification of species from various hosts **(7)**.

More recently, the differentiation between *Enterococcus lactis* and *E. faecium* has attracted some attention, particularly due to the implications for food safety and clinical microbiology **(8)**. Recent genomic analyses have provided insights into the taxonomic relationships among these species, particularly focusing on the strains classified within clade B[1] of *E. faecium*, representing community-associated (CA) strains **(9)**. *E. lactis* shares a close genetic relationship with clade B strains of *E. faecium*, as evidenced by high Average Nucleotide Identity (ANI) values exceeding 90.0% **(10)**. This genetic similarity supports the proposal to reassign clade B strains to the species

[1] The classification of *E. faecium* into different clades is addressed in section *1.3. Enterococcus faecium genomics.*

E. lactis, as they exhibit a cohesive genomic profile distinct from the more divergent clade A strains. Notably, classification of *E. faecium* or *E. lactis* can be achieved by PCR amplification of the gene *gluP* **(10)**.

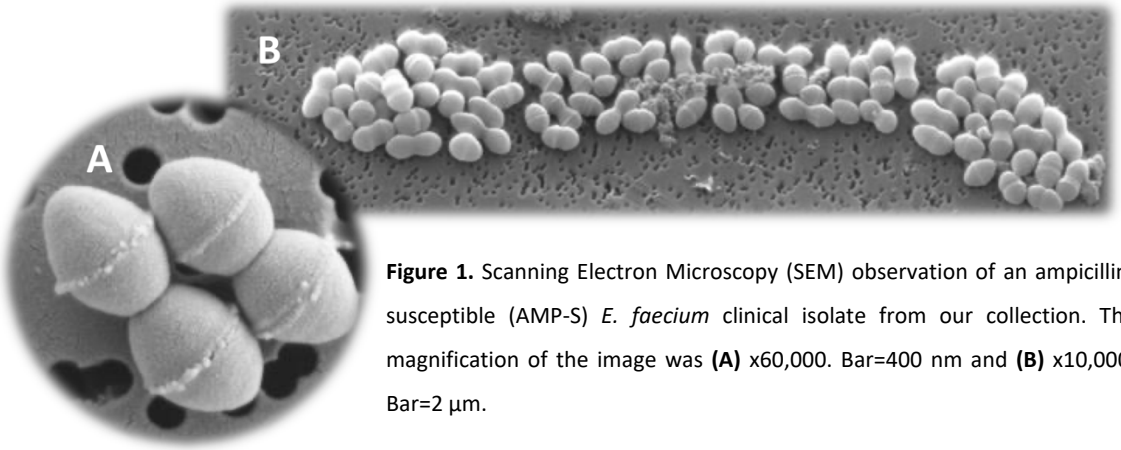


Figure 1. Scanning Electron Microscopy (SEM) observation of an ampicillin-susceptible (AMP-S) *E. faecium* clinical isolate from our collection. The magnification of the image was **(A)** x60,000. Bar=400 nm and **(B)** x10,000. Bar=2 µm.

Enterococcus species are highly resilient and can endure extreme conditions, including exposure to disinfectants and antiseptics, enabling their proliferation in selective environments such as hospitals **(11)**. While enterococcal infections were historically considered mainly endogenous—originating from bacteria present in the patient's own microbiota—, they are now recognized as transmissible within hospital settings, particularly through healthcare workers' hands and contaminated surfaces **(12,13).** Between 2010 and 2020, hospital acquired infections caused by enterococci accounted for 6.1% to 17.5% of all such infections **(14)**. Factors influencing colonization and transmission of antibiotic-resistant enterococci include prior antibiotic use, prolonged hospital stays, and exposure to colonized patients **(15)**. High-risk groups, such as those with chronic conditions or undergoing major medical treatments, are especially vulnerable to these infections **(16)**. Altogether, the rising incidence of antibiotic-resistant *E. faecium* infections represents a serious public health threat and immediate action is essential **(17)**.

1.1.2. *Enterococcus* antimicrobial resistance, tolerance, and persistence

Among the clinically significant enterococci, *E. faecium* and *E. faecalis* are of paramount importance. *Enterococcus* species employ diverse mechanisms to overcome selective pressure, which results in intrinsic and/or acquired resistance, tolerance, and persistence **[Figure 2] (18)**. Altogether, these responses enable them to withstand antibiotic treatment and adapt to challenging environments **(19)**.

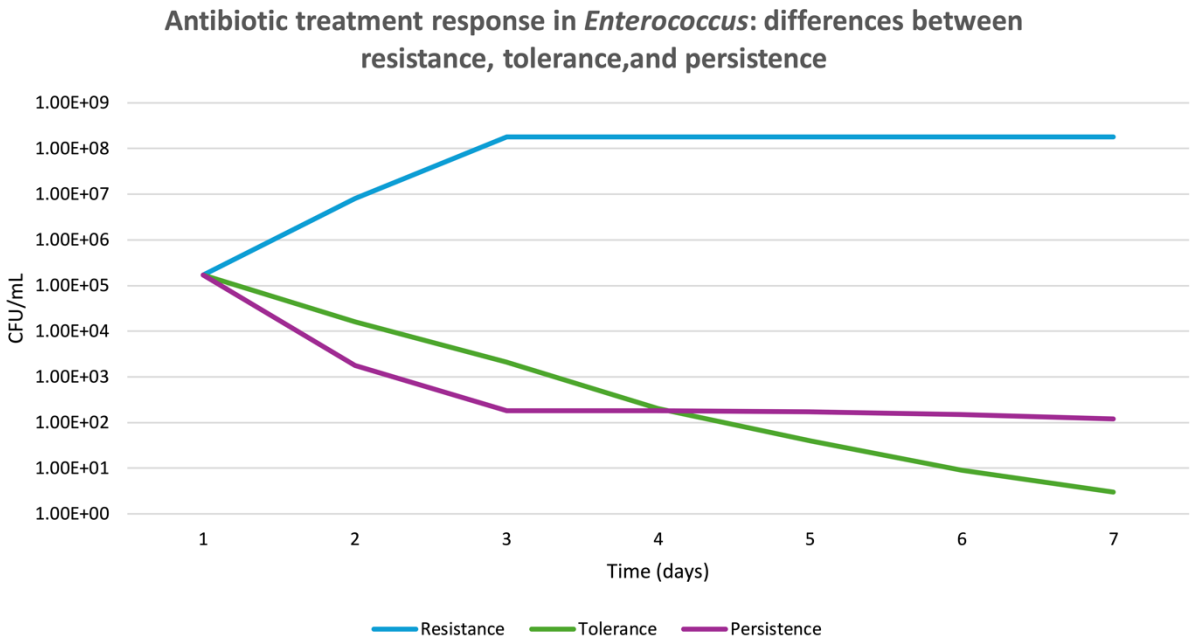


Figure 2. Different *Enterococcus* behaviours in response to antibiotic treatment where resistant strains fail to be inhibited by the antibiotic (blue); tolerant strains exhibit reduced metabolic rate, allowing them to survive in the presence of the antibiotic (green); and persistent cells, a subpopulation that withstands treatment, leading to prolonged infection risks (purple). *Source: Own elaboration.*

Bacterial resistance refers to the ability of a microorganism to survive and proliferate in the presence of sustained high antibiotic concentrations due to genetic changes that confer a survival advantage **(20)**. This is typically measured using the Minimum Inhibitory Concentration (MIC), which indicates the lowest concentration of an antibiotic that prevents bacterial growth. *Enterococcus* can exhibit resistance to nearly all antibiotics used in clinical practice, complicating treatment options. This resistance can arise through two main mechanisms: mutations and horizontal gene transfer (HGT) **(21)**.

Mutational resistance is associated with several antibiotics. The first documented descriptions of resistance to penicillin and ampicillin emerged at the end of the 20th century **(22)**. Besides, during this time, *E. faecium* and *E. faecalis* began to be documented for their intrinsic resistance to cephalosporins **(23)**. Later, it was demonstrated that the primary mechanism responsible for this intrinsic resistance is linked genetic alterations in target proteins, particularly penicillin-binding proteins (PBPs) **(24)**. Several mutational changes have been associated with tigecycline resistance by driving the overexpression of efflux pumps such as Tet(L) **(25)**. Tigecycline was introduced for clinical use in 2005, and resistance emerged rapidly; the first clinical isolate of *E. faecalis* exhibiting resistance was observed in 2008, followed by a resistant *E. faecium* isolate in 2010 **(26)**. Besides, resistance to daptomycin and linezolid was noted in the early 21st century,

typically associated with specific mutations **(27,28)**. For linezolid, resistance is often due to point mutations in the 23S rRNA gene, which alter ribosomal binding sites, diminishing the drug's effectiveness **(19,28)**. In the case of daptomycin, resistance commonly involves mutations in membrane- associated genes such as *cls*, *liaF*, *liaS*, and *liaR* **(19)**. LiaF is a key component of the three-component LiaFSR regulatory system, which plays an essential role in managing the cell envelope's defense against antibiotics. Daptomycin, which received Food and Drug Administration (FDA) approval in 2003, quickly became a concern due to reported resistance, especially among vancomycin-resistant *Enterococcus* (VRE) strains **(27)**. Similarly, linezolid encountered resistant isolates shortly after its introduction in 2000 **(28)**.

In contrast, resistance to other antibiotics in *Enterococcus* is driven by HGT, often mediated by mobile genetic elements (MGEs) such as transposons and plasmids[2] **(19)**. In addition to their intrinsic low-level resistance to aminoglycosides, with MICs typically ranging from 4 to 256 mg/L, enterococci possess the Tn5281 transposon, which confers acquired resistance to aminoglycosides, significantly elevating MICs to ≥2000 mg/L and providing resistance to nearly all aminoglycosides except streptomycin **(19,29)**. Similarly, chloramphenicol resistance is commonly conferred by "broad host-range", or Inc18, plasmids, such as plP501, and the conjugative transposon Tn1545, which also confers resistance to both chloramphenicol and tetracyclines **(30)**. Resistance to erythromycin is also associated with Inc18 plasmids in addition to the Tn5385 transposon, a large, composite transferable element that additionally carries resistance genes for tetracycline **(19)**. For tetracycline resistance, the pheromone-responsive plasmid pCF10 contains the *tet(M)*-bearing transposon Tn925. Other pheromone-responsive plasmids are similarly linked to VanB-type vancomycin resistance transfer **(31)**. Additionally, the conjugative transposon Tn916, harbouring *tet(M)*, also confers tetracycline resistance **(32)**. Vancomycin resistance, particularly of the VanA-type resistance, can be transferred between *E. faecium* and *E. faecalis* through a VanA-carrying pheromone-responsive plasmid **(33)**. Inc18 plasmids have also been associated with VanA-type resistance transfer **(19)**. Vancomycin, initially introduced in the 1950s as a treatment for aminoglycoside-resistant *Enterococcus*, initially proved effective; however, its widespread use facilitated the emergence of VRE, with the first human case reported in England in 1988 **(34)**. VRE has since become a significant cause of hospital-acquired infections, and recent epidemiological data indicate a rise in vancomycin resistance among clinical *E. faecium* isolates, with resistance rates increasing from 8.1% in 2012 to 19.0% in 2018 across European Union/European Economic Area (EU/EEA) countries **(35)**. This

[2] Transposons and plasmids are explained in section *1.3.2.1* and *1.3.2.2*, respectively.

trend is of particular concern, as vancomycin-resistant *E. faecium* (VREfm) infections contribute substantially to hospital-acquired bloodstream infections. In fact, VRE are considered by the World Health Organization (WHO) as a high-priority pathogen for which new antimicrobial therapies are needed **(36)**.

While the prevalence of VREfm in Europe remains lower than in the United States (66.0%) and Australia (47.0%), it is considerably higher than in China, where resistance rates are below 4.0% **[Figure 3] (35)**. Regional analysis indicates that vancomycin resistance rates are highest primarily in Eastern and Northern Europe, while they are lower in Western and Southern Europe, with Spain exhibiting a prevalence of around 2.8%, typically associated with sporadic hospital outbreaks **(17,35,37)**. It is important to note that the variability in resistance levels across different countries suggests that local factors, including diagnostic practices, infection control measures, and antibiotic usage, significantly influence resistance trends **(35)**. Furthermore, current data reveal a significant co-resistance of VREfm to ampicillin (>99.0%) and gentamicin (48.5%), highlighting the diminishing efficacy of these antibiotics in the empirical treatment of VREfm infections, even when administered in synergistic combinations **(35,38)**.

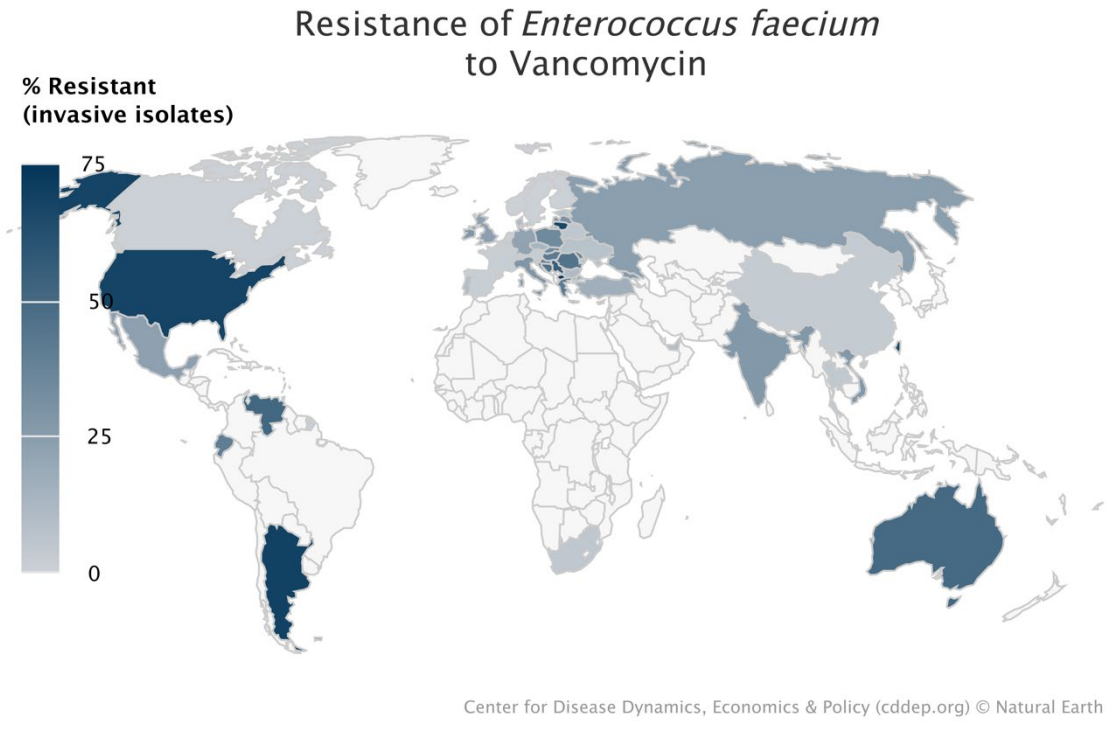


Figure 3. Global incidence of VREfm, highlighting regional variations in resistance rates. *Source: One Health Trust. Resistance Map: Antibiotic resistance. 2024. https://resistancemap.onehealthtrust.org/AntibioticResistance.php. Date accessed: Sep 22, 2024.*

Additionally, strains tolerant to antibiotics commonly used in clinical practice such as β-lactams or vancomycin, have been observed and described. Unlike resistant *Enterococcus*, tolerant strains do not grow in the presence of high concentrations of bactericidal antibiotics; however, they survive by slowing down essential processes, such as growth and metabolism **(20,39)**. This ability to evade killing temporarily results in a slow death of the bacteria. These tolerant strains demonstrate resilience against the bactericidal effects of cell-wall targeting agents, including β-lactams and vancomycin **(19)**. Consequently, prolonged high-dose antibiotic treatment is necessary to fully eradicate the infection **(40)**. The Minimum Duration Killing (MDK) is a quantitative measure used to assess bacterial tolerance to antibiotics. It represents the minimum time required to kill a specific percentage of a bacterial population when exposed to an antibiotic. This measure helps differentiate between tolerant strains, which can survive antibiotic exposure for extended periods, and resistant strains, which can grow in the presence of the antibiotic **(20)**.

On the other hand, some *Enterococcus* cells are classified as persisters, which represent a subpopulation of bacteria (less than 1.0% of the population) that can survive under lethal conditions by entering a non-growing, dormant state **(41)**. These cells remain inactive during stress but can resume growth once favorable conditions return, potentially leading to relapses and contributing to chronic infections. This makes persisters particularly problematic in clinical settings, as their presence can result in misleading perceptions of resistance. Although enterococci are usually considered extracellular pathogens, persistence can be also attributed to subpopulation of intracellular bacteria **(42)**, as it has been suggested for *Staphylococcus aureus* **(43)**. Addressing persister cells often requires more innovative treatment strategies, such as the use of combination therapies, which aim to target multiple pathways or mechanisms to effectively eradicate these resilient cells and prevent relapse of infection **(41)**.

1.1.3. Mechanisms of environmental persistence

Epidemiological studies indicate that *Enterococcus* can persist for long periods in harsh environmental conditions, including soil, water, food, sewage, plants, and hospitals **[Figure 4] (1)**. Moreover, the Environmental Protection Agency (EPA) uses enterococci as indicators of fecal contamination in brackish and marine waters, owing to their association with gastrointestinal diseases **(44)**.

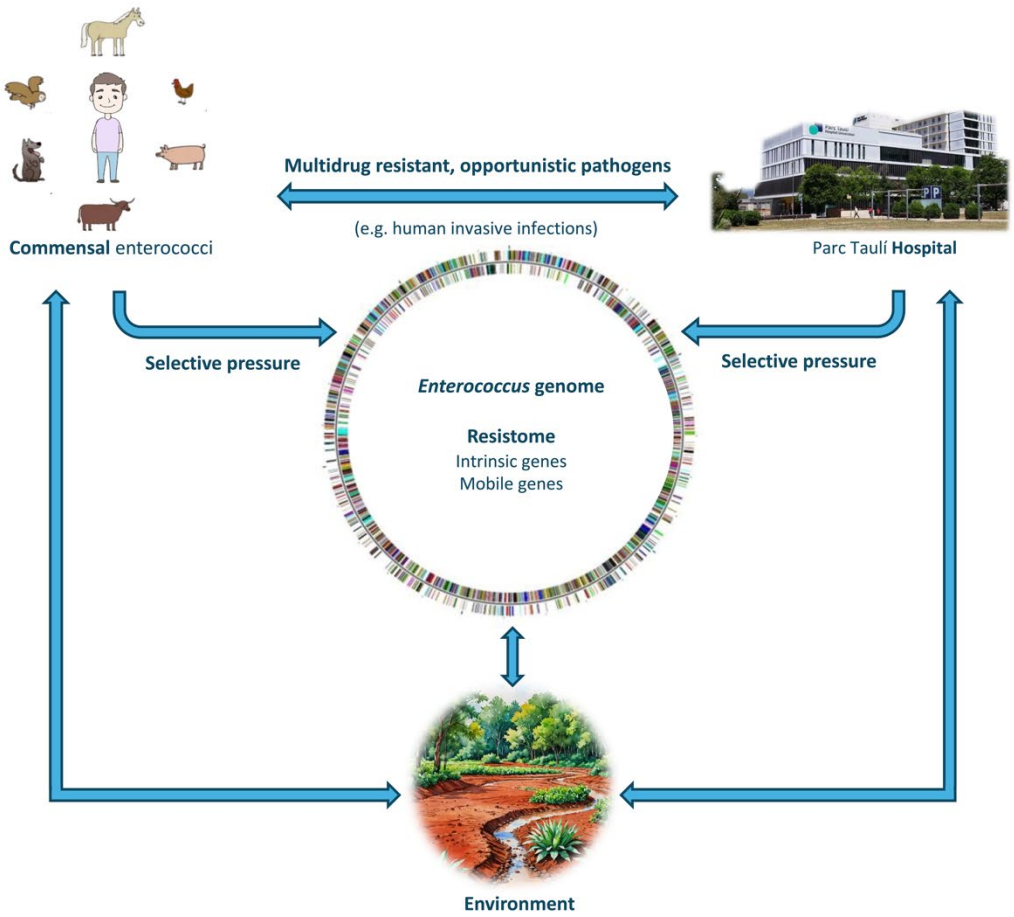


Figure 4. Schematic representation of the widespread distribution of *E. faecium* across diverse niches and the subsequent influence of selective pressure on its resistome. *Source: Own elaboration.*

Enterococci are highly adaptable to diverse environmental conditions, thriving in habitats ranging from aquatic systems to inanimate surfaces where they can persist for extended periods. They have been found to remain viable for months in oligotrophic aquatic environments and survive for years on dry surfaces **(1,45)**. Among the enterococcal species, *E. faecium* demonstrates remarkable resilience to starvation and stress by entering a dormant state as persisters or viable but non-culturable (VBNC) cells **(46)**. This adaptability is further enhanced by the multiple pathways through which enterococci are transmitted in the environment, such as sediment resuspension, runoff from agricultural fields, and fecal contamination from both urban and rural sources, with livestock and wildlife contributing significantly **(47)**. Food products that come into contact with contaminated water or soil are also potential vectors, raising concerns about foodborne transmission of antibiotic-resistant strains **(47)**. It is significant to point out that bacterial strains traverse these ecological niches often transporting plasmid-encoded antimicrobial resistance (AMR) genes, which can spread through environmental reservoirs to humans and animals. Plasmid-encoded toxin-antitoxin (TA) systems play a key role in stabilizing plasmids in bacterial populations, helping them survive in stressful environments through

persistence **(46)**. TA systems support long-term bacterial adaptation by ensuring that resistance plasmids are retained, thus facilitating the persistence and spread of antimicrobial resistance genes across environments **(48)**.

Additionally, environmental metals like copper exert selective pressures that drive the adaptation of enterococci, especially in agricultural settings **(49)**. In poultry production, copper is commonly added to feed as a growth promoter and to improve feed efficiency **(49,50)**. Rebelo and colleagues observed that the use of copper creates oxidative stress in bacteria, which enhances HGT, particularly of MGEs like plasmids, which often carry both copper resistance and antibiotic resistance genes. As a result, the selective pressure from copper not only promotes survival but also drives the co-selection of multidrug-resistant (MDR) strains.

Similarly, cationic biocides (CBs), particularly quaternary ammonium compounds (QACs), also contribute to the adaptation of *Enterococcus* spp. to environmental conditions **(51)**. While both *E. faecalis* and *E. faecium* are generally susceptible to in-use concentrations of QACs, environmental factors such as acidic pH and lower temperatures can enhance their tolerance to these biocides **(52)**. This tolerance is often associated with the acquisition or overexpression of efflux pump genes, which enable the cells to withstand the effects of biocides. As with metals, CBs tolerance genes also frequently share genetic contexts with antibiotic resistance genes, raising concerns about potential co-selection events **(51,52).** Consequently, prolonged exposure to subinhibitory concentrations of CBs can drive the selection of *Enterococcus* strains with increased resilience, thereby enhancing their persistence in ecosystems where these compounds are present **(52)**.

Given these dynamics, it is crucial to recognize the significance of the One Health approach, which emphasizes the interconnectedness of human, animal, and environmental health, advocating for collaborative efforts to address global health challenges **(53)**. This holistic approach is particularly relevant to the fight against AMR, which is closely linked to the misuse and overuse of antibiotics in each of these domains **(54)**. As mentioned, in humans, animals, and the environment, selective pressure from excessive antibiotic use promotes the acquisition of resistance mechanisms by bacteria. These bacteria can not only survive treatment but also proliferate and spread resistance genes through MGEs across species or genera **(55)**.

1.1.4. Pathogenic mechanisms

Enterococci, commonly found as commensals within the GIT, contribute to various metabolic functions, including digestion and nutrient absorption **(56,57)**. Species such as *E. faecium* and *E.*

faecalis are also utilized in probiotic formulations due to their beneficial role in maintaining gut homeostasis and enhancing the host immune response **(58)**. Despite their commensal nature, these species can act as opportunistic pathogens **(47)**. Hospitalized patients are protected by their microbiome from acquiring nosocomial infections; however, the use of broad-spectrum antibiotics, antacids that increase the gastric pH or immunosuppressive drugs are usual disruptors that favour the colonization by VRE and other microorganisms in the hospital setting **[Figure 5] (44)**. For enterococci to cause disease, they must initially overcome colonization barriers imposed by competing microbes and host defenses, including gastric acid and bile, to effectively colonize the GIT **(59)**. From this established reservoir, *Enterococcus* can then proliferate and disseminate to vulnerable sites, leading to infection. The likelihood of infection is directly related to the intestinal burden of bacteria in the gut reservoir, emphasizing the importance of understanding the factors influencing colonization and proliferation of *Enterococcus* in the host **(59)**.

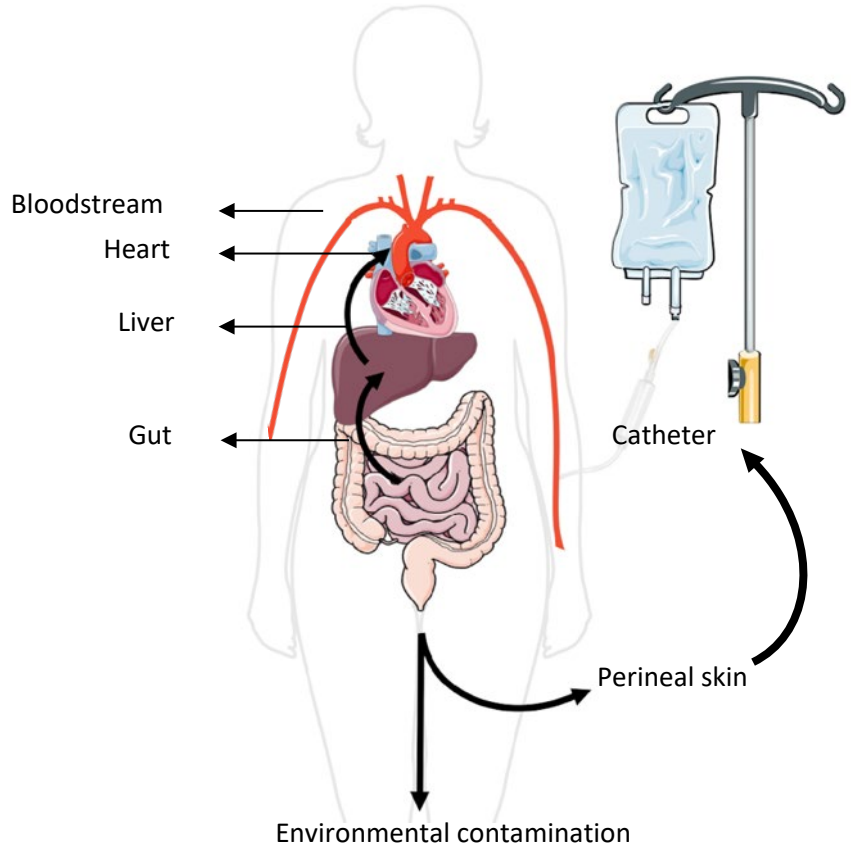


Figure 5. Representation of the pivotal role of the GIT in the initiation and dissemination of enterococcal infection. *Adapted from: Arias & Murray, 2012, Nat Rev Microbiol, 10(4), p.266.*

1.1.4.1. Human colonization

As mentioned, these bacteria are naturally present in the GIT of humans, albeit in relatively low numbers compared to other microbial species **(60)**. The two predominant species, *E. faecalis* and *E. faecium*, have likely been part of the human microbiome for a long time, possibly entering the human lineage early on through dietary sources **(59)**. Their relatively low abundance plays a crucial role in maintaining GIT colonization resistance, a protective mechanism employed by the gut microbiome which prevents the overgrowth of potentially harmful bacteria like *Enterococcus* **(61)**. Higher microbial diversity allows for competitive exclusion, where established gut microbiota outcompete newcomers, such as opportunistic pathogens, maintaining a balanced ecosystem **(59)**. When antibiotics or other disruptions occur, this balance can be disturbed, leading to increased colonization by *Enterococcus* and other pathogens, which underscores the importance of their regulated abundance in sustaining gut health **[Figure 6] (59)**.

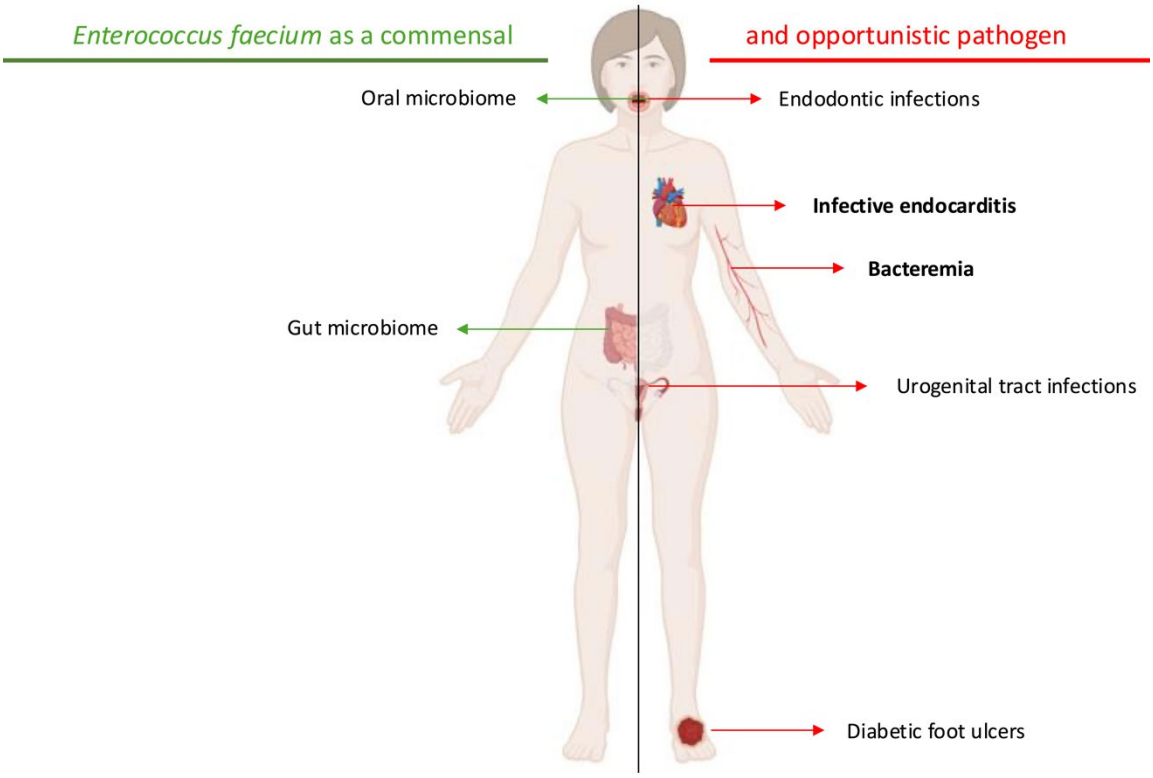


Figure 6. Scheme illustrating the role of *E. faecium* as a commensal organism and as a potential pathogen in humans. *Source: Own elaboration. Created with BioRender.com.*

Several factors have been identified as key to enable GIT colonization **(62)**. These include specific enterococcal cell surface proteins, the ability to adapt to available nutrient sources, the formation of biofilms, and the maintenance of cell envelope integrity to protect against antimicrobial and other substances produced in the intestine **(63).** Most of these functions are largely mediated by genes encoded in the core enterococcal genomes, which aligns with the concept that enterococci have evolved over millennia as GIT commensals and continue to be ubiquitous colonizers of the GIT today. Genome plasticity also plays a key role in GIT colonization, with the acquisition of accessory genes further influencing this process. This ability becomes particularly important in dysbiotic environments, such as the antibiotic-treated GIT. Additionally, gaining the capacity to produce bacteriocins offers a competitive edge, potentially displacing established enterococcal strains, which may lead to new therapeutic strategies to combat antibiotic-resistant enterococci **(62)**.

Thus, enterococci maintain a dual lifestyle, where their strong capacity for GIT colonization allows their transition to a pathogenic state **[Figure 6]**. In clinical settings, where treatments can alter the gut environment, there is an increased risk of *Enterococcus* colonization and subsequent spread to other body sites, potentially leading to infections **(62)**.

Overall, managing the balance of the human microbiome is crucial in preventing the proliferation of hospital-adapted *Enterococcus* strains and reducing the risk of hospital-adapted infections **(59)**. By understanding the dynamics of colonization and the factors that influence the gut microbiome, strategies can be developed to maintain a healthy microbial community and minimize the impact of opportunistic pathogens like *Enterococcus.*

1.1.4.2. Translocation from the gut into the bloodstream

The ability of *Enterococcus* to survive and proliferate within the GIT, as well as to interact with host cells such as intestinal epithelial cells and phagocytes, contribute to its movement across the intestinal barrier **(57)**. This process, known as translocation, enables *Enterococcus* to disseminate to other organs and establish infections **[Figure 7] (64)**. During translocation, *Enterococcus* can be captured by immune cells, including M cells and dendritic cells (DCs), which facilitate the processing of these bacteria for an immune response **(57)**. The mesenteric lymph nodes (MLNs) serve as a critical site to activate an appropriate response. If the immune system responds effectively, the bacteria may be eliminated. However, if the pathogens manage to overcome the defences of the MLNs, either to their virulence or an inadequate immune response, they may gain entry into the circulatory system, allowing for dissemination to other

organs and tissues throughout the body **(34,65)**. The risk of *Enterococcus* translocation into the bloodstream significantly increases under conditions where the gut barrier is compromised, such as during antibiotic-induced dysbiosis or in immunocompromised individuals **(59)**.

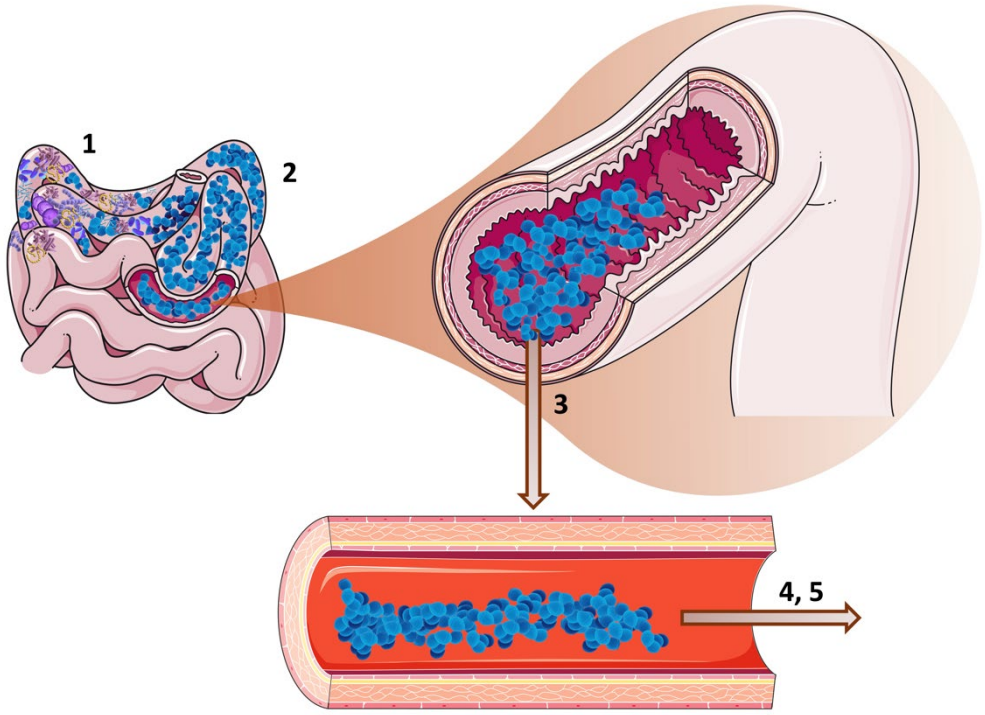


Figure 7. Key stages in the colonization and pathogenicity of *E. faecium*. **(1)** Depletion of the microbiome creates and ecological niche for *E. faecium* reducing microbial diversity and creating a niche for *E. faecium* to **(2)** colonize and dominate the GIT by outcompeting remaining cells. Once established in the GIT, *E. faecium* can **(3)** cross the intestinal barrier, especially when permeability is increased **(4)** entering the bloodstream or other tissues. *E. faecium* spreads to other tissues or organs, **(5)** leading to systemic infections. *Source: Own elaboration. Created with BioRender.com.*

1.1.5. Clinical significance of *Enterococcus* species[3]

Enterococcus species, particularly *E. faecium* and *E. faecalis*, pose a major clinical concern as pathogens capable of causing a range of infections, including urinary tract infections, bacteremia, endocarditis, and wound infections **(66)**. These infections are particularly problematic in vulnerable patient populations, such as those undergoing cancer treatment, organ transplants, or extended hospital stays, contributing to increased morbidity and mortality **(67)**. The etiology and epidemiology of these infections demonstrate their widespread presence and increasing prevalence in both hospital and community settings **(34)**.

Antibiotic resistance further complicates the management of *Enterococcus* infections, with the emergence of VRE posing significant treatment challenges. This resistance is coupled with the

[3] This topic is further elaborated in section *1.5. Infections caused by Enterococcus faecium.*

species' remarkable environmental persistence, which facilitates their survival and potential for infection across various settings **(19,68)**.

1.2. Introduction to *Enterococcus faecium* - Molecular epidemiology

E. faecium is a prominent member of the ESKAPE group (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.), which has been highlighted by the WHO as a leading cause of nosocomial and antibiotic-resistant infections over recent decades **(34)**.

E. faecium represents a significant clinical challenge, not only due to its intrinsic resistance to multiple antibiotic classes but also because of its exceptional ability to acquire additional resistance through *de novo* mutations and/or horizontal gene transfer **[Figure 8]**, enhancing its survival and persistence under selective pressure **(22,69,70)**. This adaptability is particularly evident in the context of VREfm, which has emerged as a significant pathogen in healthcare settings. However, it is essential to also consider the vancomycin-susceptible *E. faecium* (VSEfm) population, which, despite its susceptibility to vancomycin, exhibits considerable genetic diversity and epidemiological relevance **(71)**.

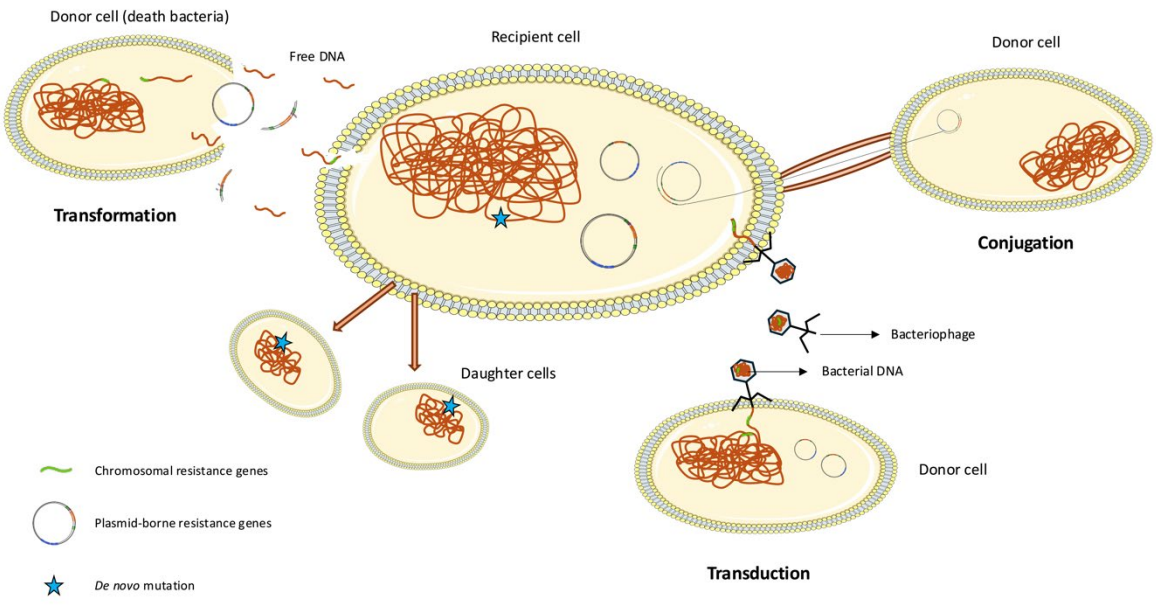


Figure 8. Mechanisms of antibiotic resistance acquisition either through *de novo* mutations or HGT, including conjugation, transduction, and transformation. *Source: Own elaboration. This figure was partly generated using Servier Medical Art, provided by Servier.*

A key aspect of understanding the epidemiology and pathogenicity of *E. faecium* lies in the analysis of sequence types (STs) identified through multi-locus sequence typing (MLST). This method allows for the classification of isolates based on variations in seven housekeeping genes (*atpA*, *ddl, gdh, purK, gyd*, *pstS,* and *adk*), providing insights into their genetic relationships and potential transmission pathways **(72)**. The identification of prevalent STs among both VREfm and VSEfm isolates reveals patterns of genetic diversity, host associations, and geographic distribution. In 2002, Homan and colleagues designed an MLST scheme that permitted identifying a total of 62 STs among the isolates analysed **(73)**. The distribution of these STs varied between VREfm and VSEfm, highlighting significant differences in their genetics and epidemiological characteristics. The most frequently encountered STs among VREfm included: (i) ST17, with a significant number derived from hospitalized patients, mainly from the United Kingdom, France, the United States, and Australia, (ii) ST6, which included representatives from both hospitalized individuals and healthy humans, as well as isolates from pigs, and they all came from the Netherlands and the United Kingdom, (iii) ST4, all of which were originated from calves in the Netherlands, and (iiii) ST16, where isolates were primarily associated with outbreaks in hospitalized patients, with representatives from the Netherlands and the United States. Moreover, Bezdicek *et al*., developed an improved MLST scheme that enabled the detection of new STs, particularly focusing on VREfm **(74)**. On the other hand, VSEfm also plays a crucial role in the overall epidemiology of *E. faecium* **(73)**. Different STs were identified by Homas, *et al*., indicating that VSEfm can be found in various ecological niches. The predominant STs identified within VSEfm strains, such as ST4, ST5, ST6, ST17, and ST44, were also shared with VREfm isolates, suggesting that there may be a genetic overlap and potential for HGT between these populations.

From a clinical perspective, MDR *E. faecium* infections are particularly concerning due to their association with vulnerable patient populations **(67)**. These infections are associated with higher mortality and recurrent bacteremia, complicating patient management and outcomes. Treatment options are notably limited and remain a subject of ongoing debate within the medical community **(75)**.

1.3. *Enterococcus faecium* genomics

1.3.1. Transmission and evolution

E. faecium strains are categorized into several clades based on their epidemiological and ecological contexts **(46,76).** Clade A includes clinical strains, further divided into A1, which are predominantly hospitalized-associated, and A2 strains, which are acquired from animals and sporadic human infections. In contrast, clade B (now re-classified as *E. lactis*, as previously mentioned) consists of commensal strains found primarily in non-clinical environments **[Figure 9]**. The interaction between different clades and environmental reservoirs significantly impacts the transmission dynamics of *E. faecium* **(46)**.

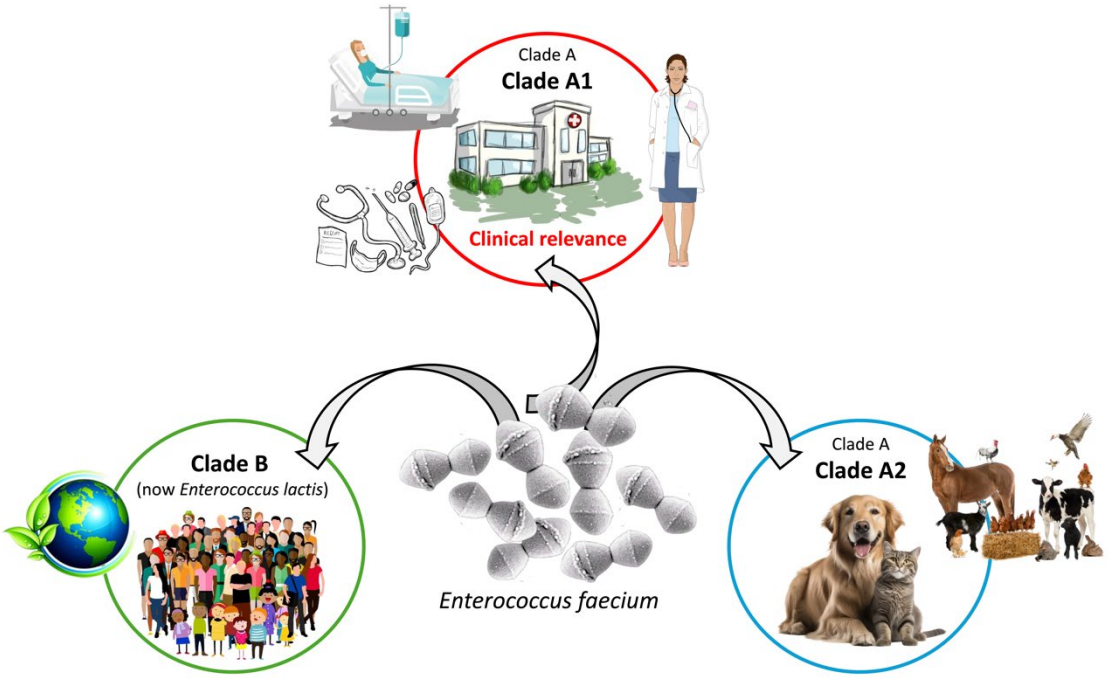


Figure 9. Diagram illustrating *E. faecium* clades (A1, A2, and B) and their respective ecological environments. *Source: Own elaboration.*

Transmission of *E. faecium* poses significant challenges in healthcare environments, especially among immunocompromised patients **(77)**. Key findings indicate that a substantial proportion of patients acquired *E. faecium* during their hospital stay. Specifically, genomic analysis revealed that 63.0% of patients who provided multiple stool samples developed carriage after admission. Epidemiological and genomic linkages were established among patients showing strong connections to at least one other patient in their immediate environment, suggesting that direct patient-to-patient transmissions, as well as environmental contamination, are significant contributors to *E. faecium* spread.

Additionally, wastewater treatment plants, which collect and mix water from diverse sources, exhibit a wide variability of *E. faecium* strains from different clades **(46)**. These settings contribute to the dissemination of various *E. faecium* strains by providing a medium through which genetic material can spread among different populations. Despite the presence of clade A1 strains in both animal and wastewater environments, transmission between A2 animal-derived strains and A1 hospital-associated (HA) strains remains limited. Additionally, geneflow between clades A1 and B is constrained, suggesting that while these clades may inhabit overlapping environments, their genetic exchange is minimal.

Regarding the genomic evolution of *E. faecium*, the process is driven by multiple factors such as gene gain, loss, and rearrangement, facilitated by horizontal gene transfer and recombination events **(46)**. Its genomic size varies from 2.23 Mbp to 3.72 Mbp, indicating considerable genomic plasticity. Among the clades, A1 shows the highest degree of genomic plasticity and harbours the most extensive collection of MGEs compared to clades A2 and B. Mutations and horizontal gene transfer are key contributors to the genomic evolution of *E. faecium*. Specific mutations, such as those in the 23S ribosomal RNA gene conferring resistance to linezolid, or mutations in the *pbp5* gene associated with β-lactam resistance, play a significant role in developing antibiotic resistance.

Transfer of chromosomal fragments is rare among bacteria, yet it appears to be quite frequent in *E. faecium*, with significant implications for antibiotic resistance and adaptability to changing environments **(78)**. Evidence suggests that the genetic region containing *pbp5* may act as a hotspot for HGT, facilitating the exchange of genetic material among strains. This region is characterized by a high frequency of insertion sequences, which could enhance recombination events **(46)**. Horizontal gene transfer enhances the genetic diversity by incorporating MGEs, such as plasmids, transposons, genomic islands, and bacteriophages, which together are referred to as the mobilome.

1.3.2. Mobile genetic elements identified in *Enterococcus faecium*

Bacteria can rapidly acquire and donate new genes and phenotypes through HGT, which involves the transfer of DNA from one organism to another **(79)**. There are three well-studied types of HGT: (i) transformation, the natural ability to take up exogenous DNA from the environment; (ii) transduction, the transfer of DNA from one cell to another via bacteriophages; and (iii) conjugation, the contact-dependent, unidirectional transfer of DNA from a donor to a recipient. Plasmids and transposons are key MGEs that contribute to the spread of antibiotic resistance

[Figure 10] (46). This section will highlight their role, emphasizing their importance in HGT and the acquisition of resistance traits in bacterial populations.

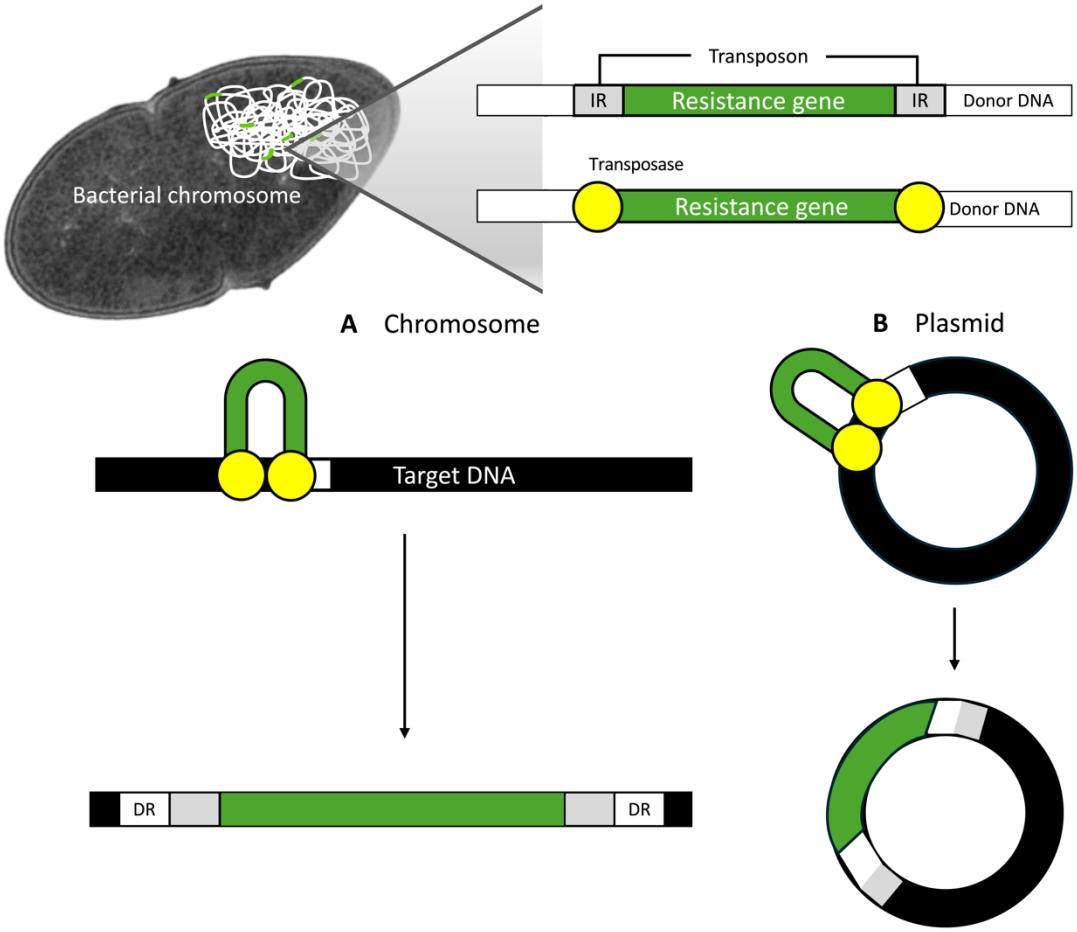


Figure 10. Role of MGE in HGT. Resistance genes located within transposons can either **(A)** integrate into different locations within its or other bacterial chromosomes, or **(B)** be transferred to plasmids. IR; Inverted Repeats, DR; Direct Repeats. *Source: Own elaboration.*

1.3.2.1. Transposons

Among the diverse range of MGEs, integrative and conjugative elements (ICEs), commonly referred to as conjugative transposons, constitute a significant category **(79)**. These elements are distinguished by their integration into the host genome and their capacity to encode a functional conjugation system which mediates their transfer to recipient cells. A prototypical example is Tn916, a well-characterized ICE that confers resistance to tetracycline, predominantly encoding efflux pumps, specifically *tet(K)* and *tet(L)*, as well as ribosomal protection proteins, such as *tet(M)*, *tet(O)*, and *tet(S)* **(79,80)**. Additionally, Tn3-type transposons represent another class of transposons, with Tn917, which encodes erythromycin resistance **(81)**. Furthermore, Tn1546, which encodes vancomycin resistance, has also been extensively

studied for its pivotal role in the dissemination of vancomycin resistance within *E. faecium* **(81,82)**.

1.3.2.2. Plasmids

Plasmids are autonomous genetic elements that can replicate independently of chromosomal DNA, acting as vehicles for antimicrobial resistance and virulence genes **(46,82,83)**. In *E. faecium*, plasmids are categorized into four main groups based on sequence homology in their replication initiator proteins (RIP): RepA_N, Inc18, RCR, and Rep_3 **(84)**. The RepA_N group encompasses a diverse range of plasmids, including both conjugative and non-conjugative types, that can vary significantly in size, ranging from approximately 3 kb to the 281 kb plasmid pLG1 found in *E. faecium*. Inc18 plasmids are important mediators of antibiotic resistance genes, such as *vanA* within Tn1546 insertions, conferring resistance to vancomycin, chloramphenicol, and MLS antibiotics. Prominent members of this group, including broad host range plasmid pAMβ1 (*E. faecalis*), plP501 (*Streptococcus agalactiae*), and sSM19035 (*Streptococcus pyogenes*), are essential in transferring vancomycin resistance from VRE to methicillin-resistant *S. aureus* (MRSA). RCR plasmids, originally described in *S. aureus* and widespread across Gram-positive bacteria, are less common in *Enterococcus*. Finally, Rep_3 plasmids include the well-characterized plasmids such as F, P1, and pSC101 and are also present in *E. faecium* as plasmids pMBB1, pDT1, and pClZ2.

To elucidate the role of plasmids in ampicillin resistance, it is crucial to investigate how these genetic elements contribute to the emergence and dissemination of this antibiotic resistance **(19)**. While the specific mechanism of plasmid-mediated conjugative transfer of ampicillin resistance remains undefined, studies have suggested potential pathways. Rice *et al*., demonstrated that chromosomally-encoded *pbp5*, a key determinant of ampicillin resistance, can be transferred between *E. faecium* strains through conjugation **(85)**. Further, although specific details of plasmid-mediated transfer are still under investigation, it has been proposed that mobilization might be facilitated by co-integrated enterococcal conjugative plasmids **(86)**.

The significance of plasmids in antibiotic resistance is further highlighted by their distribution among different clades of *E. faecium*. Strains from clade A1 are characterized by an expanded plasmidome, indicative of a higher diversity of plasmids that are crucial for antibiotic resistance, virulence, and adaptation to host environments **(46)**. The distribution of plasmids is strongly correlated with the strain's isolation environment. Additionally, the presence or absence of CRISPR-Cas systems significantly impacts genomic variability by affecting the regulation of

foreign DNA entry, which in turn contributes to the observed diversity among *E. faecium* clades. Clade A1 strains, which mostly lack CRISPR-Cas systems, possess larger genomes with a greater number of MGEs. In contrast, clade B strains (now *E. lactis*), which contain some CRISPR-Cas systems, have shorter genomes with fewer MGEs.

1.4. Stress adaptation and pathogenesis[4]

E. faecium has become a leading example of rapid bacterial adaptation to antimicrobial pressure, driven by its impressive capacity to develop resistance mechanisms **(46)**. This specie has been resistant to penicillin since the 1940s, with its MIC to this antibiotic increasing substantially over the years **(87,88)**. One of the main drivers of this resistance is the *pbp5* gene, which encodes a PBP that reduces the efficacy of β-lactam antibiotics. Mutations in the *pbp5* significantly alter the binding affinity for β-lactams, rendering *E. faecium* highly resistant to this class of drugs **(89)**. Additionally, the overexpression of *pbp5* amplifies this resistance, making treatment with β-lactams particularly challenging **(90)**.

What is particularly concerning is that *pbp5* is located on a transferable chromosomal region, which allows the horizontal transfer of resistant alleles between strains **(91)**. This has facilitated the spread of resistance within hospital settings, where *E. faecium* can exchange genetic material, leading to the emergence of MDR strains **(78)**. Phylogenetic analyses of the *pbp5* alleles have revealed that these gene clusters are a manner consistent with the evolutionary structure of their host genomes, suggesting ongoing diversification within clinical isolates of *E. faecium*.

Among the various lineages, clade A1 is particularly notorious for harbouring the higher number of antibiotic resistance genes **(46)**. Strains within this clade are often resistant to aminoglycosides and fluoroquinolones, further complicating treatment regimens. The rapid accumulation of resistance genes in this clade underscores the species' adaptability and capacity for persistence in hospital environments.

In recent years, *E. faecium* has also developed increasing tolerance to last-resort antibiotics such as daptomycin and linezolid **(92)**. Studies have identified daptomycin-resistant mutants within the gastrointestinal tracts of patients receiving intravenous daptomycin therapy, highlighting how *E. faecium* can adapt to treatment pressures in real time **(46,93)**.

[4] This has been explained in detail in section *1.1.4. Pathogenic mechanisms*.

Beyond traditional antibiotic resistance, *E. faecium* has developed tolerance to other agents such as alcohol and antiseptics like chlorhexidine[5] **(94,95)**. This ability to withstand a range of antimicrobial agents is closely tied to the species' intricate stress detection and response systems, which enable them to sense environmental threats and activate protective mechanisms, ensuring its survival in hostile conditions. The three-component system LiaFSR and the two-component-system (TCS) VanSR have been long recognized for their roles in resistance **(46,96)**. However, recent studies have highlighted additional systems involved in *E. faecium*'s stress response, such as the three-component system BsrXRS, implicated in resistance to bile salts, and several TCS, including YycFG, that contributes to daptomycin resistance, ChtRS, associated with tolerance to chlorhexidine, and CroRS, that detects cell wall stress **(96,97,98).** CroRS system is homologous to the TCS CroRS in *E. faecalis* and plays a significant role in mediating *E. faecium*'s resistance to cell wall-targeting antibiotics **(98,99,100,101)**. Kellogg S. L. and colleagues demonstrated that the deletion of CroRS orthologs in *E. faecium* results in increased susceptibility to antibiotics that target the cell wall **(100,102)**. Interestingly, some of these antibiotics were found to activate CroRS signalling in wild-type strains, indicating its role in stress response **(98,101)**. Additionally, the CroRS system is essential for *E. faecium*'s resistance to cephalosporins and ampicillin, which is mediated by the overexpression of PBP5 and the expression of a PBP5 variant that enhances resistance to β-lactams **(100)**. Therefore, it is essential for maintaining intrinsic resistance to a range of cell wall-targeting antibiotics, including β-lactams and vancomycin.

Another major challenge in controlling *E. faecium* infections is the phenomenon of cross-resistance, where resistance to one antibiotic may confer resistance to others **(103)**. This is particularly evident in tetracycline resistance, mediated by the *tet(M)* and *tet(L)* genes, which can also promote resistance to tigecycline **(46)**. Furthermore, recent clinical studies have identified daptomycin-resistant strains linked to the prophylactic use of rifaximin, a transcription inhibitor. This cross-resistance is driven by mutations in *rpoB*, a gene critical to RNA polymerase function.

Ultimately, the accumulation of multiple resistance mechanisms, combined with sophisticated stress response systems, has positioned *E. faecium* as a highly adaptable and resilient pathogen.

[5] This has been explained in detail in section *1.1.4. Pathogenic mechanisms*.

1.5. Infections caused by *Enterococcus faecium*

E. faecium causes a variety of infections, including endocarditis, urinary tract infections, wound infections as well as concurrent bacteremia **(66)**.

1.5.1. Bacteremia: Clinical features and challenges

Bacteremia, the presence of bacteria in the bloodstream, is a serious condition that can lead to systemic infections **[Figure 11]**. Enterococcal bloodstream infections (BSIs) represent the second leading cause of Gram-positive bacterial bloodstream infections in Europe and are associated with high mortality rates, particularly in hospital settings, resulting in significant in-hospital mortality **(104)**. In United States and European hospitals, *E. faecalis* and *E. faecium* account for 10.6% and 9.4% of BSIs, respectively, making them the second and the third most prevalent pathogens following *S. aureus*. Factors contributing to enterococcal bacteremia include translocation of bacteria from the gut into the bloodstream and other routes such as intravenous lines, and urinary tract infections **(59)**. The removal of infected medical devices and the detection of metastatic infection are essential for effectively addressing the infection, along with the initiation of antibiotic therapy **(105)**. The most common regimen for VREfm BSIs includes linezolid and daptomycin **(105,106)**. However, daptomycin treatment is increasingly associated with therapeutic failure, and recent studies suggest that the MIC breakpoints for daptomycin in *E. faecium* should be re-evaluated **(105,107)**. This concern is underscored by the rising incidence of enterococcal BSIs, emphasizing the need for better management strategies.

Importantly, risk factors for mortality in enterococcal bacteremia include illness severity, patient age, and specific antibiotic use **(59)**. Additionally, the specific enterococcal lineage causing BSIs may impact patient outcomes, with certain strains associated with higher mortality rates. Therefore, understanding the mechanisms and routes of enterococcal bacteremia is crucial for effective prevention and treatment. Ultimately, early detection and appropriate management are essential for improving outcomes in patients with enterococcal bloodstream infections.

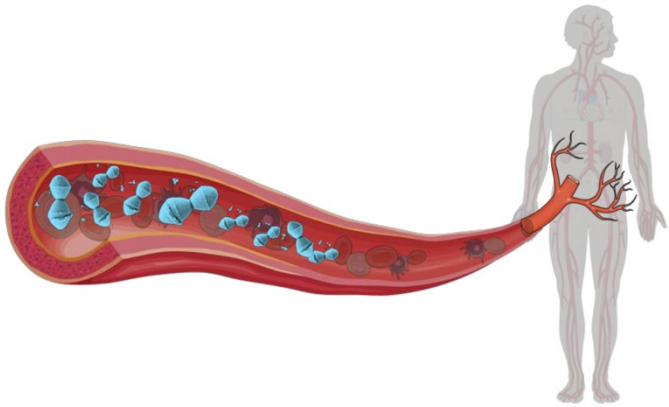


Figure 11. Representation of bacteremia, showing the presence of bacteria in the bloodstream and the subsequent dissemination of infection throughout the body. *Source: Own elaboration. This figure was partly generated using Servier Medical Art, provided by Servier.*

1.5.2. Infective Endocarditis: Clinical features and challenges

Infective endocarditis (IE) is a severe and challenging infection in which the bacteria colonize the endocardium, leading to the formation of vegetations and biofilms on heart valves **[Figure 12]**. This biofilm formation not only protects the bacteria from the host immune system but also from antibiotic treatment **(59)**. Considering that β-lactams exhibit bacteriostatic effect in enterococcal infections, an aminoglycoside is typically added to the treatment regimen **(108)**. Common first-line combinations include ampicillin or vancomycin plus gentamicin. However, care must be taken regarding the nephrotoxicity associated with aminoglycosides **(105)**. Furthermore, recent studies demonstrate the effectiveness and safety of other combination therapies, particularly in the treatment of IE caused by strains with high-level resistance to aminoglycosides (HLRA), such as the combination of ampicillin and ceftriaxone **(105)**.

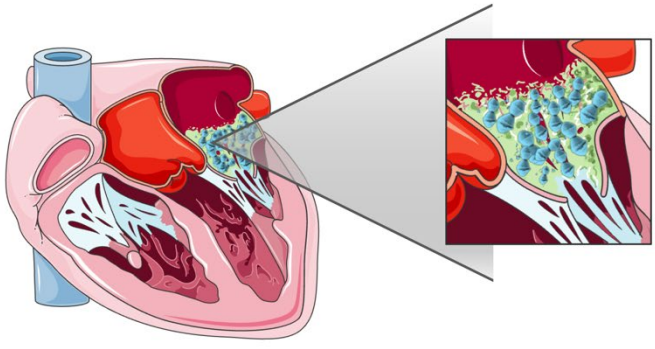


Figure 12. Representation of endocarditis, showing the bacterial colonization of heart valves and the subsequent formation of vegetations. *Source: Own elaboration. This figure was partly generated using Servier Medical Art, provided by Servier.*

In relation to the virulence of enterococci in endocarditis, *E. faecalis* exhibits several well-studied virulence factors in endocarditis, such as adhesins that facilitate adherence to heart tissue and contribute to biofilm development **(84)**. The pili encoded by the Ebp cluster are also crucial for adhesion and biofilm formation **(109)**.

Conversely, the virulence factors of *E. faecium* in IE are less well characterized. Notable factors include the surface protein Eso and the collagen-binding protein Acm, which have been implicated in pathogenicity in rat infection models. Deletion of these genes results in significantly reduced bacterial recovery in animal infections models. Additionally, antibodies against these proteins have been detected in patients with *E. faecium*-related IE. Targeting the adhesive properties and biofilm development mechanisms of *E. faecium* may offer promising therapeutic strategies for managing this infection **(110)**.

1.6. β-lactam antibiotics

1.6.1. Classification and types of β-lactam antibiotics

The rising incidence of β-lactam resistance among the ESKAPE pathogen strains, such as *E. faecium,* poses a significant public health challenge **(111).** The management of severe enterococcal infections is often complicated by both intrinsic and acquired resistance to first-line antibiotics, including β-lactams **(112)**. They are among the most widely prescribed drugs, having revolutionized the treatment of bacterial infections since their introduction in the 1940s **(113)**. Their effectiveness in combating a wide range of infections transformed the medical approach to infectious diseases, marking a significant breakthrough. However, despite their continued extensive use, antimicrobial resistance has emerged as a major global health issue, threatening their efficacy **(114)**.

β-lactam antibiotics are characterized by their highly reactive β-lactam ring **(113)**, and this group encompasses various classes, including:

Penicillins: which are bactericidal agents that inhibit bacterial cell wall synthesis. Notably, Penicillins G (benzylpenicillin) and V (phenoxymethylpenicillin) are primarily effective against Gram-positive bacteria **(115)**. Penicillin G has historically been used to treat severe streptococcal infections while penicillin V is commonly used for mild to moderate infections caused by *Streptococcus* often in outpatient or pediatric settings **(116)**. Amoxicillin and ampicillin (also known as second-generation penicillins) have broader activity, targeting enterococci and some Gram-negative bacilli like *Haemophilus influenzae* and *Escherichia coli*, with β-lactamase inhibitors extending their spectrum to include methicillin-sensitive *S. aureus* (MSSA) **(117)**. Penicillinase-resistant penicillins, also classified as second-generation penicillins, are semisynthetic antibiotics derived from natural penicillins, designed to combat β-lactamase. While less active than penicillin G, these antibiotics still inhibit bacterial cell wall synthesis and are used to treat moderate to serious infections caused by β-lactamase-producing staphylococci

(118). Lastly, third- and fourth-generation penicillins exhibit increased activity against Gram-negative bacteria, with the fourth-generation expanding its efficacy to include species such as *Klebsiella, Enterococcus, Pseudomonas aeruginosa,* and *Bacteroides fragilis* **(119).**

Cephalosporins: which are bactericidal agents with broad-spectrum activity against both Gram-positive and Gram-negative bacteria **(120)**. They are categorized into five generations, each with specific structural differences and antimicrobial activity **[Figure 13] (121)**.

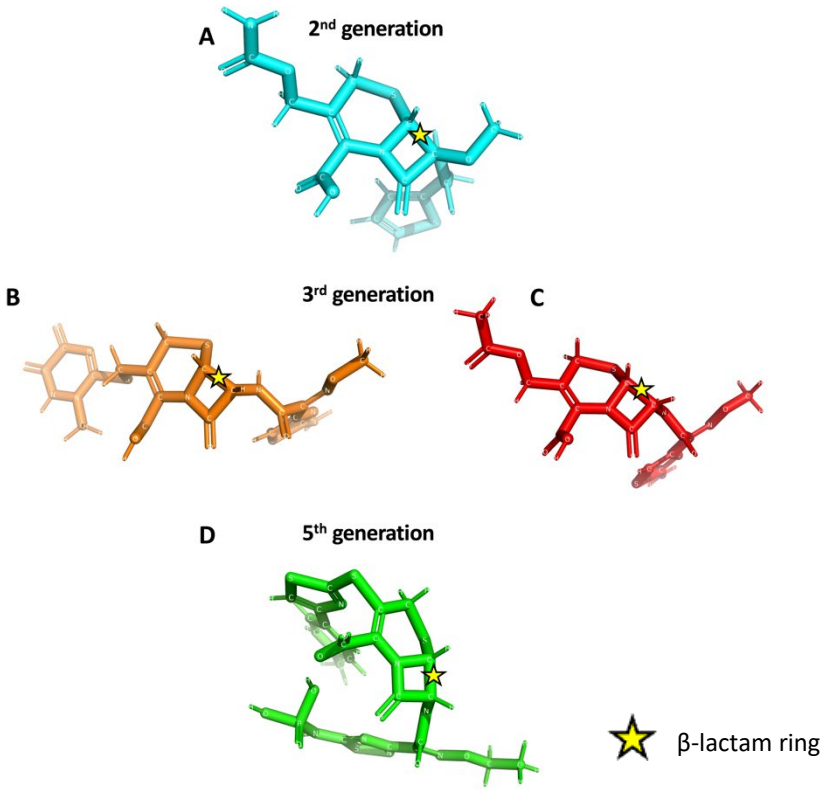


Figure 13. 3D structural representation of four cephalosporins **(A)** cefoxitin, **(B)** ceftriaxone, **(C)** cefotaxime, and **(D)** ceftaroline. These cephalosporins are selected for their relevance in this thesis. *Source: Own elaboration. Antibiotic structures were visualized and analysed using PyMOL.*

The modifications to the β-lactam ring and side chains of each generation not only influence the binding affinity to PBPs but also determine their susceptibility to β-lactamases. First-generation cephalosporins, such as cefazolin and cephalexin, are most effective against Gram-positive bacteria like *Staphylococcus* and *Streptococcus*. They are used mainly for skin infections and surgical prophylaxis **(122)**. Second-generation cephalosporins, including cefoxitin and cefuroxime, are less effective against Gram-positive bacteria than first-generation cephalosporins but expand their activity to include some Gram-negative bacteria, like *Haemophilus influenzae,* and certain anaerobes. These agents are commonly used to treat mixed infections (e.g., intra-abdominal) and respiratory tract infections. Third-generation cephalosporins, like ceftriaxone and cefotaxime, are the most commonly prescribed group,

exhibiting broad-spectrum activity on both Gram-positive and Gram-negative bacteria **(123)**. They are frequently used for serious hospital infections, including meningitis and pneumonia **(122)**. Fourth generation cephalosporins, like cefepime, show extended activity against more resistant Gram-negative bacteria, including *Pseudomonas* and *Enterobacterales* that overexpress *AmpC β-lactamases* **(121)**. Moreover, MRSA is a major cause of bloodstream infections, and fifth generation cephalosporins, such as ceftaroline and ceftobiprole, are the only β-lactams effective against it **(124)**. Additionally, ceftaroline and ceftobiprole are active against penicillin-resistant streptococci, with their activity against other Gram-positive cocci and Gram-negative bacilli being similar to that of third-generation cephalosporins **(121)**. Lastly, ceftazidime-avibactam, ceftolozane-tazobactam, and cefiderocol are novel β-lactam antibiotics developed to combat MDR Gram-negative bacteria **(125)**. Ceftazidime-avibactam and ceftolozane-tazobactam, both combining a third-generation cephalosporin with a β-lactamase inhibitor, display an antimicrobial spectrum of activity against MDR Gram-negative bacteria, including *P. aeruginosa* **(126)**. Based on results from clinical trials, these combinations demonstrated clinical efficacy in treating complicated intra-abdominal and urinary tract infections. In addition, cefiderocol is a novel siderophore cephalosporin that employs iron uptake mechanisms for bacterial entry, exhibiting broad-spectrum activity against *P. aeruginosa* and metallo-β-producing *Enterobacteriaceae* **(127)**.

Monobactams: which are parenteral β-lactams antibiotics with aztreonam being the sole available agent in this class **(128)**. Aztreonam exhibits activity comparable to ceftazidime against *Enterobacterales* that do not produce AmpC β-lactamase, extended-spectrum β-lactamase (ESBL), or carbapenemase-producing *Enterobacterales*, as well as *Pseudomona aeruginosa* **(116)**. It demonstrates synergistic effects with aminoglycosides, a characteristic shared by all β-lactams, while also exhibiting a low risk of cross-hypersensitivity with other β-lactams **(129)**. It is particularly useful for treating severe aerobic Gram-negative infections in patients with significant β-lactam allergies **(130)**.

Carbapenems: including ertapenem, imipenem, and meropenem, are parenteral β-lactam antibiotics with broad spectrum of activity against Gram-negative bacilli, anaerobes, most *Enterobacterales* (including those producing AmpC and ESBL), MSSA, and streptococci **(131)**. Notably, imipenem, and meropenem are particularly active against *P. aeruginosa* **(132)**. However, they prove to be ineffective against *E. faecium, Stenotrophomona maltophilia*, and MRSA.

1.6.2. Mechanisms of action of β-lactam antibiotics

β-lactam antibiotics exert their antimicrobial effects by targeting the synthesis of the bacterial cell wall, a crucial structure known as murein (or peptidoglycan) **(113)**. It is a crucial component of the bacterial cell wall located outside the cytoplasmic membrane in nearly all bacteria. Its primary role is to maintain cell integrity, which means that the inhibition of its synthesis, whether through mutations or antibiotics, or degradation by enzymes, leads to cell lysis **(133)**.

Peptidoglycan synthesis takes place in the cytoplasm and in the inner and outer side of the cytoplasmic membrane **(134).** It involves multiple enzymatic steps, predominantly executed by proteins encoded within the cell division operon. Crucial to peptidoglycan assembly is the cross-linking of peptide chains, facilitated by PBPs **[Figure 14]**. These PBPs catalyse the formation of cross-links by catalysing the reaction between terminal D-alanines and lysine residues on adjacent peptide chains **(135)**.

β-lactam antibiotics specifically inhibit the final step of peptidoglycan synthesis by acetylating the transpeptidase enzyme, thereby obstructing the cross-linking process **[Figure 14] (116)**. The β-lactam ring of these antibiotics closely mimics the structure of the terminal D-alanine residues, allowing them to competitively bind to PBPs. This mimicry causes the PBPs to erroneously bind to the antibiotic instead of the peptidoglycan precursor, leading to irreversible inactivation of the enzyme. Consequently, the synthesis of a functional cell wall is impaired, resulting in bacterial cell lysis and death **(113)**.

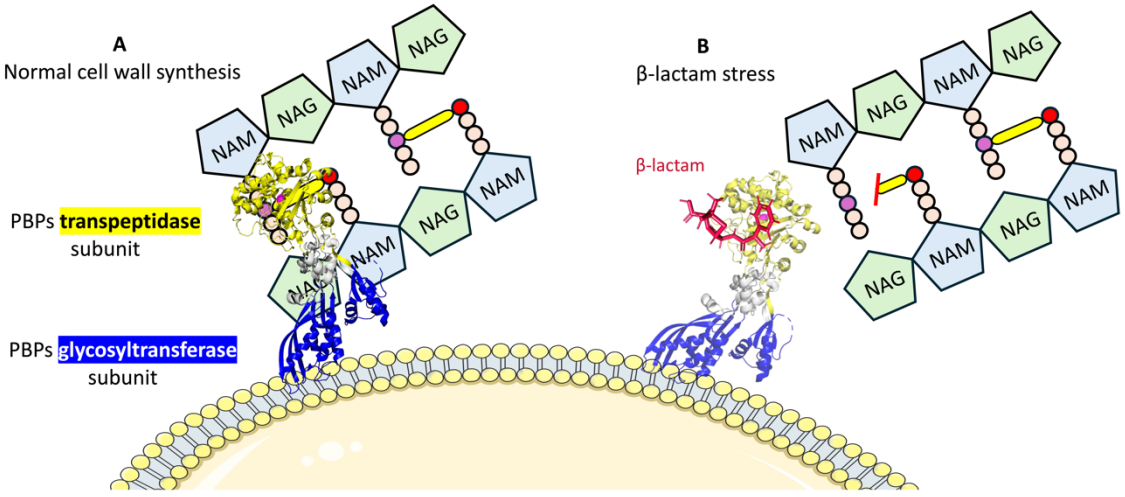


Figure 14. Cell wall formation mediated by PBPS **(A)** under normal conditions where the cross-linking of peptide chains occurs whereas **(B)** in the presence of stress, such as exposure to β-lactam antibiotics, the antibiotic mimics the natural substrate of PBPs, leading to irreversible binding at the active site serine of the enzyme, disrupting the normal synthesis of the bacterial cell wall. *Source: Own elaboration. The PDB-generated model for PBP5 (6MKG) was visualized and analysed using PyMOL.*

1.7. Mechanisms of resistance to β-lactams in *Enterococcus faecium*

E. faecium exhibits both intrinsic and acquired types of antibiotic resistance **(136)**. Intrinsic resistance refers to the inherent ability of the bacteria to resist β-lactams due to their natural biological characteristics, while acquired resistance involves the acquisition of resistance determinants through horizontal gene transfer or *de novo* mutations.

PBPs are central to bacterial cell wall synthesis and serve as the primary targets of β-lactam antibiotics **(135)**. PBPs are named for their ability to bind penicillin and other β-lactam antibiotics, which inhibit their activity. They are essential for bacterial growth and division because they are involved in the synthesis of peptidoglycan, a key component of the bacterial cell wall. PBPs are categorized into two types: type A PBPs (aPBPs) and type B PBPs (bPBPs). aPBPs possess both transpeptidase activity (responsible for cross-linking steps) and glycosyltransferase activity. In contrast, bPBPs exclusively exhibit transpeptidase activity **(137)**. Although PBPs are the principal target of β-lactams, they are not the only factors involved, as discussed in detail below.

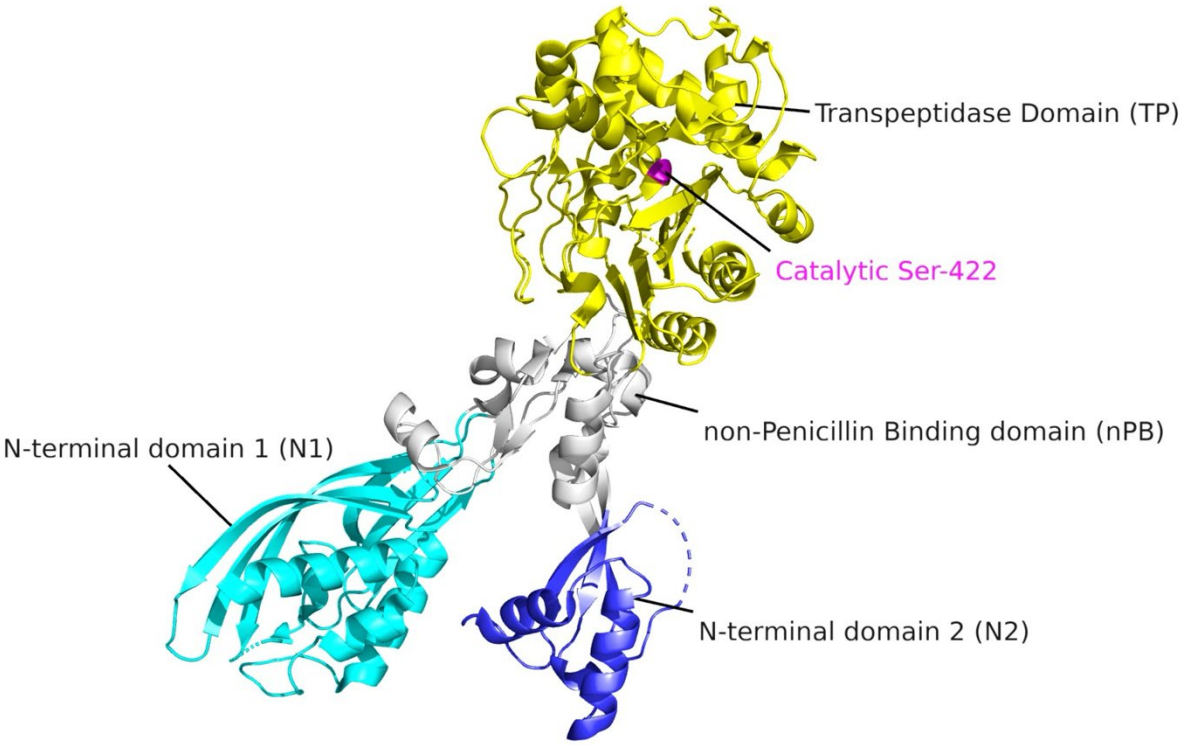


Figure 15. Representation of the crystal structure of PBP5, highlighting its four interconnected domains: N-terminal domain 1 (N1), N-terminal domain 2 (N2), non-Penicillin Binding (nPB), and the TP domain. *Source: Own elaboration. The PDB-generated model (6MKG) was visualized and analysed using PyMOL.*

Penicillin-Binding Protein 5 (PBP5), the main PBP conferring β-lactam resistance in *E. faecium*, is a low-affinity PBP that plays a crucial role, serving as the primary determinant of ampicillin resistance in *E. faecium*. Other PBP5 homologs associated with β-lactam resistance include PBP4 in *E. faecalis* and PBP2a in *S. aureus* **(111).** PBP5 consists of four interconnected domains **[Figure 15]**. The N-terminal domain 1 (N1), acts as a structural connector between two key parts of the protein. The membrane-inserting helix, which is a region that helps anchor the protein to the bacterial cell membrane, with the transpeptidase (TP) domain, that is responsible for the enzyme's activity in synthesizing the bacterial cell wall by catalysing the cross-linking of peptidoglycan strands. However, the function of the N-terminal domain 2 (N2) remains unknown. Lastly, the non-Penicillin Binding (nPB) domain connects the N1 and N2 domains to the TP domain, which contains the active site (Ser-422). Amino acid substitutions in the C-terminal transpeptidase domain have been associated with a decreased affinity for penicillin-binding, resulting in elevated β-lactam MICs **(90).** The serine residue at position 422, located at the N-terminus on helix α-2, acts as a nucleophile, providing electrons to attack the β-lactam ring of antibiotics like ampicillin **(111)**. Additionally, the active site includes an oxyanion hole that stabilizes negatively charged intermediates produced during the β-lactam ring cleavage. Moreover, the active site groove, where β-lactam antibiotics bind and react, is closed above by the "lid" and below by the C-terminal helix **[Figure 16]**.

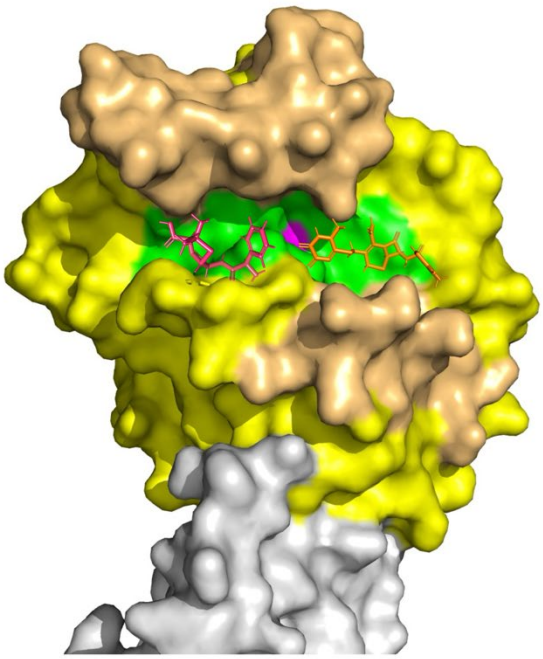


Figure 16. Visualization of the active site of PBP5, featuring the serine residue at position 422 (highlighted in purple). The active site groove, where the β-lactam antibiotics bind (indicated in green), is bounded above by the "lid" (top region marked in light orange) and below by the C-terminal helix (bottom region marked in light orange), along with the representations of ampicillin (purple molecule) and ceftriaxone (orange molecule) situated within the active site. *Source: Own elaboration. The PDB-generated model (6MKG) was visualized and analysed using PyMOL.*

Clades A1, A2, and B carry different allelic variants of *pbp5* **(138)**. Subclade A1 primarily contains the *pbp5*-R allele, which confers high ampicillin resistance (MIC≥16 mg/L), while A2 exhibits a mixture of *pbp5*-S/R alleles, associated with intermediate MIC levels, typically ranging around 4 mg/L **(139)**. Clade B, associated with lower ampicillin resistance (MIC≤2 mg/L), carries the *pbp5*-S allele **(90)**. The PBP5 proteins encoded by these alleles exhibit significant variability, particularly within the 20 to 21 amino acid positions that distinguish *pbp5*-S from *pbp5*-R **(140)**. This variability is crucial in determining the level of ampicillin resistance. Specific amino acid substitutions, specifically those near the active site of the enzyme, have been linked to increased resistance **(139,141,142,143)**. For example, substitutions such as methionine-to-alanine at position 485 enhance ampicillin MICs **(139,142,143)**, while others like valine at position 629 have a minimal impact unless combined with additional substitutions **(143)**. Furthermore, the distribution of PBP5 variants across different clades highlights the evolutionary dynamics of *E. faecium* **(78)**. The presence of mixed PBP5-S/R sequences in subclade A2 strains and unique variants in specific strains underscores the ongoing adaptation and diversification of this pathogen in response to antimicrobial pressures **(78,139)**. The identification of unique amino acid changes in highly resistant strains, such as EnGen0052 which lacks the typical high-resistance-associated substitutions, indicates that novel mechanisms may contribute to elevated ampicillin MICs **(139)**.

However, these allelic variants alone do not fully explain the observed phenotypes **(139,142)**. Strains with identical PBP5 sequences can exhibit different ampicillin MICs. Additional factors, such as the overall expression of PBP5, are also involved in resistance **(144)**. Subclade A1 and A2 ampicillin-resistant (AMP-R) strains tend to exhibit higher PBP5 expression levels. In addition, the *pbp5* gene is part of an operon that includes two upstream genes, *ftsW* and *psr* **[Figure 17]**, which further influence resistance **(145)**. Notably, the upstream region of *pbp5* has been implicated in ampicillin resistance, with alterations such as insertions and deletions affecting the promoter region and potentially impacting PBP5 expression **(90,145)**. Rice and colleagues provide evidence of extensive variation in the upstream region of *pbp5*, including both large and small DNA insertions and deletions among resistant strains. These genetic alterations are likely to affect the promoter region. Additionally, the full-length *psr* gene may function as a repressor of *pbp5* expression as, based on Rice *et al.,* 2001 study, strains exhibiting an ampicillin MIC≥8 mg/L, mutations within the *psr* gene were detected, potentially rendering the *psr* protein nonfunctional, suggesting that disruptions in this gene, or its regulatory role, could contribute to increased *pbp5* expression and, consequently, elevated ampicillin MIC values. In contrast, AMP-S strains generally retain intact *psr* sequences, without observed insertions or deletions.

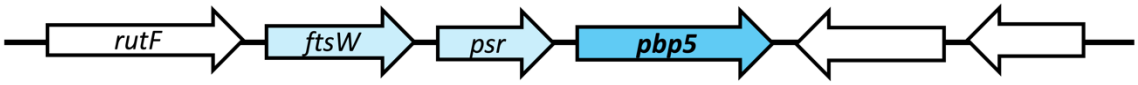


Figure 17. Genetic environment of the *pbp5* gene in *E. faecium*. *Source: Own elaboration.*

Although PBP5 is essential for peptidoglycan synthesis and conferring ampicillin resistance in *E. faecium*, alternative resistance mechanisms have also been identified, including the use of bypass enzymes and modifications in cell wall composition **(145,146)**. The *D-D-carboxypeptidase* DdcY, plays a pivotal role in the activation of the L,D-transpeptidation pathway **(147)**. This enzymatic action converts pentapeptides into tetrapeptides, which are the preferred substrates for the L,D-transpeptidase Ldtfm. By generating these tetrapeptide substrates, DdcY facilitates the bypass of PBPs, which typically utilize pentapeptides for cross-linking. For this reason, DdcY allows bacteria to maintain peptidoglycan cross-linking even when PBPs are inhibited by β-lactam antibiotics, providing an alternative pathway for cross-linking that is not recognized by these drugs **(148)**. Increased expression of DdcY is observed under ampicillin stress; however, this upregulation alone is insufficient to fully bypass PBPs activity and confer complete resistance to ampicillin. While the overexpression of DdcY is a critical response to antibiotic pressure, it must be accompanied by a specific regulatory mechanism to achieve high-level ampicillin resistance. Complete ampicillin resistance necessitates an increase of serine/threonine Ser/Thr protein phosphorylation, which is facilitated by mutations in the *stpA* gene **(149)**. These mutations lead to reduced phosphatase activity of StpA, resulting in accumulation of phosphorylated proteins, including DdcY, which is crucial for the full activation of the L,D-transpeptidation pathway for effective cell wall synthesis and antibiotic resistance.

E. faecium exhibits intrinsic resistance to cephalosporins, with PBP5 playing a critical role in this resistance, similar to its involvement in ampicillin resistance **(144,150)**. Research into this field has been advanced by the creation of artificial mutants in laboratory settings, which have allowed scientists to explore and identify some genetic factors contributing to cephalosporin resistance. Beginning with PBP5, some research indicates that it cannot confer cephalosporin resistance independently; rather, it necessitates the presence of additional aPBPs **(150,151)**. They have shown that not a single aPBP is strictly necessary for cephalosporin resistance. Nevertheless, at least one of the two aPBPs, PonA or PbpF, must be present for cephalosporin resistance to occur. This suggests that the absence of PonA can be functionally compensated by PbpF, and conversely, PbpF's absence can be offset by PonA. This functional redundancy suggests that both PonA and PbpF may possess the capability to execute familiar functions—specifically, peptidoglycan glycosyltransferase activity—necessary for resistance when

combined with PBP5 **(137,144)**. Besides, Desbonnet and colleagues identify the potential involvement of a novel protein, P5AP **(152)**. While its exact role remains undetermined, it is closely associated with PBP5 and might be involved in regulating cell wall biosynthesis or β-lactam resistance. Future research aims to clarify the structure and function of P5AP, along with its contribution to the regulatory network involving Stk, StpA, and PBP5 in *E. faecium.*

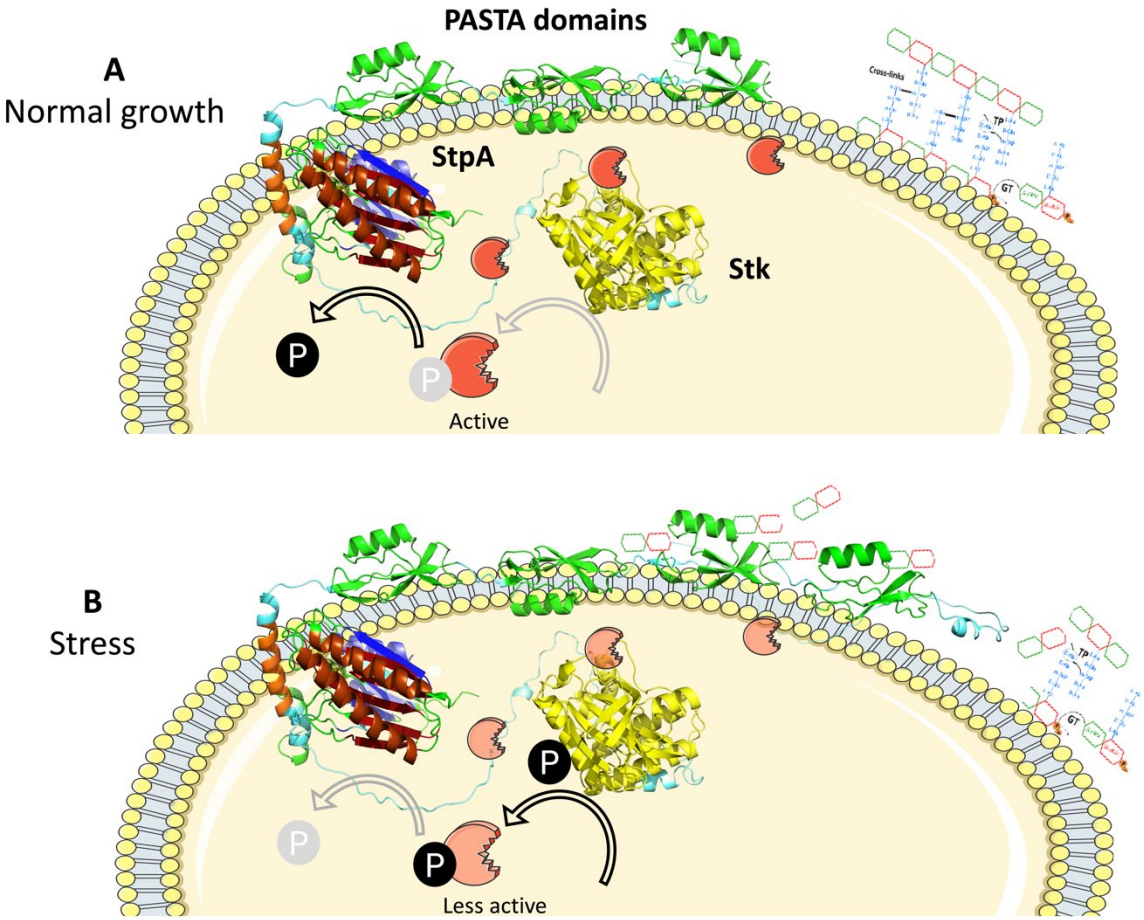


Figure 18. Interaction between Stk and StpA proteins within a bacterial cell. **(A)** Under normal growth conditions, the kinase Stk is not phosphorylated and remains less active. Meanwhile, the phosphatase StpA dephosphorylates cell wall synthesis enzymes, maintaining their normal function. **(B)** When the cell undergoes stress, muropeptides accumulate, unable to be incorporated into the cell wall synthesis process. These are detected by PASTA domains, which activate Stk. In response, Stk phosphorylates its target cell wall synthesis enzymes, downregulating their activity and slowing down cell wall synthesis. *Source: Own elaboration. The AlphaFold-generated model for CroR (A0A7U8FT31) and CroS (A0A7X9XVF2) were visualized and analysed using PyMOL.*

An additional topic to address is the ability of bacteria to sense and respond to environmental changes. This capability is essential for their survival and adaptation, enabling them to effectively navigate and thrive in fluctuating conditions **(153)**. This adaptive capacity is driven by signal transduction mechanisms involving protein kinases and phosphatases, which regulate

protein phosphorylation. Phosphorylation, a post-translational modification common across all life domains, controls target protein activity through conformational changes or protein-protein interactions.

The Ser/Thr kinase Stk and phosphatase StpA in *E. faecium* **[Figure 18]** play crucial roles in cephalosporin resistance **(152)**. These kinases are widespread in Gram-positive bacteria, impacting various functions, including virulence, antimicrobial resistance, and cell wall biosynthesis. Their interplay differs from what is observed in *E. faecalis*, where the orthologues IreK (Stk) and IreP (StpA) function similarly but follow distinct regulatory pathways. In *E. faecium*, Stk interacts with StpA in a reciprocal control mechanism that modulates cephalosporin resistance. Stk features cytoplasmic kinase domains connected to penicillin-binding protein and Ser/Thr kinase associated (PASTA) domains, which detects changes in peptidoglycan structure **(152)**. Desbonnet and colleagues found that a unique mutation in the serine-rich tail of Stk led to truncation, increasing Stk's activity, and enhancing cephalosporin resistance. This mutation may result in the constitutive activation of the kinase, highlighting its role in the bacteria's adaptation to β-lactams. They also observed that a mutation that truncates StpA increased cephalosporin resistance by disrupting its phosphatase activity, which normally regulates Stk through dephosphorylation. The absence of StpA allows for increased phosphorylation of Stk, augmenting resistance even with reduced Stk protein levels.

The $CroRS_{E.\ faecium}$ TCS in *E. faecium*, homologous to that found in *E. faecalis*, is critical for enhancing resistance to cephalosporins and ampicillin **[Figure 19] (100)**. Interestingly, it is a signaling pathway that detects cell envelope stress and triggers an adaptive response through the expression of downstream effector genes. This function has been demonstrated in *E. faecalis* **(102,154)** and later, Kellogg and colleagues showed that the same mechanism operates in *E. faecium*, where it appears to be active during normal cell growth but is further stimulated by cell wall stress, especially when exposed to antibiotics such as β-lactams **(100)**. While β-lactam exposure does not robustly activate the CroRS pathway, it is still required for resistance to these antibiotics. Research indicates that it modulates gene expression in response to stress from cell wall-targeting antibiotics, enhancing the expression of resistance-related genes upon sensing such stress. In *E. faecalis*, several genes within the CroRS regulon have been identified, primarily involving components of the enterococcal cell envelope **(98).** However, the specific composition of this regulatory network in *E. faecium* remains largely unknown.

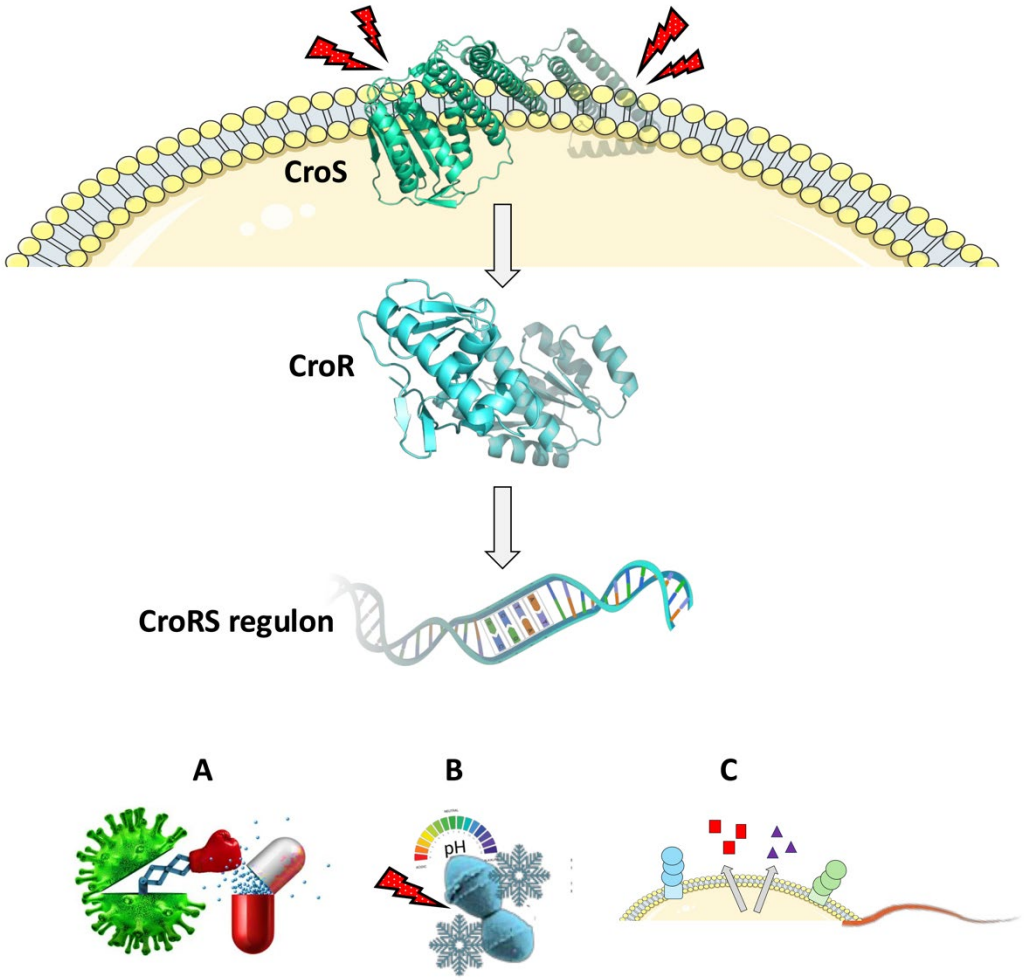


Figure 19. Representation of the TCS CroRS and the resulting phenotypic outcomes of its activation in response to cellular stress. Upon sensing stress, the CroRS system initiates a signaling cascade leading to **(A)** antibiotic resistance, **(B)** activation of stress response pathways, and **(C)** increased virulence. *The AlphaFold-generated model for CroR (A0A7U8FT31) and CroS (A0A7X9XVF2) were visualized and analysed using PyMOL.*

The *murA* gene, which initiates peptidoglycan biosynthesis of the bacteria cell wall and is the principal target of fosfomycin, plays a crucial role in cephalosporin resistance in *E. faecalis* **(24,155)**. Vesić and colleagues investigated the contribution of *murA* to cephalosporin resistance in *E. faecalis* and assessed whether this role was conserved in *E. faecium*. Their findings indicate that its function in conferring resistance to cephalosporins is preserved in *E. faecium*, paralleling its role in *E. faecalis.* They identified two homologs of *murA*, named *murAA* and *murAB*, giving *murAA* a more critical role in growth, cell wall integrity, and antibiotic resistance. Specifically, *murAA* significantly contributes to cephalosporin resistance **(24,152)**. Its deletion results in notable reduction in resistance to cephalosporins, without affecting susceptibility to ampicillin. Besides, while the catalytic activity of *murAA* is essential for cephalosporin resistance, it is not sufficient on its own. Increasing *murAA* expression can improve resistance, even in cells lacking the regulatory kinase IreK (which normally modulates *murAA*). This suggests that *murAA* has additional regulatory functions that contribute to its role in resistance.

1.8. Expected contributions and impact

This doctoral thesis advances our understanding of antibiotic resistance mechanisms in *E. faecium*. This research addresses significant gaps in the field and offers novel insights with potential implications for treatment strategies and resistance management, with the performance of three well-differentiated studies:

Evaluation of the efficacy of ampicillin and cephalosporins for ampicillin-susceptible *Enterococcus faecium*. The first study focuses on the efficacy of combining ampicillin with cephalosporins against *E. faecium*. This investigation contributes to the optimization of therapeutic strategies by identifying which combinations may be most effective in overcoming enterococcal infections, thus providing a basis for more informed clinical decision-making and potentially improving patient outcomes.

Diverse genomic and epidemiological landscapes of redundant *pbp5* genes in *Enterococcus* spp.: insights into plasmid mobilization, ampicillin susceptibility, and environmental interactions. The second study explores the phenomenon of *pbp5* gene redundancy in *E. faecium*, where redundant *pbp5* genes are present on both the chromosome and a plasmid. This research offers a significant contribution by uncovering the prevalence and functional implications of this redundancy. By examining public data and field isolates, the study sheds light on the role of *pbp5* redundancy in β-lactam resistance. This understanding could lead to new approaches in addressing resistance and may guide the development of targeted interventions or new therapeutic agents.

Single point mutations in *croS*, *nusG*, or *rpoB* genes restore cephalosporin resistance to a naturally occurring *Enterococcus faecium* susceptible isolate. The third study challenges the prevailing notion of intrinsic resistance in *E. faecium* by investigating cephalosporin (CPH) low-MIC isolates from various patients with bloodstream infections. These isolates provide a unique opportunity to examine bacterial evolutionary dynamics and identify novel resistance mechanisms. The study's contributions lie in its ability to uncover new genes and mutations associated with cephalosporin resistance, thus expanding our understanding of how *E. faecium* adapts during infection and revealing potential targets for novel treatments.

2. Hypothesis

- The efficacy of combined antibiotic therapies against ampicillin-susceptible *Enterococcus faecium* isolates is underexplored. We hypothesized that ampicillin in combination with ceftriaxone or ceftaroline could improve the efficacy of ampicillin monotherapy against ampicillin-susceptible *E. faecium* strains isolated from bloodstream infections, based on the premise that the complementary saturation of penicillin-binding proteins (PBPs) by both ampicillin and cephalosporins could lead to improved results observed *in vitro*. This hypothesis was assessed with the experimental package described in chapter I.

- The existence of *Enterococcus* strains with redundant *pbp5* genes have been occasionally described, but its prevalence, phylogenetic distribution and impact on antibiotic resistance is largely unknown. We hypothesized that redundant *pbp5* genes could play a significant role in the evolutionary adaptation of *E. faecium* to ampicillin selective pressure. This hypothesis was assessed with the experimental package described in chapter II.

- Our group identified several *E. faecium* clinical isolates NOT intrinsically resistant to cephalosporins. We hypothesized that these isolates could represent effective platforms to identify genes and mutations involved in cephalosporin resistance. This hypothesis was assessed with the experimental package described in chapter III.

3. Objectives

3.1. Main objective

The main objective of this thesis is to get a better understanding of β-lactam resistance in *Enterococcus faecium* and explore new therapeutic strategies to combat infections caused by this microorganism.

3.2. Secondary objectives

- **To assess the *in vitro* efficacy of β-lactam antibiotic combinations to treat infections caused by ampicillin-susceptible (AMP-S) *E. faecium*.** The possible synergistic activity of ampicillin in combination with two different cephalosporins (ceftriaxone or ceftaroline) will be evaluated against AMP-S *E. faecium* clinical isolates obtained from patients with bloodstream infections.

- **To investigate the occurrence of *pbp5* gene redundancy in *Enterococcus* and its possible role in evolutionary adaptation to ampicillin selective pressure in *E. faecium*.** The prevalence of *Enterococcus* strains with redundant copies of the *pbp5* gene will be assessed using public databases of complete genomes. Additionally, the diversity of genetic platforms involved in the mobilization of the *pbp5* gene will be explored, analysing their advantages for *E. faecium* adaptation to different environments. Finally, the potential functional advantage for *E. faecium* of an additional *pbp5* gene located on a transferable plasmid will be investigated.

- **To assess the possible use of *E. faecium* natural isolates with low minimum inhibitory concentrations (MICs) to cephalosporins as platforms to identify novel genes and mutations important for cephalosporin resistance.** The frequency of ceftriaxone high-MIC variants arising from an *E. faecium* isolate with low MICs to cephalosporins will be determined. The genomic differences between the cephalosporin high-MIC variants and the original *E. faecium* isolate with low MIC values will be compared to identify the genetic causes of the phenotypic switch. Additionally, the existence of global transcriptional changes in the cephalosporin high-MIC variants will be assessed in relation to the original *E. faecium* isolate with low MICs to cephalosporins.

Chapter I: Evaluation of the efficacy of ampicillin and cephalosporins for ampicillin-susceptible *Enterococcus faecium*

Ethical compliance and Data Protection: The study adhered to the relevant data protection laws: Real Decreto que la desarrolla (RD 1720/2007), Llei 14/2007, 3 de juliol, de Recerca Biomèdica i Reglament (UE) 2016/679 del Parlament europeu i del Consell de 27 d'abril de 2016 de Protecció de Dades (RGPD) y la nueva legislación en la UE sobre datos personales, en concreto el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de protección de datos (RGPD). The data were electronically anonymized to ensure that the researcher did not have access to patient identities.

4. Chapter I: Evaluation of the efficacy of ampicillin and cephalosporins for ampicillin-susceptible *Enterococcus faecium*

4.1. Abstract

Enterococcus faecium, a significant nosocomial pathogen, poses substantial treatment challenges. While combinations of ampicillin with cephalosporins are first-line therapies to treat *Enterococcus faecalis* high-mortality-rates infections, their efficacy against ampicillin-susceptible (AMP-S) *E. faecium* is less clear.

This study evaluates the effectiveness of combining ampicillin with ceftriaxone or ceftaroline against AMP-S *E. faecium* strains.

Ten AMP-S *E. faecium* bloodstream isolates from complicated infections were analysed. Susceptibility to several antibiotics, including ceftriaxone, ceftaroline, and cefotaxime, was determined using standard methods. Time-kill assays were conducted to assess the synergistic and additive effects of ampicillin in combination with ceftriaxone or ceftaroline.

Time-kill studies revealed that ampicillin combined with ceftaroline demonstrated synergistic and/or additive effects in 7 out of 10 strains analysed. In contrast, ampicillin combined with ceftriaxone showed less pronounced synergy, with only 3 out of 10 strains exhibiting synergistic and/or additive effects.

Our findings indicate that ampicillin combined with ceftaroline, or ceftriaxone provides synergistic activity against some but not all AMP-S *E. faecium* clinical isolates. The observed synergy suggests that this combination could offer a potential therapeutic option for challenging AMP-S *E. faecium* infections.

4.2. Introduction

Enterococcus faecium has become a prominent clinical pathogen, particularly associated with nosocomial infections. The antimicrobial resistance of this bacteria, driven by both acquired and intrinsic mechanisms, poses significant challenges for effective treatment and infection control **(156)**. As resistance continues to develop, including to last-resort antibiotics, there is an urgent need for new therapeutic options or effective antibiotic combinations **(36,156)**.

Ampicillin has been the cornerstone to treat infections caused by enterococci susceptible to β-lactam antibiotics due to its efficacy in inhibiting cell wall synthesis. However, *E. faecium* has developed resistance mechanisms that significantly reduce the effectiveness of ampicillin. A key mechanism involves alterations in Penicillin-Binding Proteins (PBPs), specifically Pbp5, which have a reduced affinity for β-lactam antibiotics, further contributing to resistance. The gene encoding Pbp5 exists in two primary allelic forms: *pbp5*-S and *pbp5*-R **(138,142)**. The *pbp5*-S allele is typically found in community-associated (CA) strains, which are generally susceptible to ampicillin with MICs usually ≤2 mg/L. In contrast, the *pbp5*-R allele is associated with hospital-associated (HA) strains, which demonstrate resistance to ampicillin, characterized by MICs typically >64 mg/L. The nucleotide sequences of *pbp5*-S and *pbp5*-R differ by approximately 5%, resulting in consistent variations in 20 to 21 amino acid positions between the two forms. Meanwhile, cephalosporins' effectiveness against *Enterococcus* is limited, thus they are not used as monotherapies **(144)**.

While *E. faecium* isolates classified as ampicillin-susceptible (AMP-S) are expected to respond to ampicillin monotherapy, clinical challenges persist due to the bacteriostatic nature of β-lactams in certain difficult-to-treat infections, often leading to poor outcomes **(157)**. Consequently, β-lactams are commonly combined with aminoglycosides to leverage their synergistic effects **(158)**. However, the significant risk of renal toxicity associated with aminoglycosides has prompted the search for safer alternatives. For example, the endocarditis guidelines from the American Heart Association (AHA) and the European Society of Cardiology (ESC) now recommend the use of ampicillin combined with a β-lactam from the cephalosporin group, ceftriaxone, for the treatment of *Enterococcus faecalis* endocarditis. This regimen has demonstrated high success rates without increasing the risk of renal impairment linked to aminoglycosides **(159)**. In contrast, *in vitro* studies have shown that this combination does not consistently demonstrate synergy in AMP-S *E. faecium* isolates **(161)**. Additionally, the use of another cephalosporin, ceftaroline, has been explored. While synergism between ampicillin and

ceftaroline has been documented against *E. faecalis* **(160)**, the efficacy of this combination in AMP-S *E. faecium* has yet to be investigated.

This study aims to address the gap in knowledge regarding the efficacy of ampicillin combined with cephalosporins, specifically ceftriaxone or ceftaroline, against AMP-S *E. faecium*.

4.3. Material and methods

4.3.1. Strains and culture conditions

Ten AMP-S *E. faecium* bloodstream isolates from complicated bacteremia and infective endocarditis episodes were identified at Parc Taulí Hospital (2014-2021) using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) instrument (Bruker, Billerica, MA, USA). Bacterial cultures were grown in cation-adjusted Mueller-Hinton broth (Sigma Aldrich, St Louis, MO, USA) according to the procedures of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI) **(162,163)**.

4.3.2. Multi-locus sequence typing

Multi-locus sequence typing (MLST) was employed to determine the sequence type (ST) of the genomes. The typing of seven specific genes (*atpA*, *ddl*, *gdh*, *gyd*, *pstS*, *adk*, and *purK*) were involved. Genomes with identical alleles across all seven genes were classified as belonging to the same ST.

4.3.3. Antibiotic susceptibility testing

The Minimum Inhibitory Concentrations (MICs) of different antibiotics were determined using the MicroScan system (Dade Behring, West Sacramento, CABD) from 2014 to 2019 and the Phoenix system (BD Phoenix™, Franklin Lakes, USA) from 2020 to 2021 for all antibiotics, including ampicillin, cephalosporins, daptomycin, and vancomycin. Cephalosporins and ampicillin susceptibility was further confirmed using the Etest (BioMérieux, Marcy-l'Étoile, France), following guidelines of the EUCAST **(162)** or, when not possible, the CLSI **(163)**. All tests were performed in triplicate.

4.3.4. *In vitro* time-kill curves

Time-kill curves (TKCs) were conducted to evaluate the bactericidal and synergistic effects of various combination regimens against AMP-S *E. faecium* strains. The antibiotics used were

ampicillin (Sigma Aldrich, St Louis, MO, USA), ceftriaxone (Sigma Aldrich, St Louis, MO, USA), and ceftaroline (Pfizer, New York, NY, USA), alone or combined, following the recommended methodology **(164)**. The regimens included ½ x MIC or 1 x MIC levels of the drugs, with an initial inoculum of $5x10^5$ CFU/mL. For AMP-S *E. faecium* strains with ceftriaxone MIC values >256 mg/L, experiments were performed using 150 mg/L of the antibiotic, approximately its peak serum concentration (C_{max}) after a single dose **(165)**. Culture aliquots were sampled at 0, 4, and 24 hours for each strain and condition and bacterial viability was determined (log_{10} CFU/mL). Quality controls were performed throughout to ensure antibiotic efficacy. Each experiment was conducted in triplicate. A bactericidal effect was defined as a reduction of at least 3 log_{10} CFU/mL compared to the initial inoculum, while synergistic effects were confirmed by at least a 2 log_{10} CFU/mL reduction at 24 hours compared to the most active single agent within the combination. The additive effect was considered when the combination resulted in a reduction of 1 to <2 log_{10} CFU/mL compared to the most active antibiotic. Indifference occurred when the combination showed a change ±1 log_{10} CFU/mL compared to the most active antibiotic **(164)**.

4.4. Results

4.4.1. Clinical data

Between July 2014 and August 2021, a total of ten cases of AMP-S *E. faecium* bacteremia were documented (Table 1). The sources of bacteria were predominantly biliary (40%) and urinary (20%) origins, with additional cases stemming from digestive, endocardial, and unknown foci. The patient cohort included individuals with significant comorbidities, with pancreatic and biliary neoplasms being the predominant conditions.

Treatment regimens varied according to the clinical context, with 70% of bacteremia cases treated with β-lactams. One of these patients, a case with a prosthetic mitral valve endocarditis due to *E. faecium*, was managed with a combination of ampicillin and ceftaroline. The patient was admitted on 16/09/2020, and an initial transesophageal echocardiogram on 18/09 revealed an image suggestive of vegetation. Antibiotic therapy was initiated on 19/09 with daptomycin and ceftaroline, adjusted to daptomycin and imipenem on 22/09, and finally switched to ampicillin and ceftaroline on 02/10, continuing until 28/10. The patient recovered successfully, with no signs of persistent infection, and was discharged in stable condition.

4.4.2. Sequence type

Genetic variability was observed among the strains, as they were categorized into various sequence types (STs) **[Table 1]**. This included both previously characterized STs and novel STs.

Table 1. Summary of the ten strains under study. ST, Sequence Type; Mar, March; Jun, June; Jul, July; Aug, August; Sep, September; F, female; M, male.

Strains (isolation)	ST	Age/sex	Comorbidities	Source of infection	Antibiotic therapy	Outcome (persistence/ mortality)
Efm1 (Jul 2014)	ST_{new}	79/M	Hypertension, diabetes mellitus, mild cognitive impairment. Bacteremia of biliary origin, community acquired. Treated with amoxicillin-clavulanate. Non-persistent, non-fatal.	Biliary	Before bacteremia: Levofloxacin For bacteremia: Amoxicillin-clavulanate	no/no
Efm2 (Mar 2015)	ST32	85/F	Pancreatic neoplasm. Bacteremia of biliary origin, healthcare-associated. Treated with piperacillin-tazobactam. Non-persistent, non-fatal.	Biliary	Before bacteremia: Piperacillin-tazobactam For bacteremia: Meropenem	no/no
Efm3 (Mar 2015)	ST10	66/M	Cholangiocarcinoma. Bacteremia of biliary origin, nosocomial. Treated with meropenem. Non-persistent, fatal due to neoplasm one month later.	Biliary	Before bacteremia: No For bacteremia: Amoxicillin-clavulanate	no/yes
Efm4 (Mar 2015)	ST1604	73/M	Head of pancreas neoplasm with metastasis and additional tumor spread. Bacteremia of biliary origin, healthcare-associated. Treated with meropenem. Non-persistent, non-fatal.	Hepatic abscess	Before bacteremia: No For bacteremia: Meropenem	no/no
Efm5 (May 2015)	ST29	89/F	Total dependence, cerebrovascular accident. Bacteremia of urinary origin, healthcare-associated. Treated with levofloxacin and clindamycin. Non-persistent, non-fatal.	Urinary	Before bacteremia: No For bacteremia: Levofloxacin + clindamycin	no/no
Efm6 (Jun 2015)	ST_{new}	86/F	Alzheimer's disease. Bacteremia of urinary origin, community-acquired. Treated with amoxicillin-clavulanate. Non-persistent, non-fatal.	Urinary	Before bacteremia: Levofloxacin For bacteremia: Amoxicillin	no/no
Efm9 (Sep 2020)	ST121	46/F	Crohn's disease. Bacteremia of digestive origin, healthcare-associated. Treated with linezolid. Non-persistent, non-fatal.	Digestive	Before bacteremia: No For bacteremia: Linezolid	no/no
Efm10 (Sep 2020)	ST22	74/M	Congestive heart failure, cirrhosis, valvulopathy. Bacteremia of endocardial origin, nosocomial. Treated with vancomycin. Non-persistent, non-fatal.	Endocarditis	Before bacteremia: No For bacteremia: Daptomycin + ceftaroline; daptomycin + imipenem; ampicillin + ceftaroline	no/no
Efm54 (Aug 2021)	ST2193	86/M	Oropharyngeal neoplasm with chemotherapy. Bacteremia of unknown focus, community acquired. Treated with teicoplanin and ampicillin. Non-persistent, non-fatal.	Primary care (unknown focus)	Before bacteremia: No For bacteremia: teicoplanin; ampicillin	no/no
Efm57 (Jul 2020)	ST771	64/F	Admission for cholangitis secondary to choledocholithiasis. Bacteremia of biliary origin, healthcare-associated. Treated with ceftriaxone and metronidazole. Persistent bacteremia. Non-fatal.	Biliary	Before bacteremia: Ceftriaxone + metronidazole For bacteremia: meropenem; linezolid	no/no

4.4.3. Antibiotic susceptibility

The MIC values for ampicillin, vancomycin, and daptomycin demonstrated general susceptibility among the AMP-S *E. faecium* isolates, as detailed in **Table 1**. Ampicillin displayed the lowest MIC values across all isolates, with isolate Efm54 being particularly susceptible (0.064 mg/L). Vancomycin and daptomycin also showed consistent susceptibility, with no isolates exhibiting resistance to these antibiotics. In contrast, the MIC analysis of cephalosporins—ceftaroline, ceftriaxone, and cefotaxime—revealed variability in susceptibility, as shown in Figure 1 and Table 1. Ceftaroline exhibited uniformly low MIC values across all isolates, ranging from 0.25 mg/L to 0.75 mg/L **[Figure 20; Figure 21, Table 2]**. However, ceftriaxone and cefotaxime showed more variable results **[Figure 20; Figure 21, Table 2]**. Isolates Efm1, Efm5, Efm10, and Efm54 had low MIC values (≤8 mg/L), while isolates Efm3, Efm4, Efm6, and Efm57 displayed higher MIC values (>256 mg/L for ceftriaxone and >32 for cefotaxime). Notably, isolate Efm54 was the most susceptible to all the antibiotics tested, including ceftaroline (0.064 mg/L), ceftriaxone (1 mg/L), and cefotaxime (0.75 mg/L).

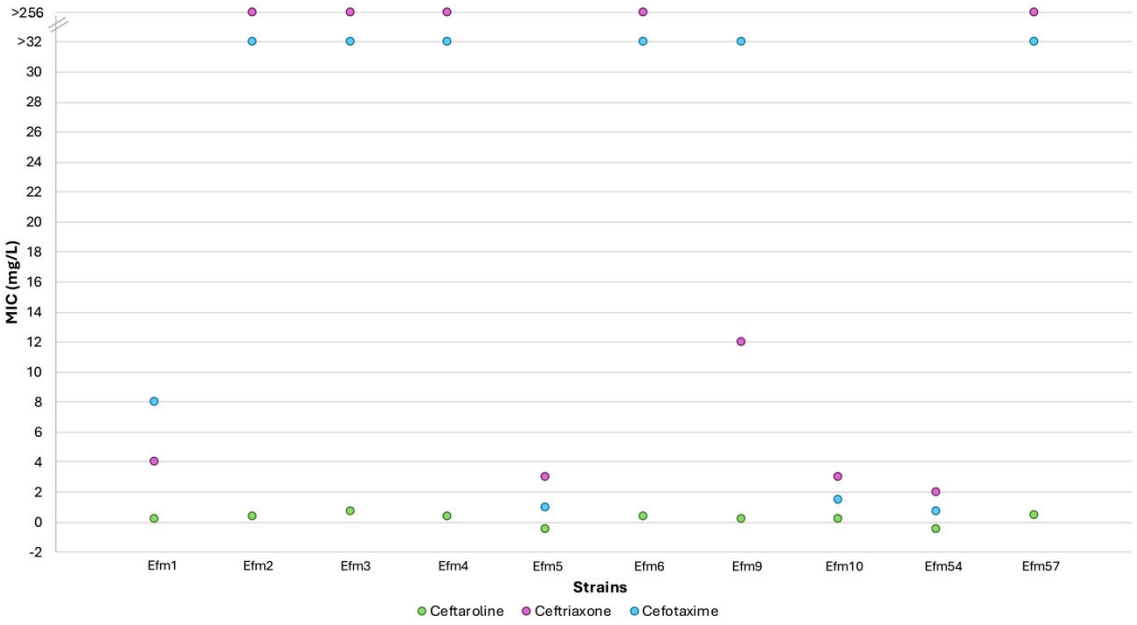


Figure 20. MICs of the cephalosporins—ceftaroline, ceftriaxone, and cefotaxime—against the ten AMP-S *E. faecium* strains. The double line over the >32 mg/L indicates that the rest of the axis beyond >256 is not scaled.

Table 2. MICs of ampicillin (AMP), ceftaroline (CTL), ceftriaxone (CTR), cefotaxime (CTA), vancomycin (VAN), and daptomycin (DAP) against the ten AMP-S *E. faecium* strains. EUCAST clinical breakpoints (version 14.0, [2024]): for AMP ≤4 mg/L susceptible (S), >8 mg/L resistant (R); for VAN ≤4 mg/L S, >4 mg/L R; for DAP ≤4 mg/L SDD*, ≥ 8 R; for cephalosporins Undetermined. *Susceptible-Dose Dependent.

MIC (mg/L)						
Strains	**AMP**	**CTL**	**CTR**	**CTA**	**VAN**	**DAP**
Efm1	0.19	0.25	4	8	1	4
Efm2	0.75	0.38	>256	>32	<=1	4
Efm3	2	0.75	>256	>32	<=1	4
Efm4	0.5	0.38	>256	>32	2	4
Efm5	0.25	0.19	3	1	4	<=0.5
Efm6	1	0.38	>256	>32	1	4
Efm9	0.75	0.25	12	>32	4	4
Efm10	0.75	0.25	3	1.5	4	4
Efm54	0.064	0.064	1	0.75	1	2
Efm57	4	0.5	>256	>32	1	2

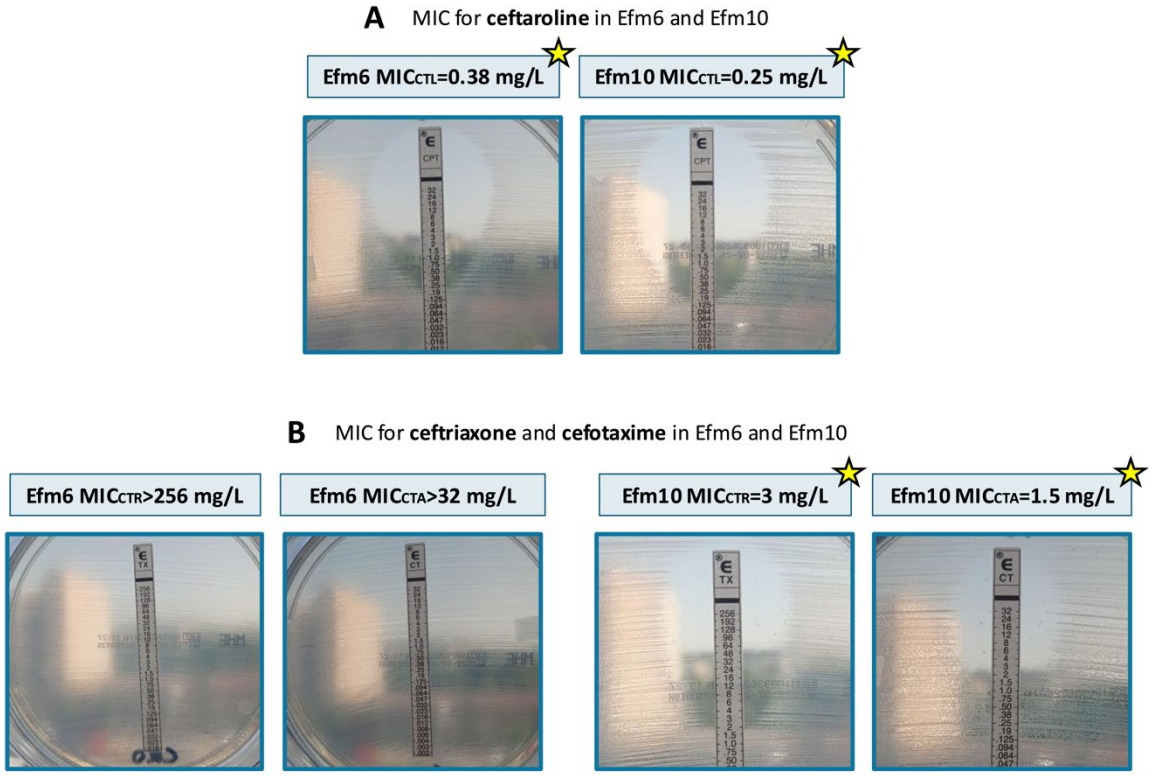
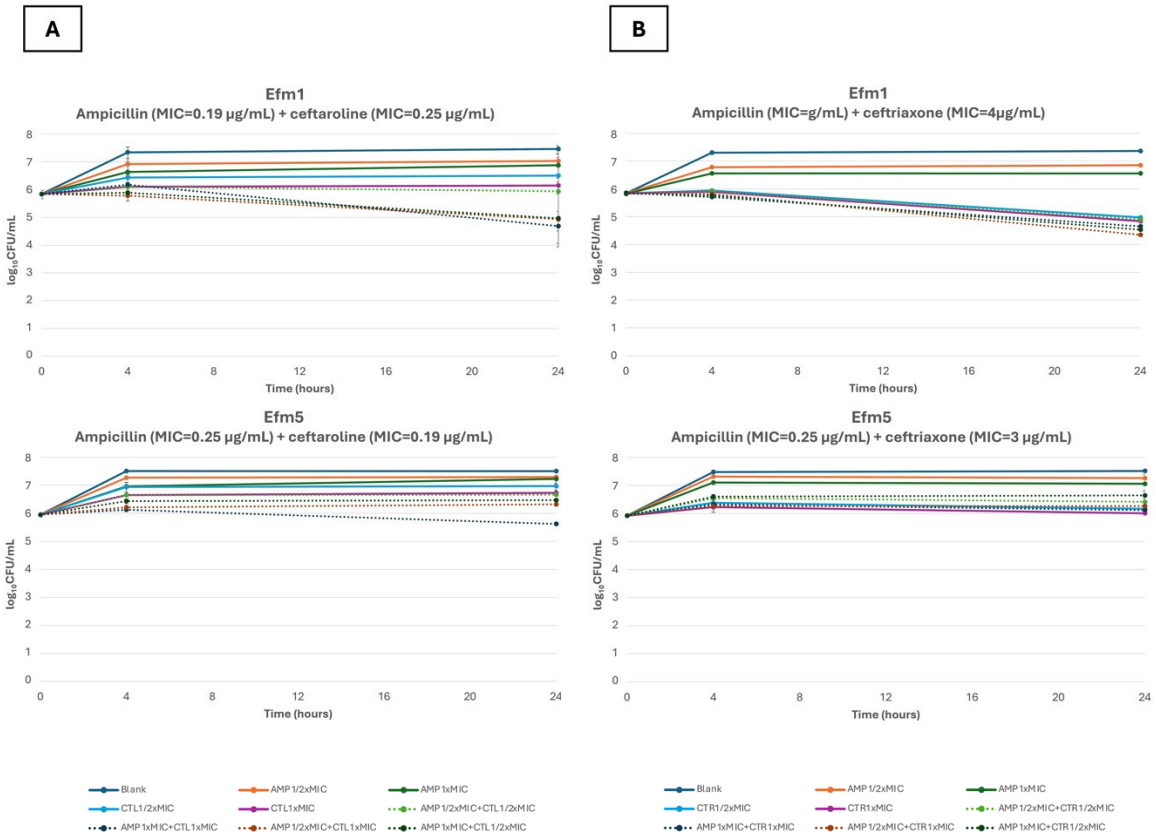


Figure 21. Etest MIC results of **(A)** ceftaroline (CTL), with Efm6 on the left and Efm10 on the right, and **(B)** ceftriaxone (CTR) and cefotaxime (CTA). The left side shows Efm6 (CTR on the left and CTA on the right), while the right-side displays Efm10 (CTR on the left and CTA on the right). The yellow star indicates the low MICs for cephalosporins.

4.4.4. *In vitro* time-kill studies

As expected, monotherapies did not exhibit bactericidal effects, as the experiments were conducted using sublethal antibiotic concentrations. The combination of ampicillin and ceftaroline against AMP-S *E. faecium* strains demonstrated varied interactions. Notably, 7 out of 10 strains exhibited either synergistic and/or additive effects **[Figure 22; Figure 23, Table 3; Table 4; Table 5; Table 6]**. Efm6, for instance, showed a marked synergy with a ΔChange of -3.05 log_{10} CFU/mL at 1xMIC of ceftaroline, indicating substantial inhibitory effect. The combination of ampicillin and ceftriaxone exhibited less effective interactions compared to the combination of ampicillin and ceftaroline. Specifically, 3 out of 10 strains showed either synergistic and/or additive effects, while the remaining strains display indifferent effects. Notably, Efm6 showed a marked synergy with a ΔChange of -3.68 log_{10} CFU/mL at 1 x C_{max} of ceftriaxone, achieving a bactericidal effect, an outcome not observed in any other strain analysed. These results suggest that while the combinations of ampicillin with either ceftaroline or ceftriaxone can enhance growth inhibition for certain strains through synergistic and additive interactions, they are generally insufficient for complete bacterial eradication at the concentrations used in our experiments.



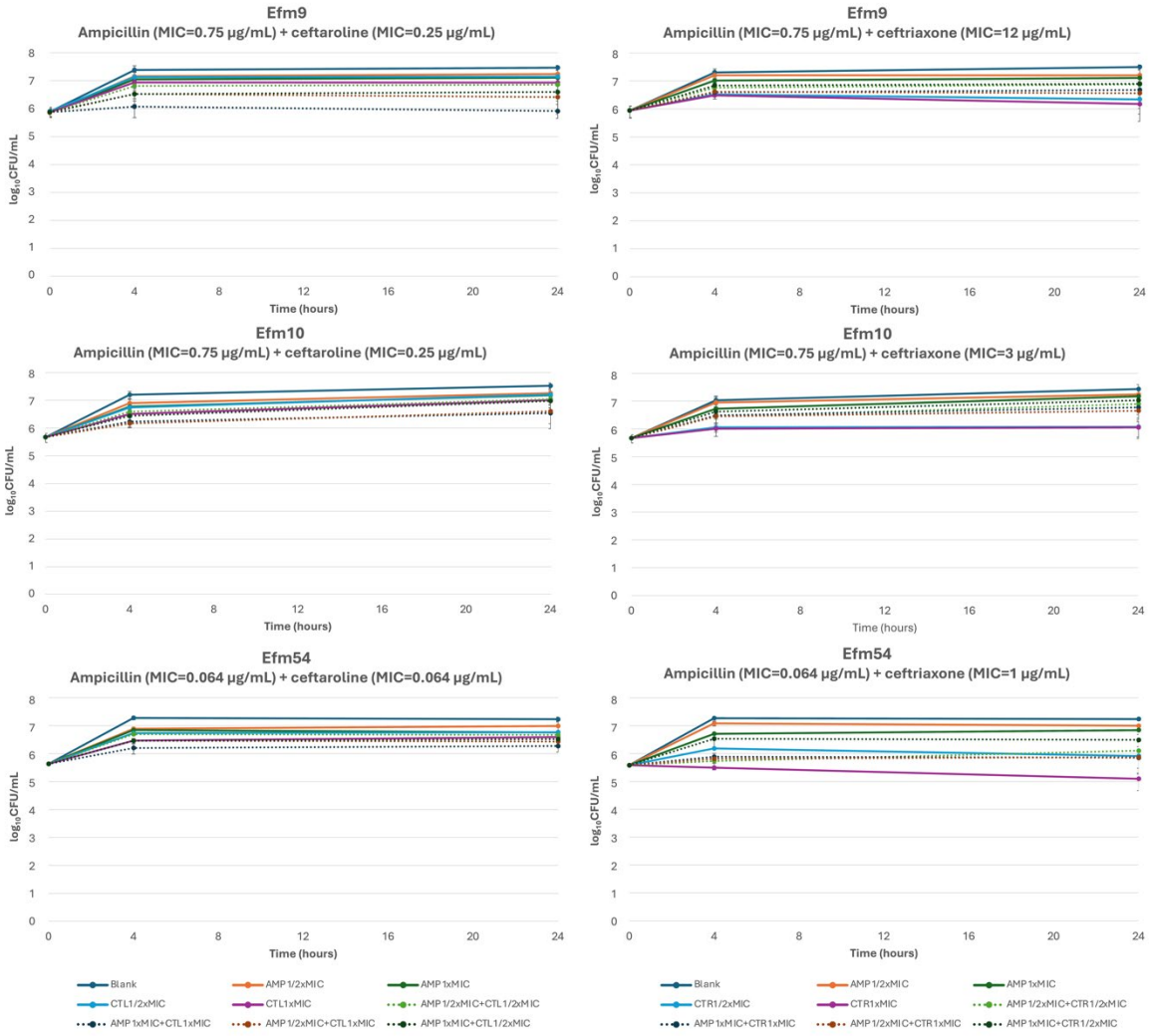
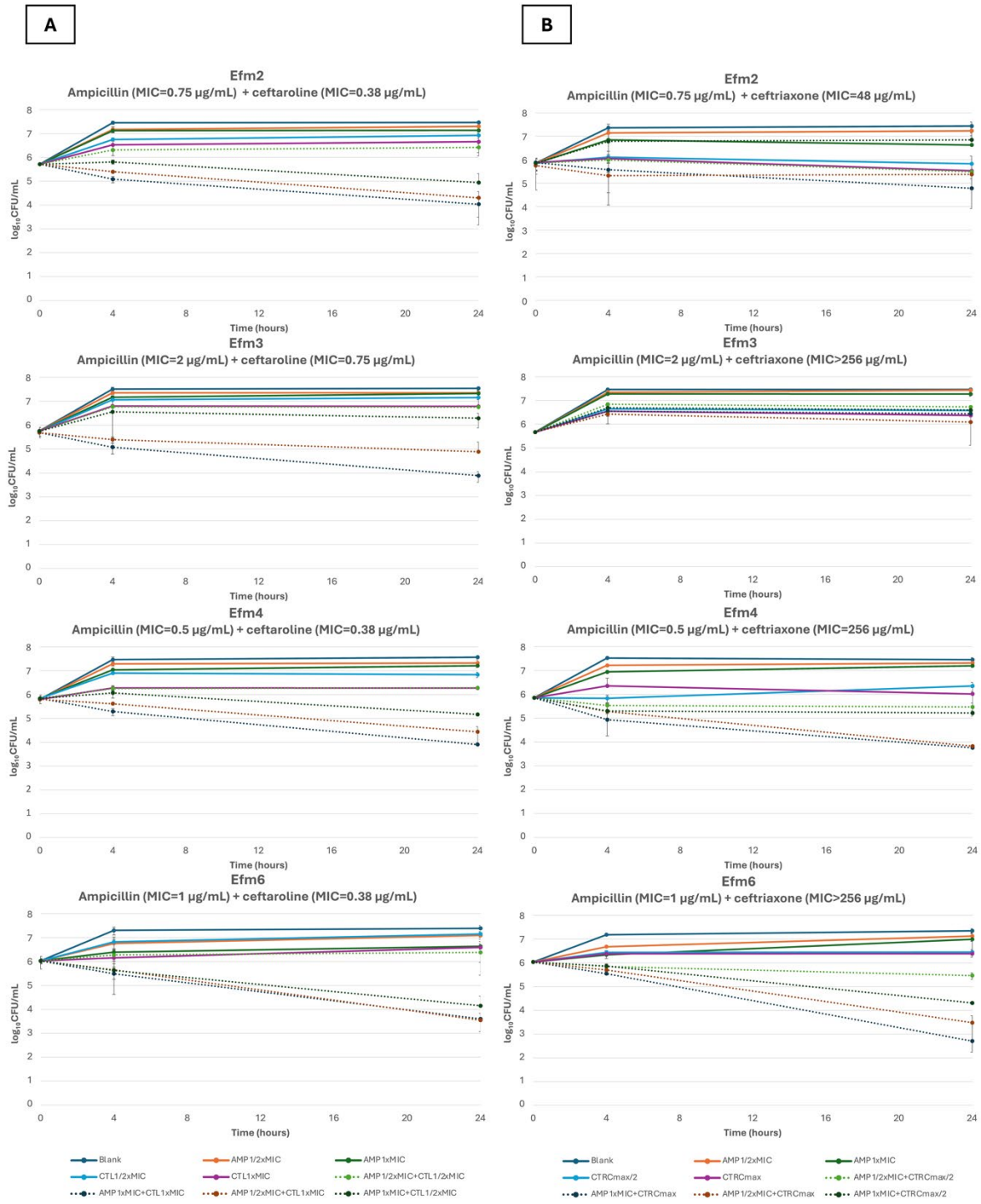


Figure 22. TKCs for combinations of ampicillin (AMP) with ceftaroline (CTL) or ceftriaxone (CTR) against the five AMP-S *E. faecium* strains with low MICs to ceftaroline and ceftriaxone. Panel A depicts the TKCs for the combination of AMP with CTL, while panel B shows the TKCs for the combination of AMP with CTR. Each curve represents the bacterial count (log_{10} CFU/mL) over time.


A
B
Efm2
Ampicillin (MIC=0.75 µg/mL) + ceftaroline (MIC=0.38 µg/mL)
Efm2
Ampicillin (MIC=0.75 µg/mL) + ceftriaxone (MIC=48 µg/mL)
Efm3
Ampicillin (MIC=2 µg/mL) + ceftaroline (MIC=0.75 µg/mL)
Efm3
Ampicillin (MIC=2 µg/mL) + ceftriaxone (MIC>256 µg/mL)
Efm4
Ampicillin (MIC=0.5 µg/mL) + ceftaroline (MIC=0.38 µg/mL)
Efm4
Ampicillin (MIC=0.5 µg/mL) + ceftriaxone (MIC=256 µg/mL)
Efm6
Ampicillin (MIC=1 µg/mL) + ceftaroline (MIC=0.38 µg/mL)
Efm6
Ampicillin (MIC=1 µg/mL) + ceftriaxone (MIC>256 µg/mL)
log10CFU/mL
Time (hours)
Blank
AMP 1/2xMIC
AMP 1xMIC
CTL1/2xMIC
CTL1xMIC
AMP 1/2xMIC+CTL1/2xMIC
AMP 1xMIC+CTL1xMIC
AMP 1/2xMIC+CTL1xMIC
AMP 1xMIC+CTL1/2xMIC
CTRCmax/2
CTRCmax
AMP 1/2xMIC+CTRCmax/2
AMP 1xMIC+CTRCmax
AMP 1/2xMIC+CTRCmax
AMP 1xMIC+CTRCmax/2

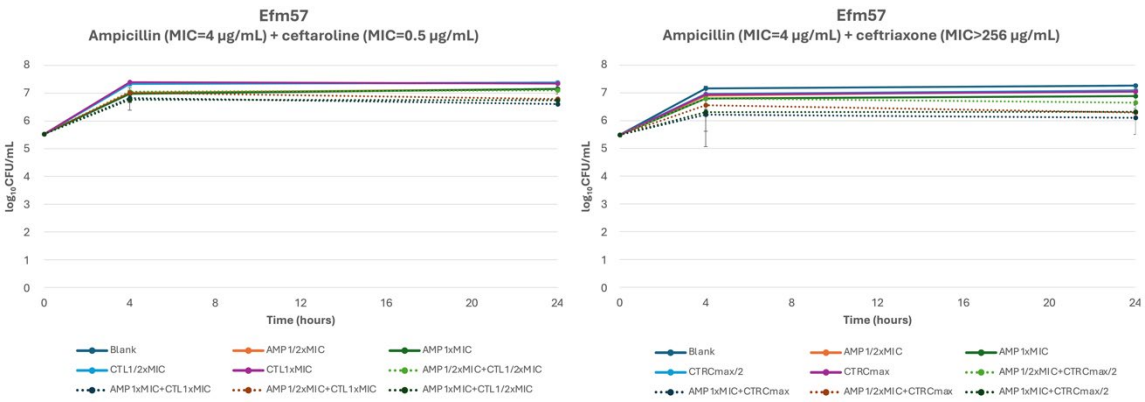


Figure 23. TKCs for combinations of ampicillin (AMP) with ceftaroline (CTL) or ceftriaxone (CTR) against the five AMP-S *E. faecium* strains with high MICs to ceftriaxone. Panel A depicts the TKCs for the combination of AMP with CTL, while panel B shows the TKCs for the combination of AMP with CTR. Each curve represents the bacterial count (log_{10} CFU/mL) over time.

Table 3. TKCs data for the ampicillin-ceftaroline combination in AMP-S Efm1 to Efm5 strains. Each row represents the bacterial count (log_{10} CFU/mL) at various time points for each treatment condition.

Ampicillin + ceftaroline						
		log_{10} CFU/ml	ΔChange (log_{10} CFU/ml)			
	Conditions	**0h**	**4h**	**24h**	**AMP+CTL**	
Efm1	Blank	5,8	+1,5	+1,6	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,8	+1,1	+1,2	AMP_{1xMIC}+CTL_{1xMIC}	Additive
	AMP_{1xMIC}	5,8	+0,8	+1,0	$AMP_{1/2xMIC}$+CTL_{1xMIC}	Additive
	$CTL_{1/2xMIC}$	5,8	+0,6	+0,7	AMP_{1xMIC}+$CTL_{1/2xMIC}$	Additive
	CTL_{1xMIC}	5,8	+0,3	+0,3		
	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	5,8	+0,3	+0,1		
	AMP_{1xMIC}+CTL_{1xMIC}	5,8	+0,3	-1,2		
	$AMP_{1/2xMIC}$+CTL_{1xMIC}	5,8	-0,1	-0,9		
	AMP_{1xMIC}+$CTL_{1/2xMIC}$	5,8	+0,0	-0,9		
Efm2	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,7	+1,7	+1,8	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,7	+1,5	+1,6	AMP_{1xMIC}+CTL_{1xMIC}	Synergy
	AMP_{1xMIC}	5,7	+1,4	+1,4	$AMP_{1/2xMIC}$+CTL_{1xMIC}	Synergy
	$CTL_{1/2xMIC}$	5,7	+1,0	+1,2	AMP_{1xMIC}+$CTL_{1/2xMIC}$	Additive
	CTL_{1xMIC}	5,7	+0,8	+0,9		
	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	5,7	+0,6	+0,7		
	AMP_{1xMIC}+CTL_{1xMIC}	5,7	-0,6	-1,7		
	AMP1/2xMIC+CTL_{1xMIC}	5,7	-0,3	-1,4		
	AMP_{1xMIC}+$CTL_{1/2xMIC}$	5,7	+0,1	-0,8		
Efm3	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,8	+1,7	+1,8	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,8	+1,6	+1,6	AMP_{1xMIC}+CTL_{1xMIC}	Synergy
	AMP_{1xMIC}	5,8	+1,4	+1,6	$AMP_{1/2xMIC}$+CTL_{1xMIC}	Additive
	$CTL_{1/2xMIC}$	5,7	+1,3	+1,4	AMP_{1xMIC}+$CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,8	+1,0	+1,0		
	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	5,7	+1,0	+1,0		
	AMP_{1xMIC}+CTL_{1xMIC}	5,7	-0,6	-1,8		
	$AMP_{1/2xMIC}$+CTL_{1xMIC}	5,7	-0,3	-0,8		
	AMP_{1xMIC}+$CTL_{1/2xMIC}$	5,7	+0,8	+0,5		
Efm4	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,8	+1,7	+1,8	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,8	+1,5	+1,5	AMP_{1xMIC}+CTL_{1xMIC}	Synergy
	AMP_{1xMIC}	5,8	+1,2	+1,4	$AMP_{1/2xMIC}$+CTL_{1xMIC}	Additive
	$CTL_{1/2xMIC}$	5,8	+1,1	+1,0	AMP_{1xMIC}+$CTL_{1/2xMIC}$	Additive
	CTL_{1xMIC}	5,8	+0,5	+0,5		
	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	5,8	+0,4	+0,4		
	AMP_{1xMIC}+CTL_{1xMIC}	5,8	-0,5	-1,9		
	$AMP_{1/2xMIC}$+CTL_{1xMIC}	5,8	-0,2	-1,4		
	AMP_{1xMIC}+$CTL_{1/2xMIC}$	5,8	+0,2	-0,7		
Efm5	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,9	+1,6	+1,6	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,9	+1,3	+1,3	AMP_{1xMIC}+CTL_{1xMIC}	Additive
	AMP_{1xMIC}	5,9	+1,0	+1,3	$AMP_{1/2xMIC}$+CTL_{1xMIC}	Indifference
	$CTL_{1/2xMIC}$	5,9	+1,0	+1,0	AMP_{1xMIC}+$CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,9	+0,7	+0,8		
	$AMP_{1/2xMIC}$+$CTL_{1/2xMIC}$	5,9	+0,7	+0,7		
	AMP_{1xMIC}+CTL_{1xMIC}	5,9	+0,2	-0,3		
	$AMP_{1/2xMIC}$+CTL_{1xMIC}	5,9	+0,3	+0,4		
	AMP_{1xMIC}+$CTL_{1/2xMIC}$	5,9	+0,5	+0,5		

Table 4. TKCs data for the ampicillin-ceftaroline combination in AMP-S Efm6 to Efm57 strains. Each row represents the bacterial count (log_{10} CFU/mL) at various time points for each treatment condition.

Ampicillin + ceftaroline						
		log_{10} CFU/ml	ΔChange (log_{10} CFU/ml)			
	Conditions	**0h**	**4h**	**24h**	**AMP+CTL**	
Efm6	Blank	6,0	+1,3	+1,4	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	6,0	+0,7	+1,1	$AMP_{1xMIC}+CTL_{1xMIC}$	Syergy
	AMP_{1xMIC}	6,0	+0,4	+0,6	$AMP_{1/2xMIC}+CTL_{1xMIC}$	Synergy
	$CTL_{1/2xMIC}$	6,0	+0,8	+1,1	$AMP_{1xMIC}+CTL_{1/2xMIC}$	Synergy
	CTL_{1xMIC}	6,0	+0,1	+0,6		
	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	6,0	+0,2	+0,4		
	$AMP_{1xMIC}+CTL_{1xMIC}$	6,0	-0,5	-2,4		
	$AMP_{1/2xMIC}+CTL_{1xMIC}$	6,0	-0,4	-2,5		
	$AMP_{1xMIC}+CTL_{1/2xMIC}$	6,0	-0,2	-1,7		
Efm9	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,9	+1,5	+1,6	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,9	+1,3	+1,4	$AMP_{1xMIC}+CTL_{1xMIC}$	Additive
	AMP_{1xMIC}	5,9	+1,2	+1,2	$AMP_{1/2xMIC}+CTL_{1xMIC}$	Indifference
	$CTL_{1/2xMIC}$	5,9	+1,2	+1,2	$AMP_{1xMIC}+CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,9	+1,1	+1,1		
	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	5,9	+0,9	+1,0		
	$AMP_{1xMIC}+CTL_{1xMIC}$	5,9	+0,2	+0,0		
	$AMP_{1/2xMIC}+CTL_{1xMIC}$	5,9	+0,7	+0,6		
	$AMP_{1xMIC}+CTL_{1/2xMIC}$	5,9	+0,6	+0,7		
Efm10	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,7	+1,5	+1,9	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,7	+1,2	+1,6	$AMP_{1xMIC}+CTL_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,7	+1,1	+1,5	$AMP_{1/2xMIC}+CTL_{1xMIC}$	Indifference
	$CTL_{1/2xMIC}$	5,7	+1,1	+1,5	$AMP_{1xMIC}+CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,7	+0,8	+1,3		
	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	5,7	+0,9	+1,4		
	$AMP_{1xMIC}+CTL_{1xMIC}$	5,7	+0,6	+0,9		
	$AMP_{1/2xMIC}+CTL_{1xMIC}$	5,7	+0,5	+0,9		
	$AMP_{1xMIC}+CTL_{1/2xMIC}$	5,7	+0,8	+1,3		
Efm54	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,6	+1,6	+1,6	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,6	+1,3	+1,4	$AMP_{1xMIC}+CTL_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,6	+1,2	+1,1	$AMP_{1/2xMIC}+CTL_{1xMIC}$	Indifference
	$CTL_{1/2xMIC}$	5,6	+1,1	+1,1	$AMP_{1xMIC}+CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,6	+0,8	+1,0		
	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	5,6	+1,1	+1,0		
	$AMP_{1xMIC}+CTL_{1xMIC}$	5,6	+0,6	+0,6		
	$AMP_{1/2xMIC}+CTL_{1xMIC}$	5,6	+0,8	+0,8		
	$AMP_{1xMIC}+CTL_{1/2xMIC}$	5,6	+0,8	+0,9		
Efm57	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTL**	
	Blank	5,5	+1,8	+1,8	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,5	+1,5	+1,6	$AMP_{1xMIC}+CTL_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,5	+1,5	+1,6	$AMP_{1/2xMIC}+CTL_{1xMIC}$	Indifference
	$CTL_{1/2xMIC}$	5,5	+1,8	+1,9	$AMP_{1xMIC}+CTL_{1/2xMIC}$	Indifference
	CTL_{1xMIC}	5,5	+1,9	+1,8		
	$AMP_{1/2xMIC}+CTL_{1/2xMIC}$	5,5	+1,5	+1,6		
	$AMP_{1xMIC}+CTL_{1xMIC}$	5,5	+1,3	+1,1		
	$AMP_{1/2xMIC}+CTL_{1xMIC}$	5,5	+1,5	+1,3		
	$AMP_{1xMIC}+CTL_{1/2xMIC}$	5,5	+1,2	+1,2		

Table 5. TKCs data for the ampicillin-ceftriaxone combination in AMP-S Efm1 to Efm5 strains. Each row represents the bacterial count (log_{10} CFU/mL) at various time points for each treatment condition.

Ampicillin + ceftriaxone						
		log_{10} CFU/ml	ΔChange (log_{10} CFU/ml)			
	Conditions	**0h**	**4h**	**24h**	**AMP+CTR**	
Efm1	Blank	5,8	+1,5	+1,5	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,8	+0,9	+1,0	$AMP_{1xMIC}+CTR_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,8	+0,7	+0,7	$AMP_{1/2xMIC}+CTR_{1xMIC}$	Indifference
	$CTR_{1/2xMIC}$	5,8	+0,1	-0,9	$AMP_{1xMIC}+CTR_{1/2xMIC}$	Indifference
	CTR_{1xMIC}	5,8	+0,1	-1,0		
	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	5,8	+0,1	-0,9		
	$AMP_{1xMIC}+CTR_{1xMIC}$	5,9	-0,2	-1,2		
	$AMP_{1/2xMIC}+CTR_{1xMIC}$	5,8	-0,0	-1,5		
	$AMP_{1xMIC}+CTR_{1/2xMIC}$	5,8	-0,1	-1,3		
Efm2	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,9	+1,5	+1,6	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,8	+1,3	+1,4	$AMP_{1xMIC}+CTR_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,8	+1,0	+0,8	$AMP_{1/2xMIC}+CTR_{1xMIC}$	Indifference
	$CTR_{1/2xMIC}$	5,9	+0,3	-0,0	$AMP_{1xMIC}+CTR_{1/2xMIC}$	Additive
	CTR_{1xMIC}	5,9	+0,2	-0,4		
	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	5,8	+0,1	-0,4		
	$AMP_{1xMIC}+CTR_{1xMIC}$	5,9	-0,3	-1,1		
	$AMP_{1/2xMIC}+CTR_{1xMIC}$	5,9	-0,4	-0,4		
	$AMP_{1xMIC}+CTR_{1/2xMIC}$	5,9	+0,9	+1,0		
Efm3	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,7	+1,8	+1,8	$AMP_{1/2xMIC}+CTR_{Cmax/2}$	Indifference
	$AMP_{1/2xMIC}$	5,7	+1,7	+1,8	$AMP_{1xMIC}+CTR_{Cmax}$	Indifference
	AMP_{1xMIC}	5,7	+1,6	+1,6	$AMP_{1/2xMIC}+CTR_{Cmax}$	Indifference
	$CTR_{Cmax/2}$	5,7	+1,0	+0,9	$AMP_{1xMIC}+CTR_{Cmax/2}$	Indifference
	$CTR_{Cmax/2}$	5,7	+0,9	+0,7		
	$AMP_{1/2xMIC}+CTR_{Cmax/2}$	5,7	+1,2	+1,1		
	$AMP_{1xMIC}+CTR_{Cmax}$	5,7	+0,9	+0,8		
	$AMP_{1/2xMIC}+CTR_{Cmax}$	5,7	+0,8	+0,4		
	$AMP_{1xMIC}+CTR_{Cmax/2}$	5,7	+1,0	+0,9		
Efm4	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,9	+1,7	+1,6	$AMP_{1/2xMIC}+CTR_{Cmax/2}$	Indifference
	$AMP_{1/2xMIC}$	5,9	+1,4	+1,5	$AMP_{1xMIC}+CTR_{Cmax}$	Synergy
	AMP_{1xMIC}	5,9	+1,1	+1,3	$AMP_{1/2xMIC}+CTR_{Cmax}$	Synergy
	$CTR_{Cmax/2}$	5,9	-0,0	+0,5	$AMP_{1xMIC}+CTR_{Cmax/2}$	Additive
	$CTR_{Cmax/2}$	5,9	+0,5	+0,2		
	$AMP_{1/2xMIC}+CTR_{Cmax/2}$	5,9	-0,3	-0,4		
	$AMP_{1xMIC}+CTR_{Cmax}$	5,9	-0,9	-2,1		
	$AMP_{1/2xMIC}+CTR_{Cmax}$	5,9	-0,6	-2,0		
	$AMP_{1xMIC}+CTR_{Cmax/2}$	5,9	-0,5	-0,6		
Efm5	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,9	+1,6	+1,6	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,9	+1,4	+1,3	$AMP_{1xMIC}+CTR_{1xMIC}$	Indifference
	AMP_{1xMIC}	5,9	+1,2	+1,1	$AMP_{1/2xMIC}+CTR_{1xMIC}$	Indifference
	$CTR_{1/2xMIC}$	5,9	+0,5	+0,2	$AMP_{1xMIC}+CTR_{1/2xMIC}$	Indifference
	CTR_{1xMIC}	5,9	+0,3	+0,1		
	$AMP_{1/2xMIC}+CTR_{1/2xMIC}$	5,9	+0,6	+0,5		
	$AMP_{1xMIC}+CTR_{1xMIC}$	5,9	+0,4	+0,2		
	$AMP_{1/2xMIC}+CTR_{1xMIC}$	5,9	+0,3	+0,3		
	$AMP_{1xMIC}+CTR_{1/2xMIC}$	5,9	+0,7	+0,7		

Table 6. TKCs data for the ampicillin-ceftriaxone combination in AMP-S Efm6 to Efm57 strains. Each row represents the bacterial count (log_{10} CFU/mL) at various time points for each treatment condition.

Ampicillin + ceftriaxone						
		log_{10} CFU/ml	ΔChange (log_{10} CFU/ml)			
	Conditions	**0h**	**4h**	**24h**	**AMP+CTR**	
Efm6	Blank	6,0	+1,2	+1,3	$AMP_{1/2xMIC}$+$CTR_{Cmax/2}$	Indifference
	$AMP_{1/2xMIC}$	6,0	+0,6	+1,1	AMP_{1xMIC}+CTR_{Cmax}	Synergy
	AMP_{1xMIC}	6,0	+0,3	+1,0	$AMP_{1/2xMIC}$+CTR_{Cmax}	Synergy
	$CTR_{Cmax/2}$	6,0	+0,4	+0,4	AMP_{1xMIC}+$CTR_{Cmax/2}$	Synergy
	CTRCmax/2	6,0	+0,3	+0,3		
	$AMP_{1/2xMIC}$+$CTR_{Cmax/2}$	6,0	-0,2	-0,6		
	AMP_{1xMIC}+CTR_{Cmax}	6,0	-0,5	-3,3		
	$AMP_{1/2xMIC}$+CTR_{Cmax}	6,0	-0,3	-2,6		
	AMP_{1xMIC}+$CTR_{Cmax/2}$	6,0	-0,2	-1,7		
Efm9	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,9	+1,4	+1,6	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,9	+1,3	+1,3	AMP_{1xMIC}+CTR_{1xMIC}	Indifference
	AMP_{1xMIC}	5,9	+1,1	+1,2	$AMP_{1/2xMIC}$+CTR_{1xMIC}	Indifference
	$CTR_{1/2xMIC}$	5,9	+0,6	+0,4	AMP_{1xMIC}+$CTR_{1/2xMIC}$	Indifference
	CTR_{1xMIC}	5,9	+0,5	+0,2		
	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	5,9	+0,8	+0,9		
	AMP_{1xMIC}+CTR_{1xMIC}	5,9	+0,7	+0,7		
	$AMP_{1/2xMIC}$+CTR_{1xMIC}	5,9	+0,7	+0,6		
	AMP_{1xMIC}+$CTR_{1/2xMIC}$	5,9	+0,9	+1,0		
Efm10	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,7	+1,4	+1,8	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,7	+1,3	+1,6	AMP_{1xMIC}+CTR_{1xMIC}	Indifference
	AMP_{1xMIC}	5,7	+1,1	+1,5	$AMP_{1/2xMIC}$+CTR_{1xMIC}	Indifference
	$CTR_{1/2xMIC}$	5,7	+0,4	+0,4	AMP_{1xMIC}+$CTR_{1/2xMIC}$	Indifference
	CTR_{1xMIC}	5,7	+0,3	+0,4		
	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	5,7	+0,8	+1,2		
	AMP_{1xMIC}+CTR_{1xMIC}	5,7	+0,8	+1,1		
	$AMP_{1/2xMIC}$+CTR_{1xMIC}	5,7	+0,8	+1,0		
	AMP_{1xMIC}+$CTR_{1/2xMIC}$	5,7	+1,0	+1,4		
Efm54	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,6	+1,7	+1,7	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	Indifference
	$AMP_{1/2xMIC}$	5,6	+1,5	+1,4	AMP_{1xMIC}+CTR_{1xMIC}	Indifference
	AMP_{1xMIC}	5,6	+1,1	+1,3	$AMP_{1/2xMIC}$+CTR_{1xMIC}	Indifference
	$CTR_{1/2xMIC}$	5,6	+0,6	+0,3	AMP_{1xMIC}+$CTR_{1/2xMIC}$	Indifference
	CTR_{1xMIC}	5,6	-0,1	-0,5		
	$AMP_{1/2xMIC}$+$CTR_{1/2xMIC}$	5,6	+0,2	+0,5		
	AMP_{1xMIC}+CTR_{1xMIC}	5,6	+0,3	+0,3		
	$AMP_{1/2xMIC}$+CTR_{1xMIC}	5,6	+0,2	+0,3		
	AMP_{1xMIC}+$CTR_{1/2xMIC}$	5,6	+0,9	+0,9		
Efm57	**Conditions**	**0h**	**4h**	**24h**	**AMP+CTR**	
	Blank	5,5	+1,7	+1,8	$AMP_{1/2xMIC}$+$CTR_{Cmax/2}$	Indifference
	$AMP_{1/2xMIC}$	5,5	+1,4	+1,6	AMP_{1xMIC}+CTR_{Cmax}	Indifference
	AMP_{1xMIC}	5,5	+1,3	+1,4	$AMP_{1/2xMIC}$+CTR_{Cmax}	Indifference
	$CTR_{Cmax/2}$	5,5	+1,5	+1,6	AMP_{1xMIC}+$CTR_{Cmax/2}$	Indifference
	CTRCmax/2	5,5	+1,5	+1,6		
	$AMP_{1/2xMIC}$+$CTR_{Cmax/2}$	5,5	+1,3	+1,2		
	AMP_{1xMIC}+CTR_{Cmax}	5,5	+0,7	+0,6		
	$AMP_{1/2xMIC}$+CTR_{Cmax}	5,5	+1,1	+0,8		
	AMP_{1xMIC}+$CTR_{Cmax/2}$	5,5	+0,8	+0,8		

4.5. Discussion

This study investigates the impact of combining ampicillin with either ceftriaxone or ceftaroline against clinical AMP-S *E. faecium* strains as potential therapeutic strategies for challenging difficult-to-treat AMP-S *E. faecium* infections. Reduced susceptibility to β-lactams in *E. faecium*, predominantly within the CC17 clonal complex **(166,167)**, has been associated with the overexpression of the low-affinity Pbp5 **(145,168)** and, more recently, to other PBPs such as PbpF, PonA, or PbpA **(137,152)**. However, approximately 7.5% of *E. faecium* clinical isolates in our region remain susceptible to ampicillin **(17)**.

Some AMP-S *E. faecium* strains under study exhibited low MICs for ceftaroline, ceftriaxone, and cefotaxime. This observation is noteworthy because *E. faecium* has traditionally been considered intrinsically resistant to all cephalosporins **(136)**. Cephalosporin resistance in enterococci has been associated with the reduced binding affinity of these antibiotics to the Pbp5 protein, a key penicillin-binding protein. Additionally, previous studies have shown that mutants in the CroRS two-component system (TCS) or the class B penicillin-binding protein PbpA exhibit cephalosporin (CPH) low-MIC values **(100,137)**. Importantly, all these mutant strains were genetically engineered in the laboratory, making our current finding of naturally occurring CPH low-MIC clinical isolates particularly novel. Further work is needed to determine the genetic causes of the CPH low-MIC phenotype in our clinical isolates.

While ampicillin plus ceftriaxone is a first-line therapy for *E. faecalis* endocarditis **(159,160)**, data on its efficacy against AMP-S *E. faecium* infections is limited. Synergism between amoxicillin and cefotaxime against *E. faecium* strains has shown to be inconsistent **(163)**. A recent study found synergism between ampicillin and ceftriaxone in only 3 out of 9 (33.3%) AMP-S *E. faecium* isolates, showing MICs for ampicillin between 0.25 and 0.5 mg/L **(161)**. Although low MIC values for ampicillin in AMP-S *E. faecium* isolates might suggest potential susceptibility to synergistic effects when combined with ceftriaxone, our time-kill assays do not consistently support this trend. This indicates that MIC values alone may not reliably predict the presence or absence of synergism between ampicillin and cephalosporins.

Previous studies have demonstrated that ampicillin plus ceftaroline exhibits synergistic effects against *E. faecalis,* which is susceptible to ampicillin **(169)**. This synergy is also well-documented in *E. faecium* strains that are typically resistant to ampicillin. Our research found significant synergistic effects of ampicillin and ceftaroline against nearly half of the *E. faecium* isolates studied, which are ampicillin-susceptible. This finding suggests that the effectiveness of the

ampicillin-ceftaroline combination may extend beyond strains with established ampicillin resistance, supporting its potential as a therapeutic option for infections caused by AMP-S *E. faecium*.

Importantly, the combinations tested in our assays at MIC concentrations did not display bactericidal activity against AMP-S *E. faecium*, with the exception of one strain. This lack of widespread bactericidal effect, despite observed synergistic or additive interactions, may be attributed to several factors. Firstly, the MIC concentrations used in the assays might be insufficient for achieving complete bactericidal eradication. The concentrations required for bactericidal activity often exceed those needed merely to inhibit bacterial growth. Additionally, the strains possess mechanisms of resistance or adaptive responses that allow survival even in the presence of effective antibiotic combinations. The effectiveness of the combinations at higher concentrations, which more accurately simulate physiological conditions, remains unexplored. In the same line, the use of *in vitro* models simulating human pharmacokinetics could provide further insights into the clinical effectiveness of β-lactams at concentrations higher than the MIC. Moreover, while our study was not designed to investigate the distribution of different PBPs in the studied strains, this limitation, along with the relatively small number of strains analysed, restricts the generalizability of our results. Despite these constraints, our findings suggest that ampicillin and ceftaroline could exhibit synergistic activity against AMP-S *E. faecium* strains and may represent a viable therapeutic option for challenging difficult-to-treat infections caused by AMP-S *E. faecium*. Future studies focusing on the clinical correlation of these *in vitro* observations are required.

Chapter II: Diverse genomic and epidemiological landscapes of redundant *pbp5* genes in *Enterococcus* spp.: insights into plasmid mobilization, ampicillin susceptibility, and environmental interactions

5. Chapter II: Diverse genomic and epidemiological landscapes of redundant *pbp5* genes in *Enterococcus* spp.: insights into plasmid mobilization, ampicillin susceptibility, and environmental interactions

5.1. Abstract

Genetic redundancy in bacteria plays a crucial role in enhancing adaptability and accelerating evolution in response to selective pressures, particularly those associated with rapid environmental changes. Aminopenicillins like ampicillin are important therapeutic options for *Enterococcus* infections in both humans and animals, with resistance mostly associated with *pbp5* gene mutations or overexpression. While the occurrence of redundant *pbp5* genes has been occasionally reported, the advantages for the host bacteria have not been explored in detail. During a whole-genome sequencing project of *Enterococcus faecium* from bacteremic patients, we identified an ST592 strain (Efm57) with redundant *pbp5* genes. This presented an opportunity to investigate the prevalence and implications of multiple *pbp5* acquisitions in diverse *Enterococcus* species across various sources, geographical regions, and timeframes. The analysis of 618 complete *Enterococcus* genomes from public databases revealed that 3.2% harboured redundant *pbp5* genes, located on chromosomes or plasmids across different species from diverse epidemiological backgrounds. The proteins encoded by these genes showed homologies ranging from 51.1% to 97.5% compared to native copies. Phylogenetic analysis grouped redundant PBP5 amino acid sequences into three distinct clades, with insertion sequences (mostly IS6-like) facilitating their recent spread to diverse plasmids with varying genetic backbones. The presence of multiple antibiotic resistance genes on *pbp5*-plasmids, including those conferring resistance to linezolid, underscores their involvement in co-selection and recombination events with other clinically-relevant antibiotics. Conjugation experiments confirmed the transferability of a specific 24 kb *pbp5*-plasmid from the Efm57 strain. This plasmid was associated with higher minimum inhibitory concentrations of ampicillin and conferred bacteria growth advantages at 22°C. In conclusion, the widespread distribution of redundant *pbp5* genes among *Enterococcus* spp. highlights the complex interplay between genetic mobility, environmental factors, and multidrug resistance in overlapping ecosystems emphasizing the importance of understanding these dynamics to mitigate antibiotic resistance spread within the One Health framework.

5.2. Introduction

Aminopenicillins are extensively used in human medicine and veterinary practice to treat a variety of infections including those caused by *Enterococcus*, to which these antibiotics are among the few treatment options available **(170**,**171)**. Ampicillin resistance in *Enterococcus faecium* is mainly linked to the overexpression or mutation of Penicillin-Binding Protein 5 (PBP5), encoded by the chromosomal intrinsic gene *pbp5* **(78)**. *E. faecium* clinical strains globally exhibit high rates of ampicillin resistance due to PBP5 mutations **(172,173,174)**, while variable resistance rates are found among *Enterococcus* spp. from non-clinical settings **(175,176,177,178,179,180,181)**. Despite its chromosomal location, it has been shown that *pbp5* has occasionally been mobilized through conjugative transposons or large chromosomal genetic platforms among *E. faecium* from diverse clonal lineages and sources **(78,182,183)**. However, plasmid-borne *pbp5* genes are rarely described and instances are limited to an *Enterococcus hirae* from the pig gut and a clade B *E. faecium* (now reclassified as *Enterococcus lactis*) from human feces **(184,185)**. In both chromosomal and plasmid *pbp5* mobilization, redundant chromosomal *pbp5* copies were maintained and associated with diverse ampicillin susceptibility phenotypes **(78,183,186)**.

Genetic redundancy in bacteria, such as duplicated genes, is achieved by homologous recombination and horizontal gene transfer **(187)**. Indeed, the presence of duplicated genes may provide functional diversification, allowing bacteria to rapidly adapt to changing environmental conditions or stress, accelerating evolution within microbial communities across diverse niches without compromising essential functions **(188,189)**. *Enterococcus* spp. often undergo horizontal gene transfer and recombination, leveraging their flexible genome to adapt across different environments and hosts **(83,190,191)**. However, the full extent of occurrence of redundant *pbp5* genes among *Enterococcus*, diversity of mobilization genetic platforms and the benefits for niche and host adaptations are underexplored. During a project involving whole-genome sequencing of several *E. faecium* clinical isolates from bacteremic patients, we identified a strain with redundant *pbp5* genes on its chromosome and a plasmid. This third description of *pbp5* redundancy among *Enterococcus* spp. presented an opportunity to explore the prevalence, genomic context, and functional implications of this phenomenon in *Enterococcus* spp., using both public genome data and a field isolate.

5.3. Material and methods

5.3.1. Clinical context of *Enterococcus faecium* 57 (Efm57) strain

An *E. faecium* clinical strain, designated Efm57, was isolated from a 64-year-old patient with a bloodstream infection associated with acute cholangitis at Parc Taulí University Hospital (Sabadell) in July 2020 **[Figure 24]**. The patient received empirical treatment with ceftriaxone and metronidazole for 8 days. Following post-endoscopic retrograde cholangiopancreatography complications, the antibiotic was escalated to meropenem. Based on diagnostics, treatment was switched to linezolid for 7 days, successfully curing the infection. The etiological agent was identified by Gram staining and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker MS).

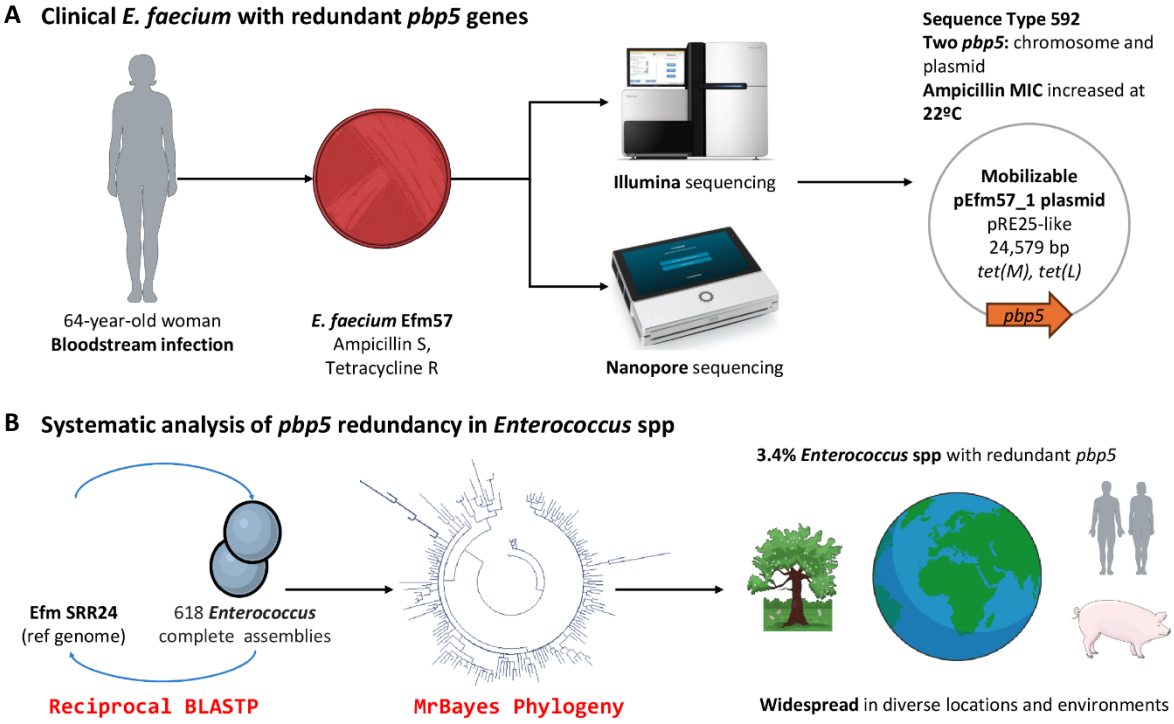


Figure 24. Summary of the study investigating redundant *pbp5* genes in *Enterococcus* species. **(A)** An *E. faecium* strain (Efm57) was isolated from a patient at Hospital Parc Taulí. Whole-genome sequencing was performed using Illumina for the genome and Nanopore for the plasmid. **(B)** Following this, a broader investigation was carried out, analysing 618 complete *Enterococcus* genomes from public databases to explore the occurrence of redundant *pbp5* genes across various species, geographical locations, and time periods.

5.3.2. DNA extraction and whole-genome sequencing

Efm57 isolate was grown on Columbia Agar plates with 5% Sheep Blood (bioMérieux) at 37°C. Total DNA was purified using the DNeasy Blood & Tissue Purification Kit (Qiagen), and DNA quality was assessed using a Qubit® 2.0 fluorometer (Thermo Fisher Scientific). Libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina). Whole-genome sequencing (WGS) was performed using paired-end sequencing on an Illumina HiSeq 2500 sequencer available at the Genomics Unit of the Centre de Regulació Genòmica (CRG, Barcelona). Quality of the raw sequencing reads was checked using FastQC (v0.11.9 https://github.com/s-andrews/FastQC, accessed in June 2023). Read pre-processing and filtering were performed with TrimGalore v0.6.6 **(192)** shaving the sequencing adapters, trimming the initial 20 bp poor-quality positions, and applying a Phred score >= 20 limit. The trimmed paired-end reads were assembled *de novo* using SPAdes v3.13.1 **(193)**, and assembly quality was then assessed using CheckM v1.2.2 **(194)**. Assembled scaffolds were annotated with prokka v1.14.6 **(195)** against the Clusters of Orthologous Genes (COG) **(196)**, High-quality Automated and Manual Annotation of Proteins (HAMAP) **(197)**, and Pfam **(198)** databases. To assess strain clonality, multi-locus sequence typing (MLST; Sequence Type-ST) was performed **(199)**. Putative antibiotic resistance genes were predicted with ABRicate v1.0.1 (https://github.com/tseemann/abricate, accessed in June 2023) using the in-built NCBI AMRFinderPlus **(200)** database. Prediction of the ampicillin resistance phenotype due to *pbp5* mutations was done by ResFinder v4.5.0 **(201)**, and virulence genes were assessed with Virulence Finder v2.0 **(202)** from the Center for Genomic Epidemiology (CGE).

Plasmids were extracted from Efm57 using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific), and DNA quality was assessed with a Qubit® 2.0 fluorometer (Thermo Fisher Scientific). Libraries were prepared using the SQKRBK004 Rapid Barcoding Kit (Oxford Nanopore Technologies) and sequenced using a FLO-MIN106 flow-cell on a Mk1c device (MinION, Oxford Nanopore Technologies). Guppy v5.0.11 was used for basecalling (SUPERIOR mode), demultiplexing, and for read filtering (Q>=10 limit). The resulting filtered reads were *de novo* assembled using flye v2.9.1 for plasmid reconstruction **(203)**. Plasmid scaffolds were identified using MOB-recon and mobility was predicted based on the MOB-typer module bot available at the MOB-suite v3.1.9 **(204)**. Plasmid replicases were predicted with Plasmid Finder 2.1 (CGE) **(205)**.

5.3.3. Comparative genomics and phylogenetic inference

PBP5 homologs were compiled from the Efm57 genome and from 618 complete *Enterococcus* spp. assemblies available in the NCBI database (accessed on June 2023) through modified reciprocal BLASTP (BLASTP v2.12.0) **(206)** using the *E. faecium* SRR24 PBP5 protein [WP_086321967] sequence as the query, a conservative e-value of <1e–20 and query coverage of >75% **[Figure 24]**. In canonical reciprocal BLAST, two genes residing in different genomes were considered homologs if their protein sequences found each other as the best BLASTP hit in the other genome **(207)**. In this case, reciprocal BLASTP rationale was modified to accept *Enterococcus* PBP5 paralogs by taking more than one hit in the first BLASTP. These hits were only accepted as paralogs if they found *E. faecium* SRR24 PBP5 sequence as their best hit in the second BLASTP. Plasmid-borne PBP5 described by Morroni *et al.* **(186)** was manually included in the panel of predicted PBP5 homologs. Genetic arrangements of predicted PBP5 homologs were downloaded and compared using Biopython v1.81 **(208)**. Scripts used for obtaining and analysing genomic data are available in the GitHub repository (https://github.com/Bacterial-Blood-Pathogens/efm_pbp5).

For phylogenetic inference, we performed a T-COFFEE v12.00 multiple sequence alignment using the non-identical panel (<100% identity) of predicted PBP5 homologs, combining three CLUSTALW v2.1 protein sequence alignments with variable (5, 10, 25) gap open penalties **(209)**. The non-redundant set of PBP5 sequences was generated by clustering all predicted homologs using USEARCH v11.0.667 with a 100% similarity threshold and otherwise default parameters **(210)**. PBP5 encoded in genomes with redundant *pbp5* copies were maintained, despite being identical. The alignment was then trimmed with Gblocks v0.91b using the “half gap” setting and otherwise default parameters **(211)**. Bayesian phylogenetic inference on the trimmed alignment was performed with MrBayes v3.2.6 **(212)**. Four Metropolis-coupled Markov chain Monte Carlo simulations with four independent chains were run for 5,000,000 generations, using a mixed four-category gamma distributed rate plus proportion of invariable sites model [invgamma] and a JTT (Jones–Taylor–Thornton) amino acid substitution model. A consensus tree was generated and visualized using iTOL v6 **(213)**. *Vagococcus fluvialis* 36B2 PBP5 protein sequence was used as the root. All plasmid-borne *pbp5* genes were considered as redundant. Also, chromosomal variants in genomes containing multiple *pbp5* copies were considered as the redundant variants when they mapped to a well-supported clade alongside plasmid-associated *pbp5* genes. Epidemiological background data of all *Enterococcus* spp. included in the tree were retrieved from Bacterial and Viral Bioinformatics Resource Center **(214)**.

5.3.4. Antibiotic susceptibility testing

Antibiotic susceptibility tests for Efm57 were performed using the BD Phoenix system (BD Phoenix™, Franklin Lakes, USA) (levofloxacin, vancomycin, teicoplanin, daptomycin, linezolid), Etest (ampicillin, tetracycline, rifampicin) or disk diffusion (ciprofloxacin, erythromycin, quinupristin-dalfopristin, chloramphenicol, gentamicin, or streptomycin) following guidelines for clinical breakpoints interpretation of the European Committee on Antimicrobial Susceptibility Testing **(162)** or, when not possible, the Clinical and Laboratory Standards Institute **(163)**. Minimum inhibitory concentration (MIC) of ampicillin was also determined by Etest diffusion under modified conditions, using incubation temperatures of 22°C.

5.3.5. Conjugation assays

Conjugation assays using Efm57 as the donor strain for the *pbp5*-plasmid [also carrying *tet(M)* and *tet(L)* genes] were conducted through the filter-mating method, maintaining a 1:1 ratio of donor to recipient. *E. faecium* 64/3, *E. lactis* BM4105RF, and *E. faecalis* JH2-2 were used as recipient strains **(78,215)**. They are all resistant to rifampicin and fusidic acid but susceptible to tetracycline. Transconjugants were recovered on Brain Heart Infusion agar (37°C for 24-48 hours) supplemented with 30 mg/L rifampicin, 25 mg/L fusidic acid, and 16 mg/L tetracycline. The resistance profiles to rifampicin, fusidic acid, and tetracycline of several recovered transconjugants was confirmed by disk diffusion. The presence of the *tet(M)* gene was verified by PCR amplification using previously described primers and conditions **(216)**. Ampicillin susceptibility of both transconjugants and recipient strain was evaluated by the Etest diffusion method under standard and modified conditions as described above.

5.3.6. Kinetic assays

Growth curves were performed to Efm57, transconjugants and their recipient strain on 96-well microtiter plates and determined using a Multiskan FC microplate photometer (Thermo Fisher Scientific). Absorbance at 550 nm (A550) was recorded every 15 minutes for 24 hours. Experiments were conducted at temperatures of 22°C or 37°C in Brain Heart Infusion broth, both in the absence and presence of ampicillin at concentrations equal to ½ of the MIC, as determined under standard conditions for each strain tested **(162)**. Each time-point was tested in triplicate, and three biological replicates were conducted for each strain and condition to ensure biological reproducibility. Blank wells were included for control measurements. To determine the doubling time of bacterial cultures, a nonlinear regression analysis was performed specifically on the exponential growth phase. The exponential phase was identified based on the visual consistency

of the slope observed in the growth curve. The growth rate was derived from the best-fit parameters of the exponential growth model. To ensure the appropriateness of the selected data points, the R^2 value was calculated by adding an exponential trendline to the graph and selecting the option to display the R^2 value in Excel 16.88. An $R^2 > 0.9$ was used as the validation criterion, ensuring a high level of accuracy for the model.

5.3.7. Data availability

Sequence data from this study have been submitted to the NCBI database and are available under the BioProject PRJNA1132796. The nucleotide sequence of the pEfm57_1 plasmid has been deposited in GenBank under the accession number PP974444.

5.4. Results

5.4.1. Antibiotic susceptibility and genetic characterization of *Enterococcus faecium* Efm57

Antibiotic susceptibility tests indicated that Efm57 was susceptible to ampicillin (MIC=4 mg/L) and to most of all other antibiotics tested, and resistant to tetracycline and ciprofloxacin **[Table 7]**. Efm57 was classified as ST592 according to PubMLST for *E. faecium* (Bezdicek *et al.,* 2023). The isolate carried diverse virulence factors, including gene variants typically associated with clinical (*ccpA*, *fms14*, *sgrA*) and non-clinical (*acm*, *bepA*, *fnm, sagA, scm*) *E. faecium* strains **[Table S1]**. MOB-suite identified two plasmids: pEfm57_1 (24,579 bp; GenBank accession number PP974444), a *rep2* broad host range pRE25-like plasmid; and pEfm57_2 (34,956 bp), a *rep17* narrow-host pRUM-like plasmid. Both plasmids were predicted to be mobilizable (MOBV family relaxase). Although no complete conjugation modules were identified in any plasmid, pEfm57_1 was predicted to encode the conjugative transposon protein TcpC, and pEfm57_2 contained TraE-encoding gene protein, which is part of the Type IV Secretion System (T4SS). However, further studies are needed to confirm whether interactions between these elements are enough for facilitating conjugation.

Table 7. Antibiotic susceptibility profile of Efm57 strain. MIC, minimum inhibitory concentrations; S, susceptible; R, resistant; mm, millimetres; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute. *****Guidelines used for antibiotic susceptibility test interpretation: EUCAST, 2024 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_14.0_Breakpoint_Tables.pdf) and CLSI, 2020 (CLSI Document M100. 30th ed).

Antibiotic (Method)	**Phenotype (EUCAST/CLSI)***
Ampicillin (Etest)	MIC= 4 mg/L (S)
Tetracycline (Etest)	MIC >256 mg/L (R)
Rifampicin (Etest)	MIC= 0.023 mg/L (S)
Ceftaroline (Etest)	MIC= 0.5 mg/L (S)
Ceftriaxone (Etest)	MIC >256 mg/L (R)
Cefotaxime (Etest)	MIC >32 mg/L (R)
Levofloxacin (Microdilution)	MIC= 4 mg/L (S)
Vancomycin (Microdilution)	MIC= 0.5 mg/L (S)
Teicoplanin (Microdilution)	MIC <=1 mg/L (S)
Daptomycin (Microdilution)	MIC= 4 mg/L (S)
Linezolid (Microdilution)	MIC= 2 mg/L (S)
Ciprofloxacin (5 μg; disk diffusion)	13 mm (R)
Erythromycin (15 μg; disk diffusion)	34 mm (S)
Quinupristin-dalfopristin (15 μg; disk diffusion)	34 mm (S)
Chloramphenicol (30 μg; disk diffusion)	24 mm (S)
Gentamicin (30 μg; disk diffusion)	21 mm (S)
Streptomycin (300 μg; disk diffusion)	22 mm (S)

Of note, pEfm57_1 encoded a *pbp5* gene variant flanked by *psr* (potential role in cell wall metabolism) **(217)** and a truncated version of *napA* (a monovalent cation:proton antiporter-2 family protein missing the initial 675 nucleotides), each surrounded by complete sequences of the IS6 family IS1216E. PBP5 sequence encoded in pEfm57_1 showed 97.6% amino acid identity with their chromosomal native counterpart. Known PBP5 mutations predicted to be related to ampicillin resistance were detected in the chromosomal (V24A, S27G, E100Q, K144Q, T172A, N496K, A499T, and E525D) but not in the plasmid variant according to ResFinder. The pEfm57_1 plasmid also harboured tetracycline resistance genes [*tet(M)*, *tet(L)*] and multiple instances of recombinases and transposases of the IS3, IS6 and IS30 families **[Figure 25]**. No antibiotic resistance genes were predicted in the pEfm57_2 plasmid.

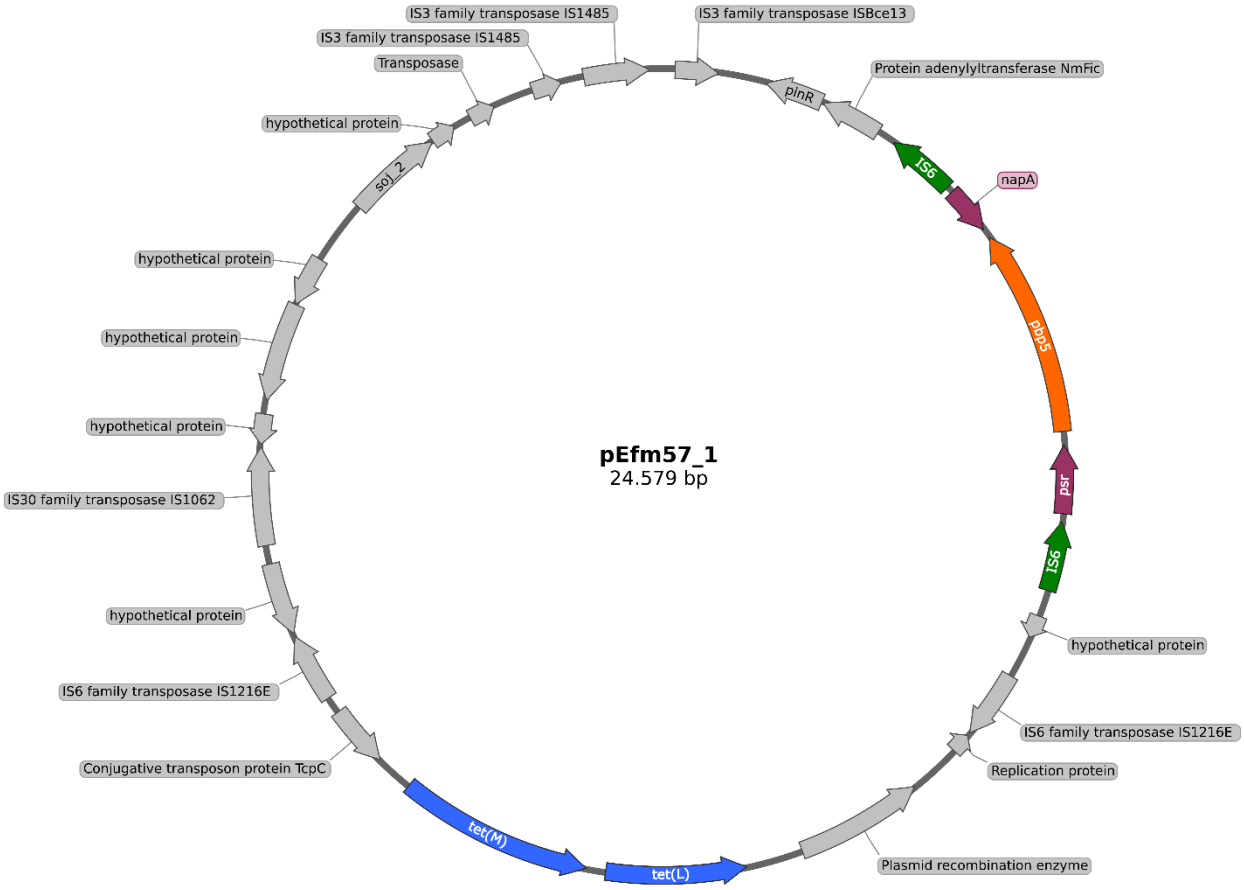


Figure 25. Schematic map depicting the genetic architecture of pEfm57_1 plasmid. The figure was constructed with SnapGene.

5.4.2. Comparative genomics and phylogenetic analysis of *pbp5* genes

In order to gain evolutionary insights into the mobilization and redundancy of *pbp5* genes across the *Enterococcus* genus, we searched for PBP5 homologs in all the NCBI *Enterococcus* complete genome assemblies. Our analysis determined that 20 genomes (3.2%) belonging to different *Enterococcus* spp. carried redundant *pbp5* genes, with two genomes harbouring three copies of the gene. These redundant *pbp5* were either chromosomal (n=6) or plasmidic (n=17) **[Figure 26]**. In order to confirm that the redundant *pbp5* genes were mobilized from an *Enterococcus* background, we performed a simple BLASTP analysis against one randomly-selected RefSeq genome per bacterial genus (n=1,530) and 11 additional RefSeq genomes of different *Enterococcus* species encoding a single *pbp5* gene. The results revealed that top BLASTP hits for redundant PBP5 sequences were always all the *Enterococcus* genomes included in the analysis.

To explore the phylogenetic relationship between the redundant *pbp5* genes and their native counterparts, Bayesian phylogenetic analysis was conducted on the predicted *Enterococcus* PBP5 sequences. The tree inferred by MrBayes software **[Figure 26]** revealed that the proteins

encoded by the redundant *pbp5* genes mapped to three well-supported clades, hereafter referred to as Clades I, II and III. PBP5 protein sequences of the different clades showcased remarkable diversity, encompassing sequences with pairwise amino acid sequence identities ranging from 44.8% to 56.5%.

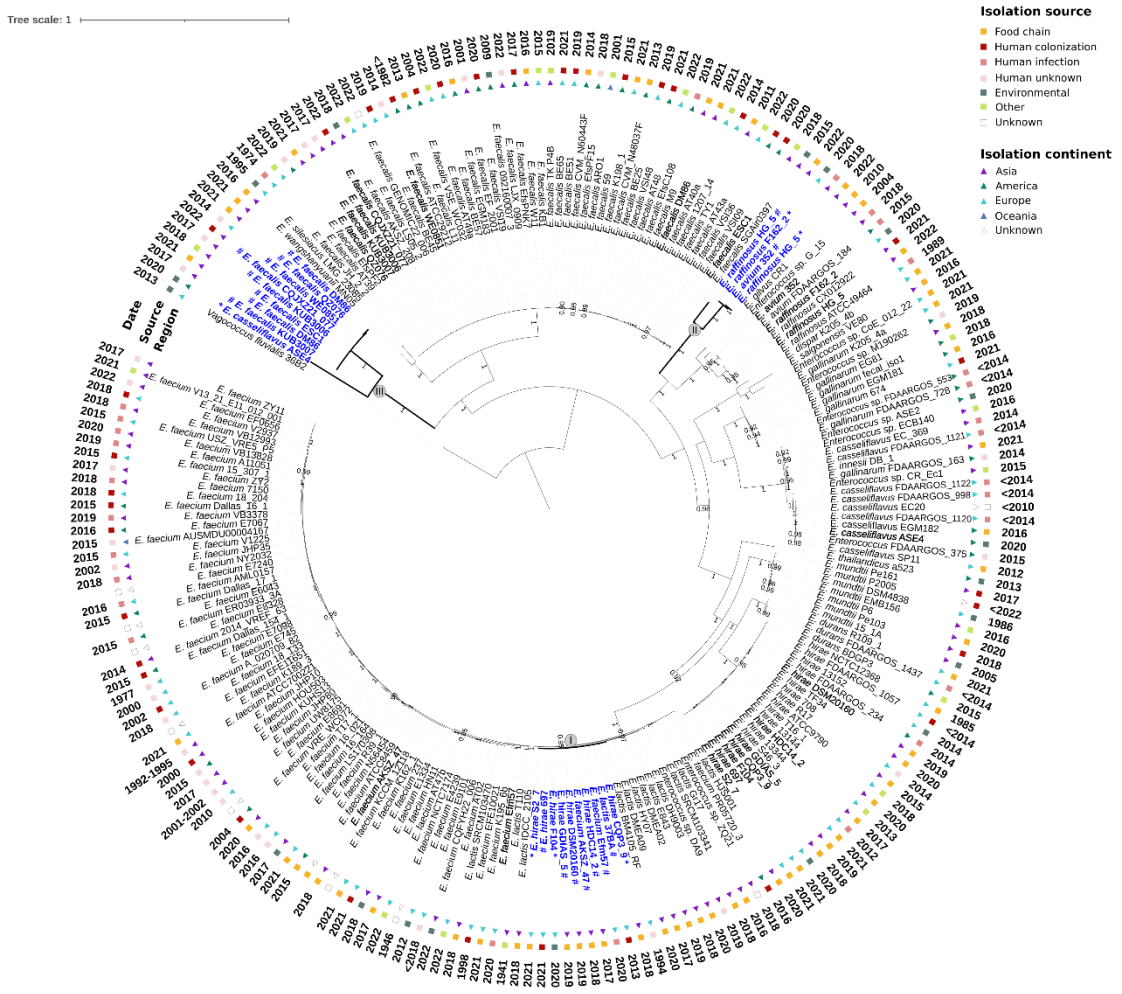


Figure 26. Rooted consensus tree of PBP5 protein sequences. Support values are shown for branches with posterior probability values higher than 0.9. Clades I, II and III, including redundant PBP5 identified in *Enterococcus* spp., are denoted by circles and shaded in blue. PBP5 sequences within these clades are marked with '*' if chromosomal and '#' if plasmid-borne. Chromosomal counterparts of redundant PBP5 sequences are highlighted in bold. *V. fluvialis* 36B2 PBP5 sequence is used as the root.

The three clades included only redundant PBP5 sequences and not their native counterparts. For the PBP5 variants of the three clades ResFinder did not predict any known mutations conferring ampicillin resistance. The redundant *pbp5* sequences across the three clades were found on both plasmids and chromosomes, each in unique genetic contexts specific to their respective clades. The *pbp5*-containing plasmids from all clades often carried diverse antibiotic resistance genes. Although redundant PBP5 were found in isolates from several origins, they were often detected in isolates from humans and the food chain in the three clades. The specificities of each cluster were analysed below, focusing on their amino acid identity, genetic contexts, associations with other antibiotic resistance genes, and epidemiological context.

5.4.2.1. Genomic insights into redundant PBP5 sequences across different clades

Clade I comprises the *pbp5* gene encoded in pEfm57_1 as well as plasmid-encoded *pbp5* variants (n=6) from another *E. faecium* (*rep2*+*repUS43*; 43,116 bp; ST22), *E. lactis* (*rep2*; 40,396 bp) and *E. hirae* (two plasmids with *repUS15*, one with *rep2*+*repUS43* or one with *repUS1*; 53,013-136,848 bp) isolates, plus redundant *pbp5* chromosomal copies of *E. hirae* (n=2) **[Figure 26]**. PBP5 sequences of this clade showed a mean amino acid identity of 99.9% (± 0.1 SD). When comparing these redundant PBP5 to their native PBP5 sequences, amino acid identity ranged from 78.5% (± 0.2 SD) in *E. hirae* to 97.5% (± 0.2 SD) in *E. faecium* and *E. lactis.* Genetic environments of Clade I *pbp5* were all similar to that described for pEfm57_1 *pbp5* variant, involving truncated versions of *napA* and in one case of *psr*, and two complete copies of IS6 family (IS*1216*) transposases **[Figure 27]**. This genetic arrangement (*psr*-*pbp5*-*napA*) was also observed in the native chromosomal *pbp5* copies of all analysed *E. lactis*, *E. hirae* and *Enterococcus durans,* in 88% of *E. mundtii*, and in 78% *E. faecium*. The cluster comprising Clade I PBP5 protein sequences and native PBP5 of *E. faecium* and *E. lactis* appeared to be closely related to native PBP5 encoded in *E. hirae*, *E. durans* and *E. mundtii* genomes **[Figure 26]**, further supported by the conservation of their genetic synteny **[Figure 27]**.

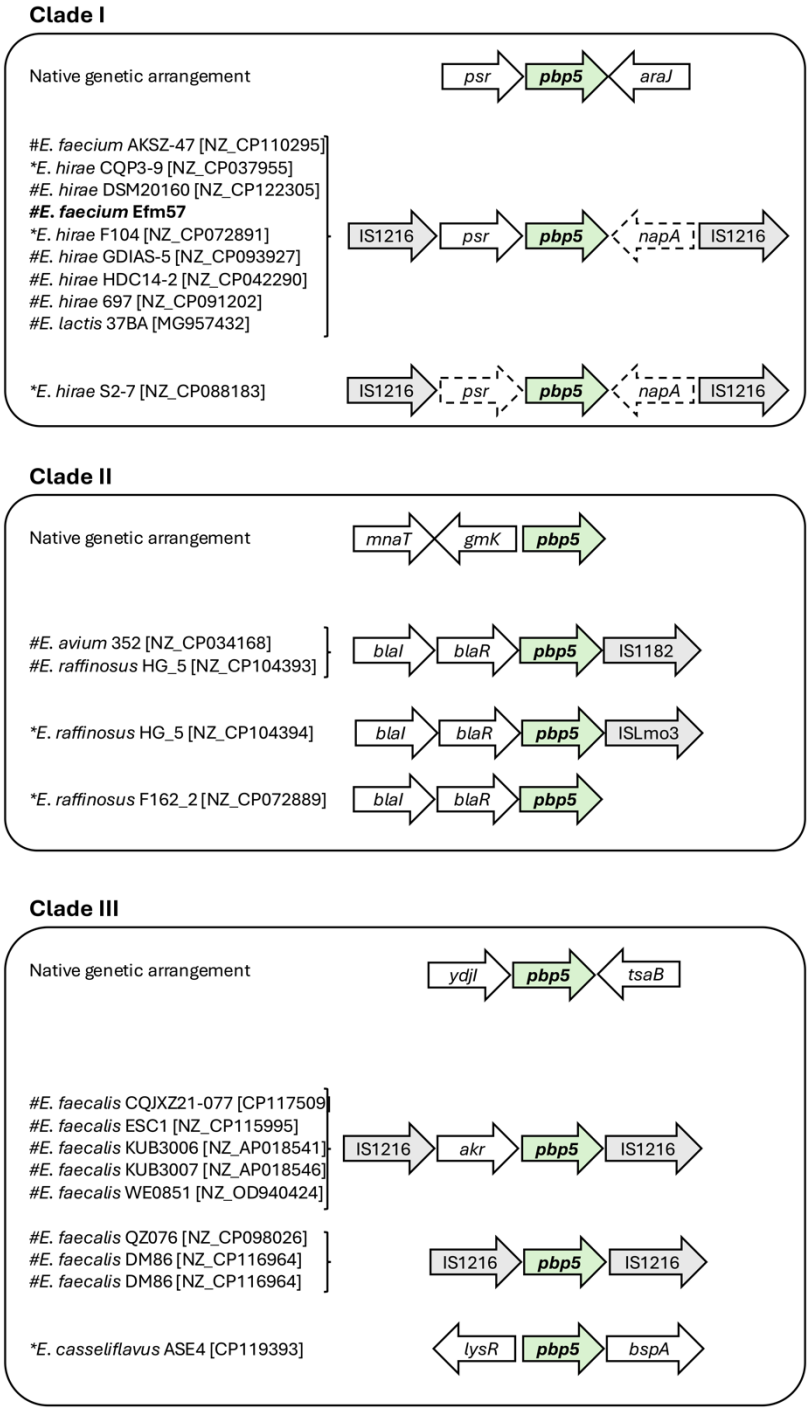


Figure 27. Graphical representation of genetic arrangements encompassing all identified redundant PBP5-encoding genes. Arrow shading corresponds to *pbp5* (green) and mobilization genes (grey). Dashed arrows indicate pseudogenes. PBP5 sequences within these clades are marked with '*' if chromosomal and '#' if plasmid-borne. GenBank accession numbers are also provided.

Clade II comprises two plasmid-borne *pbp5* genes from *Enterococcus avium* (*rep1*; 87,704 bp) and *Enterococcus raffinosus* (unknown *rep*; 79,977 bp), plus two additional chromosomal *pbp5* copies of *E. raffinosus* strains **[Figure 26]**. Of note, *E. raffinosus* HG_5 encoded two more copies of *pbp5* (75.5% amino acid identity) located either on the chromosome or on another pHG52

plasmid, being positioned in different branches of Clade II. PBP5 sequences of this clade showed a mean amino acid identity of 87.6% (± 12.0 SD). This identity value decreased to 57.9% (± 0.7 SD) when comparing Clade II PBP5 with their native counterparts. The genetic environments of Clade II encompass *blaI* and *blaR1* homologs located upstream of *pbp5* genes, and their putative mobilization accounted for complete sequences of IS6 (ISLmo3) or IS1182 family transposases. No transposases were predicted in the genetic background of the redundant *pbp5* gene in the *E. raffinosus* F162_2 genome **[Figure 27]**. The topology of the tree indicated that Clade II PBP5s are more related to the native PBP5 homologs encoded in *E. avium, E. raffinosus* and *Enterococcus gilvus* than to those of other species **[Figure 26]**. Contrasting with Clade I, the genetic environments of these native *pbp5* did not align with those of Clade II suggesting that *blaI* and *blaR1* genes were acquired after the mobilization of *pbp5.*

Clade III is mainly composed of eight plasmid-borne *pbp5* genes from *E. faecalis* (three *rep1,* one *repUS11* one *rep9b* and two with unknown *rep*; 25,721-65,961 bp; ST21, ST256, ST314, ST480, ST729, STnew) and a chromosomal PBP5-encoding gene of *Enterococcus casseliflavus* **[Figure 26]**. Of note, *E. faecalis* DM86 harboured two *pbp5* genes (99.7% amino acid identity) in pDM86-*optrA* plasmid. *E. faecalis* Clade III PBP5 sequences showed a mean amino acid identity of 99.8% (± 0.2 SD). This value decreased to 51.1% (± 0.2 SD) when comparing *E. faecalis* Clade III PBP5 with their native counterparts. Except for *pbp5* copies encoded by pDM86-*optrA* and pQZ076-1 plasmids, their genetic environments also presented a fragment of a gene encoding an aldo/keto reductase (AKR). Similarly to Clade II and in contrast to Clade I, this genetic environment was not found in any of the native *pbp5* genetic environments of analysed *Enterococcus* genomes **[Figure 27]**. The *E. casseliflavus* ASE4 strain, presented a redundant PBP5 with 47.1% amino acid identity when compared to its native PBP5 **[Figure 26]**. This PBP5 sequence showed a remarkably lower amino acid identity (62.4%± 0.1 SD) compared to the rest of Clade III PBP5 proteins. Although no mobilization genes were found in its genetic environment, this PBP5-encoding gene was considered the redundant variant since it did not map with the rest of chromosomal *E. casseliflavus* PBP5-encoding genes **[Figure 26]**. Notoriously, the genetic context of this *pbp5* variant included a *lysR* gene encoding a transcriptional regulator and a gene encoding a putative BspA family leucine-rich repeat surface protein **[Figure 27]**. Finally, the arrangement of the tree indicates that PBP5 within Clade III are related to native PBP5 copies found in *E. faecalis*, *Enterococcus silesiacus* and *Enterococcus wangshanyuanii* genomes than to those of other species **[Figure 26]**.

5.4.2.2. Antibiotic resistance genes predicted in pbp5-carrying plasmids in Enterococcus

A deeper analysis of the genetic context of plasmidic *pbp5* showed that plasmids from the three clades harboured genes coding for resistance to aminoglycosides, macrolides, streptogramins of group B, or lincosamides. Additionally, *pbp5*-plasmids from Clade I also carried genes coding for resistance to streptothricin, trimethoprim, phenicols, and linezolid (two *E. hirae* with *poxtA*). Clade III *pbp5*-plasmids included genes for resistance to fosfomycin, phenicols, linezolid (three *E. faecalis* with *cfr* or *optrA*), and the chemotherapeutic drug bleomycin. Resistance to oxazolidinones was also detected in isolates from all clades at locations other than the *pbp5*-carrying plasmids. This included five *E. hirae* or *E. faecium* isolates with *optrA* and/or *poxtA* in Clade I, one *E. raffinosus* isolate with *optrA* in Clade II, and seven *E. faecalis* isolates in Clade III carrying *optrA, cfr, cfr(B), cfr(D)*, or *poxtA*.

5.4.2.3. Epidemiological context of Enterococcus carrying redundant pbp5

Isolates carrying *pbp5* in plasmids from Clades I, II and II were isolated in recent years (2013-2022) from several sources, namely human, food chain and environment from Asia and Europe **[Figure 26]**. To identify a possible epidemiological focus for the Efm57 *pbp5* mobilization (Clade I) as well as of other PBP5 of Clades II and III a BLASTP search against the *nr* database (including non-complete genomes) was performed. PBP5 sequences identical to that of pEFM57_1 were detected in six species, with 48 isolates identified as *E. faecium*. Similarly to those strains included in the Clade I, 69.9% (n=51) of the strains carrying Efm57 PBP5 variant were from the food chain, particularly of pig origin (49%), being mostly recovered in Asia (45.1%) and North America (43.2%) between 2001-2023. On the case of Clade II, the BLASTP search against the *nr* database only identified three more PBP5 sequences identical to the plasmidic variant of two representative isolates of Clade II (*E. avium* 352 and *E. raffinosus* HG5) isolated from human colonization and food chain from Asia, between 2014 and 2022. The BLASTP search against the *nr* database with representative PBP5 of Clade III (*E. faecalis* DM86 and QZ076) further identified 46 PBP5 sequences in isolates from Asia (33.6%; pets, food-chain, human colonization or infection) and Brazil (50.8%; water and clinical isolates) recovered between 2013-2022.

5.4.3. *pbp5* transfer and ampicillin susceptibility under diverse environmental conditions

Several attempts to transfer pEfm57_1 plasmid from Efm57 to *E. lactis* BM4105RF or *E. faecalis* JH2-2 were unsuccessful. In contrast, transconjugants were successfully obtained using *E. faecium* 64/3 as a recipient strain, with a conjugation frequency of 3.8×10^{-6}. Two transconjugants (TC1 and TC2) selected for further analysis exhibited a MIC for ampicillin of 3-4 mg/L, which is

similar to that of Efm57 (4 mg/L) and higher than that of the recipient strain *E. faecium* 64/3 (1.5 mg/L) **[Figure 28]**. At 22°C, the MICs for ampicillin increased by one and a half- to fourfold for the transconjugants (6-16 mg/L), two- to threefold for Efm57 (8-12 mg/L) and twofold (2-3 mg/L) for strain *E. faecium* 64/3 **[Figure 28]**.

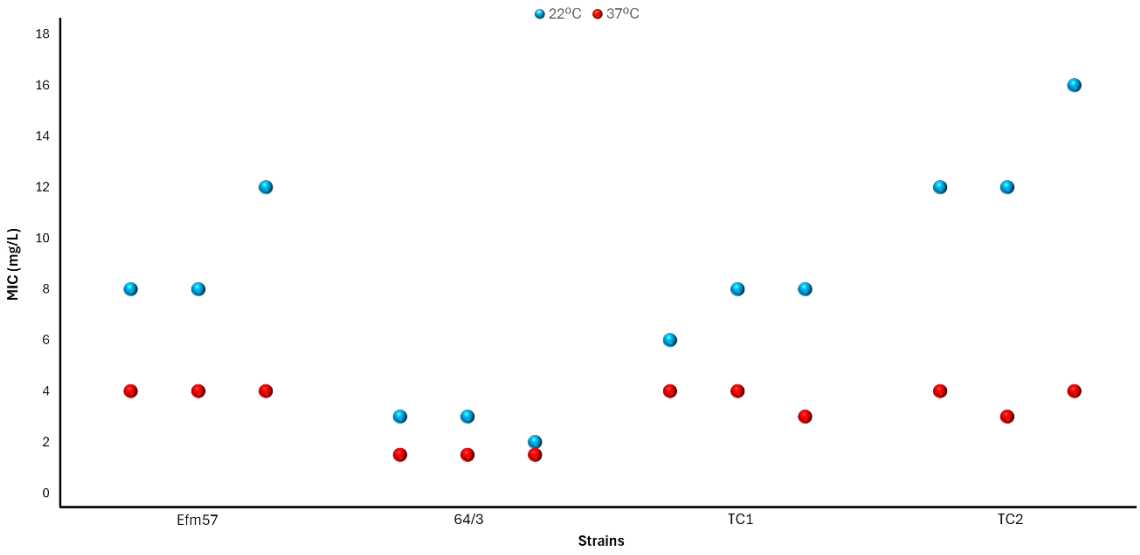
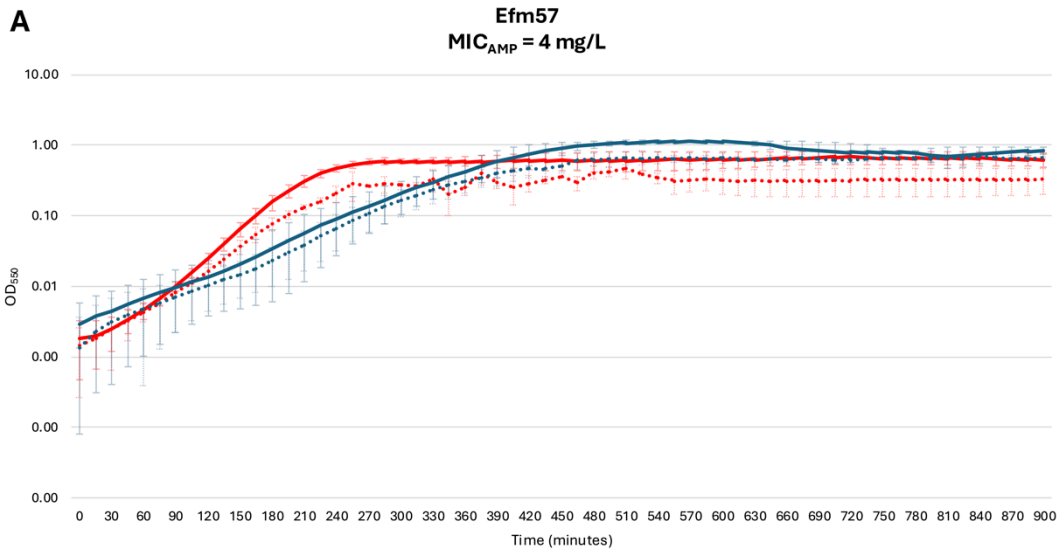
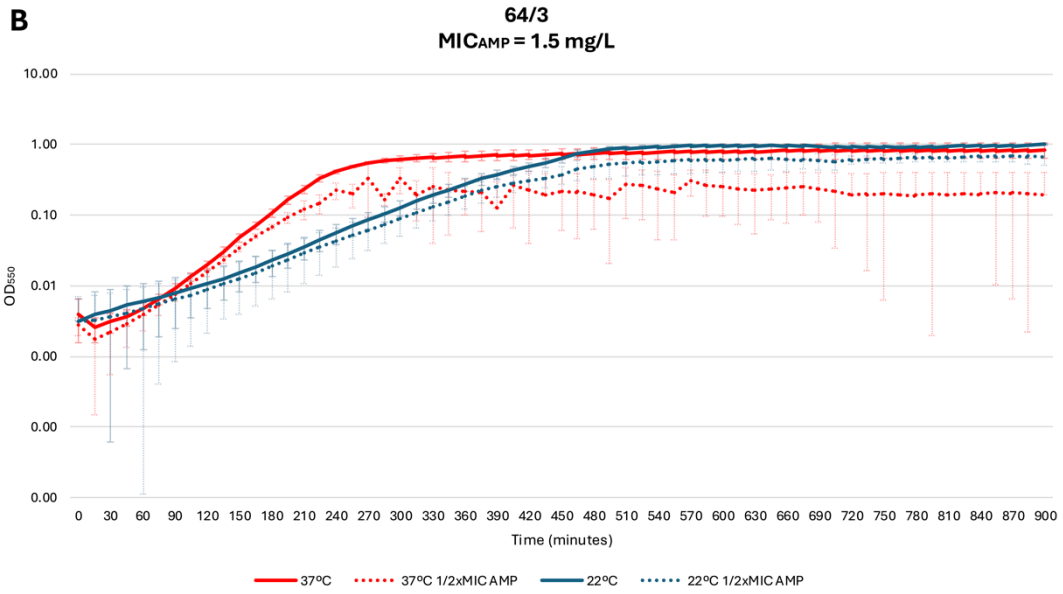


Figure 28. MIC values of ampicillin determined by Etest diffusion assay at 22°C and 37°C for the wild-type strain Efm57, the recipient strain *E. faecium* 64/3, and the transconjugants TC1 and TC2 that acquired *pbp5*-plasmid. Three replicates were performed for each strain.

5.4.4. Effect of the *pbp5*-plasmid on growth dynamics

To get further knowledge on the impact of pEfm57_1 plasmid and temperature on overcoming ampicillin selective pressure, growth curves of Efm57, 64/3, TC1, and TC2 were obtained with or without ampicillin (½ x MIC) at 37°C and 22°C **[Figure 29]**. Regardless of their genetic background, all strains exhibited a slower overall growth rate and higher doubling times at 22°C compared to 37°C as expected **[Table 8; Figure 30]**. At 37°C, the presence of ampicillin prompted an important growth delay for all strains. However, at 22°C, this decline of the growth rate was only observed in strain 64/3, which is devoid of pEfm57_1 plasmid. In contrast, at 22°C, all strains carrying pEfm57_1 exhibited comparable growth rates and doubling times with or without ampicillin.


A
Efm57
MIC$_{AMP}$ = 4 mg/L
10.00
1.00
0.10
0.01
0.00
0.00
0.00
OD$_{550}$
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510 540 570 600 630 660 690 720 750 780 810 840 870 900
Time (minutes)


B
64/3
MIC$_{AMP}$ = 1.5 mg/L
10.00
1.00
0.10
0.01
0.00
0.00
0.00
OD$_{550}$
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510 540 570 600 630 660 690 720 750 780 810 840 870 900
Time (minutes)
37ºC
37ºC 1/2xMIC AMP
22ºC
22ºC 1/2xMIC AMP

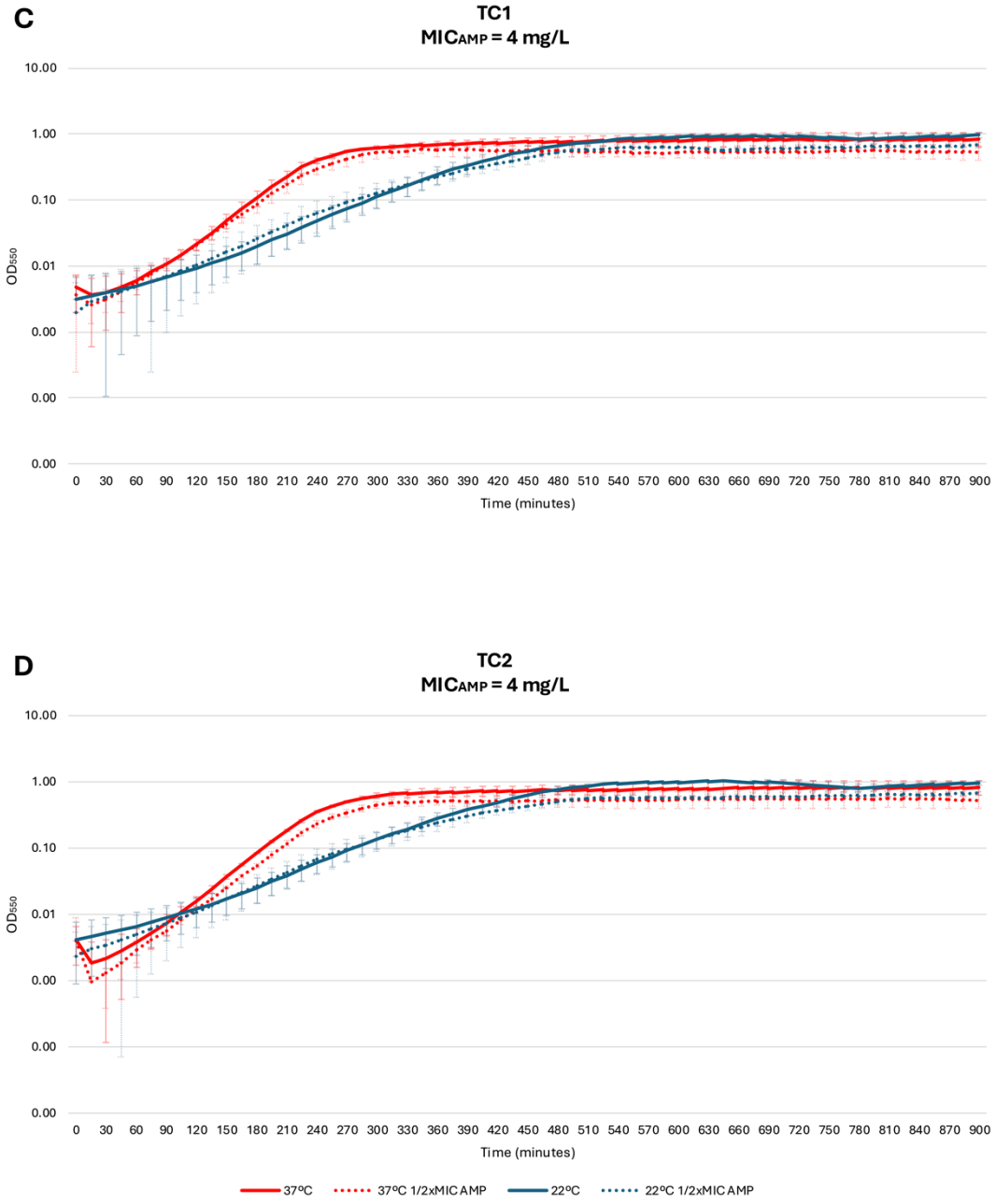


Figure 29. Growth kinetics of *E. faecium* isolates with (Efm57, TC1 and TC2) or without (64/3) the pEfm57_1 plasmid. Growth was monitored at 37°C (red lines) and 22°C (blue lines) in the absence (dark red and blue lines) or presence (light red and blue lines) of ampicillin. The ampicillin concentrations used at 37°C and 22°C correspond to 1/2xMIC determined by the Etest diffusion method under standard conditions, following EUCAST guidelines. Readings were taken every 15 minutes over 24 hours but for clarity, the values shown on the graph are presented every 30 minutes.

Table 8. Selected time points from the maximum and linear exponential growth phases, used for subsequent calculation of the growth rate and doubling time for each strain and condition. For further details on the growth rate calculations, refer to **Figure 30**. MIC, minimum inhibitory concentration; Amp, ampicillin; min, minutes; OD, optical density.

Strain	Time (min)	OD_{550}	Growth rate	Doubling time (min)	Time (min)	OD_{550}	Growth rate	Doubling time (min)
	37°C/without antibiotic				37°C 1/2xMIC AMP			
Efm57	90	0.01			105	0.01		
	105	0.02			120	0.02		
	120	0.02	0.47	88.75	135	0.02	0.40	104.23
	135	0.04			150	0.04		
	150	0.06			165	0.05		
64/3	90	0.01			75	0.01		
	105	0.01			90	0.01	0.36	115.72
	120	0.02	0.41	100.80	105	0.01		
	135	0.03			120	0.02		
	150	0.05						
TC1	105	0.01			60	0.01		
	120	0.02	0.40	104.28	75	0.01		
	135	0.03			90	0.01	0.34	122.28
	150	0.05			105	0.01		
					120	0.02		
					135	0.03		
TC2	90	0.01			75	0.00		
	105	0.01			90	0.01		
	120	0.02	0.41	101.29	105	0.01	0.37	111.83
	135	0.02			120	0.01		
	150	0.04			135	0.02		
	165	0.06			150	0.03		

Strain	Time (min)	OD_{550}	Growth rate	Doubling time (min)	Time (min)	OD_{550}	Growth rate	Doubling time (min)
	22°C/without antibiotic				22°C 1/2xMIC AMP			
Efm57	150	0.02			180	0.02		
	165	0.03			195	0.03		
	180	0.03	0.26	162.46	210	0.04	0.26	154.49
	195	0.04			225	0.05		
	210	0.06			240	0.07		
64/3	165	0.02			150	0.01		
	180	0.02			165	0.02	0.21	202.67
	195	0.03	0.22	187.51	180	0.02		
	210	0.04			195	0.02		
	225	0.04						
	240	0.06						
TC1	180	0.02			120	0.01		
	195	0.02	0.22		135	0.01		
	210	0.03		182.57	150	0.02	0.23	181.61
	225	0.04			165	0.02		
					180	0.03		
TC2	150	0.02			90	0.01		
	165	0.02			105	0.01		
	180	0.03	0.21	202.48	120	0.01	0.21	198.42
	195	0.03			135	0.01		
	210	0.04			150	0.02		

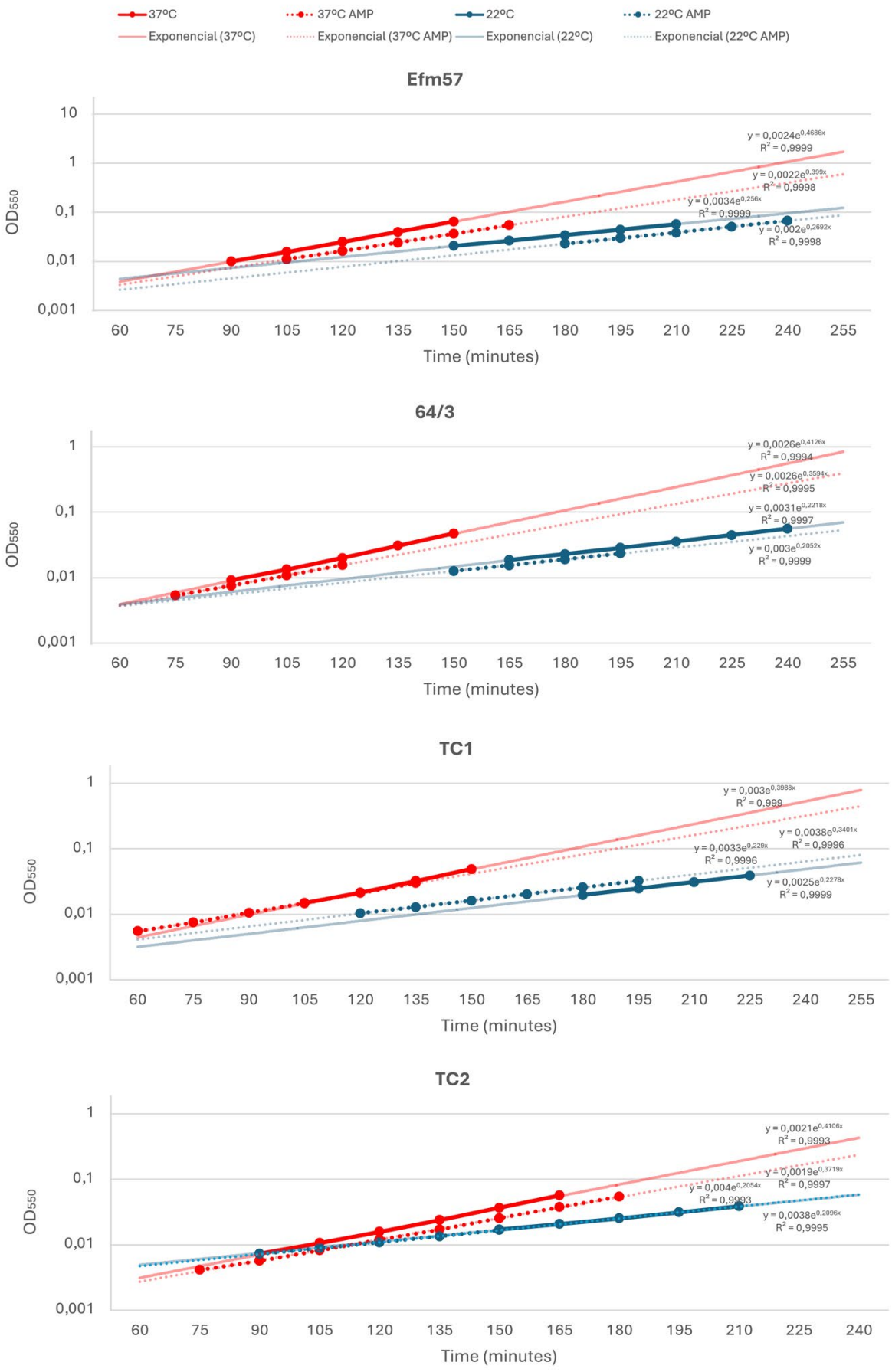


Figure 30. Selected points from the maximum exponential and linear growth phases used to calculate the growth rate and doubling time. R^2 value indicates the fit and accuracy of the selected points.

5.5. Discussion

This study highlights the widespread distribution of redundant *pbp5* genes, mainly located on plasmids, across *Enterococcus* spp. from different epidemiological contexts. Redundancy and dissemination of *pbp5* across *Enterococcus* genomes from diverse species occur more often than previously anticipated **(184,186)**, affecting isolates from different origins. The topology of the phylogenetic tree **[Figure 26]** supports an evolutionary scenario wherein chromosomal *pbp5* genes underwent at least three independent mobilization events, leading to the emergence of redundant *pbp5* variants forming the Clades I, II and III. Spread of these gene clusters appears to be predominantly mediated by complete ISs in *Enterococcus* represented in all clades, mainly IS6-like elements **(218)**, suggesting a recent acquisition and propagation of these variants by diverse plasmids. The similarity between native and plasmid-encoded PBP5 sequences of *E. faecium* and *E. lactis* in Clade I further supports the recent mobilization of *pbp5* to plasmids in these species, whereas in Clades II and III, the greater dissimilarity between native and acquired *pbp5* genes, alongside different genetic environments, suggests diversification post-mobilization or acquisition from unidentified enterococcal hosts **(187,219)**.

Plasmids are believed to play a significant role in promoting genetic redundancy, facilitating microbial adaptation through horizontal gene transfer within microbial communities **(83,191,220)**. However, persistence of redundant antibiotic resistance genes on plasmids may need ongoing selection with gene expression for their maintenance, as without it, reversion to a gene single-copy state may occur **(187,189,221)**. Our results indicate that the conjugative pEfm57_1 plasmid facilitates growth in the presence of ampicillin and, remarkably, this effect is more pronounced at 22°C. Therefore, it is tempting to speculate that specific environmental conditions, such as low temperatures, enhance the potential of plasmid-borne redundant *pbp5* genes to overcome antibiotic selective pressure at low temperatures.

These data raise concerns about the challenges in detecting strains with temperature dependent phenotypes in standard antibiotic susceptibility tests during surveillance studies in diverse ecological niches. Additionally, they highlight the potential of these strains to be selected, maintained, and spread in environments with fluctuating β-lactam concentrations and temperature variations, especially in the context of climate change. Such factors could significantly affect bacteria circulation across overlapping environments, including clinical setting **(222,223)**. Despite its clinical origin, Efm57 exhibits molecular and phenotypic characteristics divergent from typical hospital outbreak-associated *E. faecium* strains (clade A1) **(46)**, suggesting patient's gut as the possible source and an unknown community origin. Further

investigation is needed to determine whether the temperature-dependent effect on the decrease of ampicillin susceptibility is specific to the Efm57 *pbp5*-plasmid or applicable to other *pbp5*-plasmids. Previous findings with a conjugative multidrug-resistant (MDR) plasmid encoding a Clade I *pbp5* gene in *E. lactis* 37BA (previous identified as *E. faecium*) showed stable ampicillin resistance in antibiotic-free media for 30 days at 37°C **(186)**, suggesting that *pbp5*-plasmid persistence within bacterial populations and susceptibility phenotypes may be influenced by plasmid backbones, host genetic background, and diverse environmental conditions.

The presence of multiple antibiotic resistance genes on Clades I, II, and III *pbp5*-plasmids highlights the involvement of such plasmids in co-selection and recombination events with abundant genetic modules occurring in metagenomes from environments where antibiotics are extensively used. Genes conferring resistance to tetracyclines, macrolides, lincosamides, chloramphenicol, or aminoglycosides, often widespread in Bacillota from the food chain and other settings **(224,225)**, were prevalent in these plasmids across various geographical regions. Notably, *optrA*, *poxtA*, and *cfr* genes, which provide resistance to linezolid, were frequently found on *pbp5*-plasmids considering Clades I and III, indicating a heightened risk of *pbp5* mobilization also along with clinically significant resistance genes under intense antibiotic selection pressures. This aligns with a recent study that analysed redundant antibiotic resistance genes across 18,938 complete bacterial genomes, revealing their enrichment in bacteria isolated from humans, livestock, and environments associated with antibiotic use **(221)**.

In conclusion, the widespread distribution of redundant *pbp5* genes among *Enterococcus* spp. highlights the intricate relationship between genetic mobility, environmental factors, and multidrug resistance. Environmental conditions, such as temperature and antibiotic fluctuations, may trigger the replication of *pbp5*-MDR plasmids, facilitating the spread and new recombination of antibiotic resistance genes. Whether the mobilization of *pbp5* plasmids and/or *pbp5*-genes-clusters signifies an emerging concern or remains contained to few detected transitory events requires further investigation in the coming years. A deeper understanding of how multiple environmental conditions contribute to the dissemination of multidrug resistance in diverse settings is imperative to mitigate antibiotic resistance spread within the framework of One Health as well as in the context of climate change.

5.6. Data availability

Sequence data generated in this study have been submitted to the NCBI database under the BioProject accession number PRJNA1132796. The nucleotide sequence of the pEfm57_1 plasmid has been deposited in GenBank under the accession number PP974444.

Scripts used for obtaining and analysing genomic data are available in the GitHub repository (https://github.com/Bacterial-Blood-Pathogens/efm_pbp5).

Chapter III: Single point mutations in *croS*, *nusG*, or *rpoB* genes restore cephalosporin resistance to a naturally occurring *Enterococcus faecium* susceptible isolate

6. Chapter III: Single point mutations in *croS*, *nusG*, or *rpoB* genes restore cephalosporin resistance to a naturally occurring *Enterococcus faecium* susceptible isolate

6.1. Abstract

Enterococcus faecium exhibits natural resistance to cephalosporins (CPH), but the molecular basis of this phenotype is not completely understood. So far, all reported *E. faecium* with low minimum inhibitory concentrations (MIC) to cephalosporins have been genetically engineered. At the Parc Taulí University Hospital (Sabadell), we identified an ampicillin-susceptible *E. faecium* clinical isolate (Efm5) exhibiting low MICs to ceftaroline (0.19 mg/L), ceftriaxone (3 mg/L) and cefotaxime (1 mg/L). Isolates with high MIC to ceftriaxone (MIC_{CRO}>256 mg/L) and cefotaxime (>32 mg/L) were also recovered after exposure of Efm5 to 100 mg/L of ceftriaxone but retained low MICs to ampicillin and ceftaroline. Genomic comparison of Efm5 and isolates with high MIC_{CR} only revealed single nucleotide polymorphisms (SNPs) associated with non-synonymous mutations in three different genes: *croS*, *nusG* and *rpoB*.

RNA-sequence assays showed that in the *croS* mutated variants, the *pbp5* operon was markedly upregulated, which is compatible with an activation of the CroRS system. *nusG* and *rpoB* mutants exhibited extensive transcriptional changes with a substantial overlap in the differentially expressed genes associated with diverse metabolic networks. Time-killing curves assays in the presence of ceftriaxone, and electron microscopy analyses also supported a phenotypic impact of the identified mutations of the three genes. Our data show that *Enterococcus* with low MICs to CPH may occur in nature and that intrinsic resistance to these antibiotics is associated with multiple and new genes beside those previously described. These naturally occurring isolates may represent suitable platforms to better understand bacterial evolution against antibiotics within the clinical setting.

6.2. Introduction

Enterococcus faecium exhibits natural resistance to β-lactam antibiotics. Accordingly, most *E. faecium* strains are resistant to ampicillin (MIC>8 mg/L), which is primarily achieved by the production of a low-affinity penicillin-binding protein (PBP) called PBP5 **(46)**. Particular PBP5 variants and PBP5 overexpression have also been related with high level ampicillin resistance (AmpR) **(78,90)**. *E. faecium* Clade A, often hospital-associated, typically has high ampicillin MICs (≥16 mg/L) and is characterized by the presence of *pbp5*-R alleles, which encode PBP5 variants enhancing AmpR. Within Clade A, subclade A1 is associated with clinical settings, while subclade A2 includes animal-associated and sporadic human isolates that sometimes display ampicillin-susceptibility (AmpS). Clade B, which is primarily associated with community settings and generally ampicillin-susceptible (AmpS) **(76,139,186)**, carries *pbp5*-S alleles and it has recently been reclassified as *Enterococcus lactis* **(10)**. Additionally, hybrid *pbp5*-S/R patterns, observed in some strains, suggest an evolutionary progression from AmpS to AmpR **(139)**. However, these hybrid forms do not consistently affect MIC values, as reported in recent studies.

Intrinsic resistance of *E. faecium* to cephalosporins (CPHs), another group of β-lactam antibiotics commonly used to treat bacterial infections, relies on multiple molecular mechanisms that are not fully understood. One significant contributor to cephalosporin resistance in *E. faecium* is also PBP expression **(144,150)**. *E. faecium* possesses six PBPs, including three with bifunctional transglycosylation and transpeptidation activity (class A) and three performing only transpeptidation reactions (class B) **(78)**. PBP5, a class B PBP (bPBP) demonstrates low affinity for β-lactam antibiotics and plays a crucial role in *E. faecium*'s resistance against β-lactam antibiotics, including cephalosporins and ampicillin **(111,144)**. Mutants lacking PBP5 (Δ*pbp5*) exhibit significantly reduced resistance to β-lactams due to its crucial role in maintaining cell wall integrity **(137)**. PBPA, another bPBP, has recently been associated with β-lactam resistance. Strains lacking PBPA (Δ*pbpA*) exhibit decreased cephalosporin resistance and display phenotypic defects in growth kinetics, cell wall integrity, peptidoglycan synthesis, and cellular morphology, indicating PBPA's pivotal role in peptidoglycan synthesis. Conversely, Δ*pbpA* strains are comparatively less impacted in terms of ampicillin resistance compared to Δ*pbp5* strains. When both PBP5 and PBPA are absent, *E. faecium* becomes highly susceptible to cephalosporins and ampicillin, underscoring the intricate interplay between these proteins in β-lactam resistance.

In addition to PBPs, several two-component systems (TCSs) and early wall synthesis enzymes have also been related to cephalosporin resistance **(22,144)**. CroRS is a TCS that regulates various genes involved in cell wall synthesis. When the kinase CroS senses imbalances in

peptidoglycan synthesis and degradation at sites of nascent peptidoglycan, it phosphorylates the regulator CroR, leading to changes in gene expression **(98,100,101,226)**.

The eukaryotic-like kinase/phosphatase StK-StpA system has also been implicated in cephalosporin resistance in *E. faecium* **(152)**. StK is a self-phosphorylating kinase which acts as a sensor detecting modifications in nascent peptidoglycan, while StpA is a phosphatase that modulates the activity of StK **(149,227)**. By phosphorylating target proteins, StK activates signalling pathways that promote cephalosporin resistance. Mutations in StK have been shown to increase its activity, leading to enhanced resistance to cephalosporins. Similarly, mutations in StpA that impair its phosphatase function can lead to increased StK activity, promoting also higher levels of cephalosporin resistance **(152)**. The absence of StpA appears to be a primary determinant of cephalosporin resistance in *E. faecium*.

MurAA is an UDP-N-acetylglucosamine 1-carboxyvinyltransferase involved in the early steps of peptidoglycan synthesis and plays a crucial role in resistance antibiotics, particularly cephalosporins **(24)**. Deletion of *murAA* in *E. faecium* results in a significant decrease in cephalosporin resistance, aligning with observations in *E. faecalis*. Synergy between ceftriaxone and fosfomycin, a small molecule inhibitor of MurAA, has been demonstrated in *E. faecalis*, underscoring the critical involvement of this protein in cephalosporin resistance.

To date, all documented *Enterococcus* strains with reduced MICs to cephalosporins have been genetically engineered in the laboratory. *In E. faecalis* strains, separate deletions of *pbp5* and *pbpA* dramatically reduce the MICs to cephalosporins, affecting a range of cephalosporins across the 2nd, 3rd, 4th, and 5th generations **(101,137,150)**. Similarly, double mutants lacking the aPBPs *ponA* and *pbpF* in *E. faecalis* JH2-2, as well as in *E. faecium*, also become susceptible to ceftriaxone, a phenotype further observed in triple mutants with deletions of aPBPs *ponA*, *pbpF*, and *pbpZ* **(152)**. Furthermore, deletions of *ireK* (the orthologous of *stk* in *E. faecium*), as well as in double mutants Δ*ireK* and Δ*ireP* (the orthologous of *stpA* in *E. faecium*), render *E. faecalis* susceptible to cephalosporins **(228)**. Moreover, in *E. faecium*, deletion of the *croRS* regulatory genes results in a pronounced decrease in the MIC for ceftriaxone **(100)**. Notably, when PBP5 is constitutively expressed in the Δ*croRS* mutant, the MIC values do not revert to the resistance levels observed in the wild-type strains. This reveals that the presence of PBP5 alone is insufficient to restore ceftriaxone resistance and highlights the critical importance of the CroRS regulatory system in facilitating the functional activity of PBP5 in conferring resistance.

Until now, all CPH low-MIC *E. faecium* strains have been obtained *in vitro*, with no documentation of naturally occurring isolates susceptible to cephalosporins. Herein, we

describe the isolation of a cephalosporin (CPH) low-MIC *E. faecium* natural isolate from a patient with bacteremia, challenging the current paradigm of intrinsic resistance. These distinctive *E. faecium* isolates provide an opportunity to explore bacterial evolutionary dynamics during infection and uncover new genes and mutations linked to cephalosporin resistance in *E. faecium*.

6.3. Material and methods

6.3.1. Isolation and clinical context of the *Enterococcus faecium* Efm5 strain

An *E. faecium* clinical strain, designated Efm5, was isolated from an 89-year-old female patient with bacteremia at Parc Taulí Hospital in 2016. The patient, with a history of total dependence and a previous cerebrovascular accident, presented with a bacteremia likely originating from a urinary tract infection. The acquisition of the infection was associated with healthcare-related factors. The patient was initially empirically treated with a combination of levofloxacin and clindamycin for 24-48 hours. A sample from the positive blood culture was seeded on a Columbia Agar with 5% Sheep Blood plate (bioMérieux), resulting in the growth of multiple colonies with similar morphology. A representative colony underwent species identification using mass spectrometry technology (MALDI-TOF MS, Bruker), which confirmed it as *E. faecium*. Upon receiving the culture results, the treatment was switched to cefuroxime, which was subsequently administered as the definitive therapy. The clinical outcomes were favourable, with no persistence of infection and no mortality.

6.3.2. Antibiotic susceptibility testing

Antibiotic susceptibility tests were conducted using Etest for ampicillin, ceftaroline, ceftriaxone, cefotaxime, cefoxitin, vancomycin, and rifampicin. Clinical breakpoints for ampicillin, vancomycin, and rifampicin were interpreted following the Clinical and Laboratory Standards Institute **(163)**. Notably, clinical breakpoints are not available for cephalosporins.

6.3.3. Recovery of CPH high-MIC isolates

As *E. faecium* is considered intrinsically resistant to cephalosporins, we wondered whether CPH high-MIC variants could be readily isolated from our CPH low-MIC isolate. To this end, one hundred microliters of an Efm5 suspension (10^8 CFU/mL), derived from an overnight culture without antibiotic selection, were seeded onto Brain Heart Infusion (BHI) agar supplemented with 100 mg/L ceftriaxone **(152)** and incubated at 37ºC for 48 hours. CPH high-MIC colonies were subsequently selected and subcultured onto plates supplemented with 100 mg/L

ceftriaxone. Frequency of CPH high-MIC variants was determined by dividing the number of colonies growing on BHI agar supplemented with 100 mg/L ceftriaxone by the total number of colonies grown on BHI agar without antibiotics.

6.3.4. Whole Genome Sequencing

6.3.4.1. DNA extraction

Total was purified using the DNeasy Blood & Tissue Purification Kit (Qiagen), and DNA quality was evaluated using a Qubit® 2.0 fluorometer (Thermo Fisher Scientific).

6.3.4.2. DNA sequencing and quality control

Libraries were generated with the Nextera XT DNA Sample Preparation Kit (Illumina). Whole genome sequencing (WGS) was performed through paired-end sequencing on an Illumina HiSeq 2500 sequencer available at the Genomics Unit of the Centre de Regulació Genòmica (CRG, Barcelona). The quality of the raw sequencing reads was assessed using FastQC (https://github.com/s-andrews/FastQC, accessed in June 2024). Pre-processing and filtering of reads were carried out with TrimGalore **(192)**, which involved removing sequencing adapters, trimming the first 20 bp poor-quality positions, and applying a Phred score limit of ≥20.

6.3.4.3. De novo assembly and annotation

The trimmed paired-end reads were then assembled *de novo* using SPAdes **(193)**, and the quality of the assembly was evaluated using CheckM2 **(229)**. To determine strain clonality among Efm5 and CPH high-MIC isolates, multilocus sequence typing (MLST) was conducted using the MLST software **(199)**. Putative antibiotic resistance genes were predicted with ABRicate (https://github.com/tseemann/abricate, accessed in June 2024) using the in-built NCBI AMRFinderPlus **(200)** database, and virulence genes were assessed with Virulence Finder **(202)** from the CGE. Prediction of the ampicillin resistance phenotype due to *pbp5* mutations was performed with ResFinder **(201)**.

6.3.4.4. SNP Calling

In order to elucidate the genetic variants responsible for CPH high-MIC values, sequencing reads of CPH high-MIC isolates were mapped against the Efm5 genome with snippy (https://github.com/tseemann/snippy, accessed in June 2024). Synonymous Single-Nucleotide Polymorphisms (SNPs) were discarded for further analysis. Predicted mutations were confirmed

using Sanger sequencing (Servei de Genòmica i Espectroscòpia de Biomolècules, Universitat Autònoma de Barcelona).

6.3.5. Comparative genomics

To identify potential mutations responsible for the low MIC to CPH observed in the Efm5 strain, a BLASTP analysis was performed using following genes as the queries: *pbp5* (E6A31_RS06825), *pbpA* (E6A31_RS03475), *croR* (E6A31_RS13520), *croS* (E6A31_RS13525), *stpA* (E6A31_RS12560), *stk* (E6A31_RS12555) and *murAA* (E6A31_RS09060), previously reported as key contributors to intrinsic resistance to CPH **(24,100,137,152)**. The analysis specifically aimed to detect transposon insertions or premature stop codons that could disrupt the function of these resistance-associated genes.

On the other hand, in order to elucidate the genomic variations responsible for the increased MICs to CPH in Efm5 spontaneous mutants, sequencing reads of CPH high-MIC variants were mapped against the Efm5 genome with Snippy (https://github.com/tseemann/snippy, accessed in June 2024). Synonymous Single-Nucleotide Polymorphisms (SNPs) were discarded for further analysis. Predicted mutations were confirmed using Sanger sequencing (Servei de Genòmica i Espectroscòpia de Biomolècules, Universitat Autònoma de Barcelona).

To gain insights into the global distribution of observed mutations across the *Enterococcus* genus, CroS, NusG and RpoB homologs were compiled from the 767 complete *Enterococcus* spp. assemblies available in the NCBI database (accessed in October 2024) through reciprocal BLASTP **(207)** using the *E. faecium* Efm5 CroS, NusG, and RpoB protein sequences as the queries, a conservative e-value of <1e–20 and query coverage of >75%, similar to previously described **(230)**. Protein multiple sequence alignments of predicted homologs were performed with CLUSTALW using default parameters **(209)**.

6.3.6. *In vitro* time-kill curves

Time-Kill Curves (TKCs) were conducted in Mueller-Hinton Broth (MHB) with (10 mg/L) and without ceftriaxone (Sigma Aldrich, St Louis, MO, USA), using an initial inoculum of 10^6 CFU/mL. The cultures were incubated at 35ºC and 150 rpm. Aliquots of 100 µL were taken at 0, 2, 4, 6, and 24 hours for each strain and condition, and 10 µL of each aliquot was plated in duplicate on BHI agar, which was then incubated at 37ºC for 24 hours to determine bacterial viability, expressed as CFUs/mL. Experiments were performed in triplicate following standard procedures, and the average of counts was considered **(231)**.

6.3.7. Transcriptomic analysis

6.3.7.1. RNA extraction

Exponential cultures of *E. faecium* strains (Efm5, *croS*-R1, *nusG*-R3 and *rpoB*-R5) were grown on Mueller-Hinton Broth (MHB) without antibiotics at 37ºC and 150 rpm. Prior to extraction, cells were treated with Killing Buffer at a 1:2 ratio relative to the culture column, followed by gentle inversion of the tube, as previously described **(98)**. Cell lysis was achieved by pre-incubating culture pellets in Phosphate-buffered saline (PBS) containing 15 mg/L lysozyme (Merck) at 37ºC for 20 minutes. Total RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. At the end, RNA was treated with TURBO DNase (Thermo Fisher) according to the manufacturer's recommended protocol. Concentration and purity of the extracted RNA were assessed using an RNA Nano Chip (Agilent Technologies).

6.3.7.2. Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR were employed to assess gene expression. cDNA synthesis was carried out using the iScriptTM cDNA Synthesis Kit (Bio-Rad) and random primers. qPCR was performed with iTaq polymerase (Bio-Rad) and SYBR green in a CFX96 PCR instrument (Bio-Rad). Relative gene expression was calculated using the Pfaffl method **(232)**. Differential gene expression was judged based on the common arbitrary 2-fold cutoff using *adk*, *gyrB* and *ddl* as housekeeping genes. Data presented in the manuscript correspond to the analysis of RNAs isolated from five independent biological repeats. Primers used for qPCR were designed using Primer3 software and they are listed in **[Table 9]**.

6.3.7.3. RNA sequencing and quality control

For RNA sequencing (RNAseq) of *E. faecium* strains Efm5 and CPH high-MIC variants, the RNA library was prepared using the Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10bp unique dual indices. The sequencing was carried out on a NovaSeq X Plus (SeqCenter, USA), generating paired-end 150bp reads. The quality of raw sequencing data was assessed using FastQC (https://github.com/s-andrews/FastQC, accessed in June 2024), followed by trimming using TrimGalore (https://github.com/FelixKrueger/TrimGalore, accessed in June 2024). Residual rRNA reads were then eliminated using the SortMeRNA software **(233)**. Five biological replicates were analysed per strain.

6.3.7.4. Differential expression analysis

Filtered reads were mapped using Bowtie2 **(234)**, and the alignments were quantified against the Efm5 genome using FADU **(235)**. Finally, differential gene expression analysis was conducted using DESeq2 **(236)**. Genes exhibiting $-1.5 \leq \log_2$ fold-change ≥ 1.5, with an adjusted p-value <0.05, were considered as differentially expressed.

Table 9. List of primers used in this study.

Locus_tag	Gene	Primer sequence (5' – 3')
efm5_01477	*adk*	F – GCTCTGGACGCAATGCTAAA
	adk	R – CGCAACGATCACATGTGTCT
efm5_01413	*gyrB*	F – CCGTACATTGCTGCTGACTC
	gyrB	R – GGTTTTGGTGAGGCAGGAAG
efm5_01757	*rutF*	F – ACCCATCTTATCCTTGGCGA
	rutF	R – TCAGTCAGGTACAGTTCGCT
efm5_01759	*ftsW*	F – GGCTAATCGGTGCATTGTGT
	ftsW	R – GCGATACTTCCCACGTTCAT
efm5_01760/61	*psr*	F – CAGCAGCAAGTGATCAAAGC
	psr	R – CCAACTGCCGTCTACTGGTA
efm5_01762	*pbp5*	F – ATCATTGATGGAGCAACGCC
	pbp5	R – GTCCCACGAAGATCCTTATCA
efm5_01392	*croR*	F – GGACGGGATGCAAGTAGTGA
	croR	R – GGCTGATCTGGGGCAATCT

6.3.8. Electron microscopy

6.3.8.1. Scanning Electron Microscopy (SEM)

For SEM examination, exponential-phase cultures of *E. faecium* Efm5 and of CPH high-MIC variants, grown in BHI broth without antibiotics for 2.5 hours at 37ºC and 150 rpm, were fixed in a solution containing 2.5% glutaraldehyde in 0.1M phosphate buffer (PB) for 2 hours at 4ºC. Then, they were post-fixed in a solution containing 1% osmium tetroxide with 0.8% potassium ferrocyanide for an additional 2 hours. Subsequently, samples underwent dehydration using a series of increasing ethanol concentrations (50%, 70%, 90%, 96%, and 100%). After dehydration, chemical drying was performed using hexamethyldisilazane (HMDS) (EMS, Hatfield, PA, USA), followed by sputter coating with a thin layer of PdAu for 4 minutes at 20 mA. Finally, the samples were visualized using a SEM Merlin (Zeiss MErlin, Germany) operated at 5 kV. At least 20 micrographs of each strain were taken and analysed.

6.3.8.2. Transmission Electron Microscopy (TEM)

For TEM examination, Efm5 and the CPH high-MIC variants were grown, fixed, post-fixed and dehydrated following the same procedure as described for SEM. Next, the cell pellets were embedded in EPON Epoxy Resin (EMS, Hatfield, PA, USA) and polymerized at 60ºC for 48 hours. Thin sections with a thickness of 70 nm were obtained using a Reichert-Jung Ultracut E ultramicrotome. These sections were then strained with 2% uranyl acetate and Reynold's solution (0.2% sodium citrate and 0.2% lead nitrate), followed by examination under a Hitachi H-7000 transmission electron microscope operating at a voltage of 75 kV. All chemicals and reagents were procured from Sigma Chemical Co. (St. Louis, Mo, USA), unless otherwise specified. At least 20 micrographs of each strain were taken and analysed. Given the particular features observed in the *rpoB*-R5 variant, independent preparations were analysed.

6.4. Results

6.4.1. Antibiotic susceptibility profile of Efm5

Antibiotic susceptibility testing revealed that E. faecium strain Efm5 exhibited low MICs to several β-lactam antibiotics, including ampicillin (0.25 mg/L), ceftaroline (0.19 mg/L), ceftriaxone (3 mg/L), and cefotaxime (1 mg/L). MICs to other cell wall inhibitors, such as vancomycin (3 mg/L; susceptible) were also low. Efm5 was resistant to rifampicin (8 mg/L) **[Table 10]**.

Table 10. Minimum Inhibitory Concentrations (MICs) (mg/L) to several antibiotics of strains Efm5, *croS*-R1, *nusG*-R3, and *rpoB*-R5. N/A; not applicable.

	β-lactam						
Strains	**Ampicillin**	**Ceftaroline**	**Ceftriaxone**	**Cefotaxime**	**Cefoxitin**	**Rifampicin**	**Vancomycin**
Efm5	0.25	0.19	3	1	48	8	3
EfmR1	1	0.5	>256	>32	128	N/A	4
Efm5R3	0.38	0.19	>256	>32	64	N/A	2
Efm5R5	1.5	0.5	>256	>32	64	>32	2

6.4.2. Genetic data of *Enterococcus faecium* Efm5

WGS allowed the classification of Efm5 as ST29 according to PubMLST for *E. faecium*. Abricate predicted genes conferring resistance to aminoglycosides (*aac(6')-Ii*), chloramphenicol (*catA8*), macrolides (*msrC*), and tetracyclines (*tetL, tetM*). Similarly, VirulenceFinder software identified several virulence factor-encoding genes, including *acm, bepA, ccpA, empABC, fms13, fms14,*

fms15, fms17, fnm, sagA, and *scm*. Of note, genes involved in cephalosporin resistance (*pbp5*, *pbpA*, *croRS*, *stpA-stk*, *murAA*) were predicted without any transposon insertions or nonsense mutations that would cause the premature protein termination. However, despite antibiotic susceptibility testing results, known PBP5 mutations predicted to be related to ampicillin resistance were detected in Efm5 genome (V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I and E525D) according to ResFinder.

6.4.3. Isolation of ceftriaxone high-MIC variants

In assays designed to isolate CPH high-MIC variants, five colonies were observed, giving a frequency of CPH high-MIC variants isolation of $5x10^{-7}$. In all cases, Etests confirmed the increased MICs for ceftriaxone (>256 mg/L), cefotaxime (MIC >32 mg/L), and cefoxitin (>64 mg/L) as compared to the parental Efm5 strain **[Table 10]**. However, all variants remained susceptible to ampicillin and vancomycin and with low MICs observed for ceftaroline. In addition, one of the CPH high-MIC variants (*rpoB*-R5) increased by at least 4-fold its MIC to rifampicin **[Table 10]**.

6.4.4. Identification of single nucleotide polymorphisms in CPH high-MIC variants

WGS followed by comparative genomics revealed that each CPH high-MIC variant harboured a unique single nucleotide polymorphism (SNP) with respect to the parental Efm5 strain **[Figure 31]**. Specifically, the R1 and R2 variants harboured single point mutations in the *croS* gene, resulting in V171A (*croS*-R1 strain) and R343H (*croS*-R2 strain) CroS substitutions. Structural analysis using AlphaFold predictions indicated that residues V171 and R343 are located near the phosphoryl-accepting H173 of CroS **[Figure 32] (102)**.

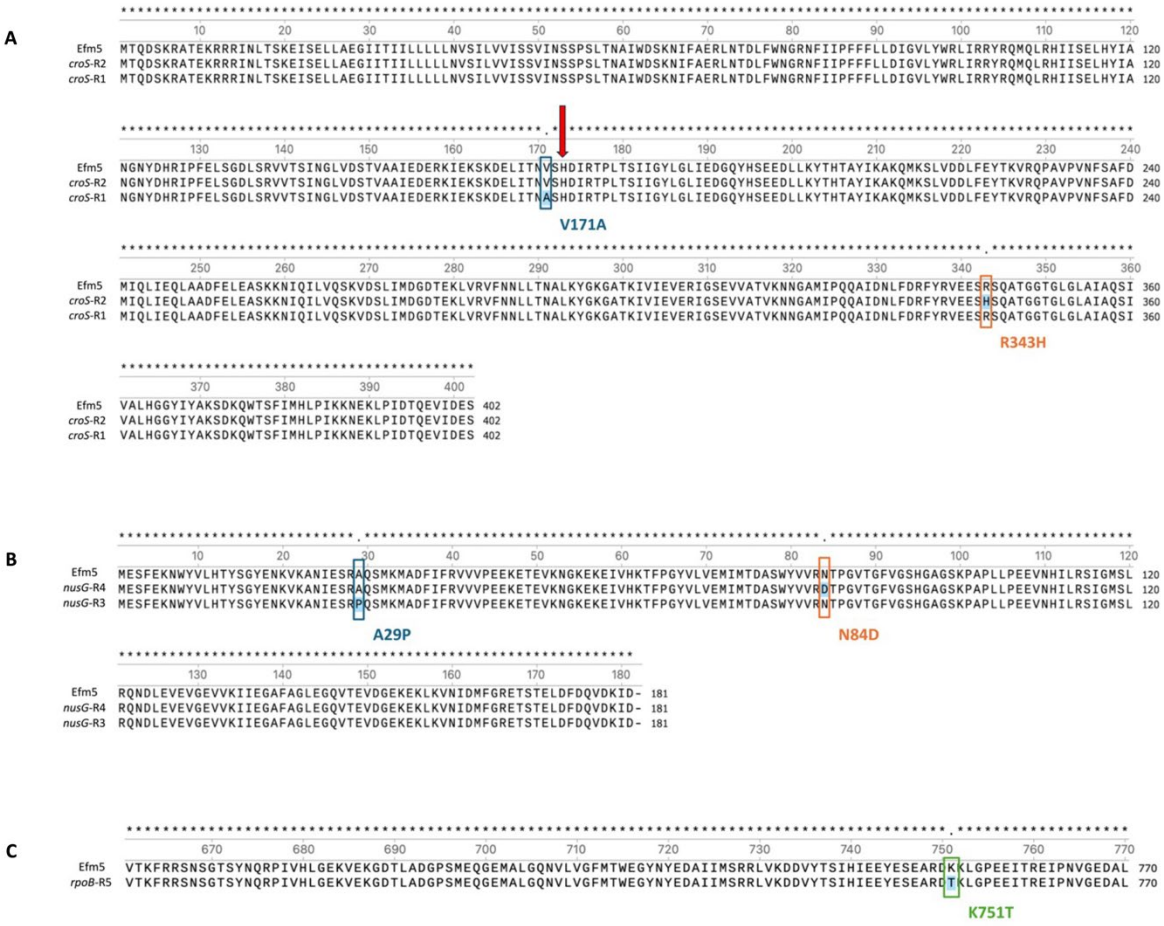


Figure 31. (A) Sequence alignment depicting two segments of the CroS protein with the single point mutations found in *croS*-R1 (V171A) and *croS*-R2 (R343H). **(B)** Sequence alignment of the NusG protein with the single point mutations found in *nusG*-R3 (A29P) and *nusG*-R4 (N84D). **(C)** Sequence alignment of the RpoB protein with the single point mutation found in *rpoB*-R5 (K751T).

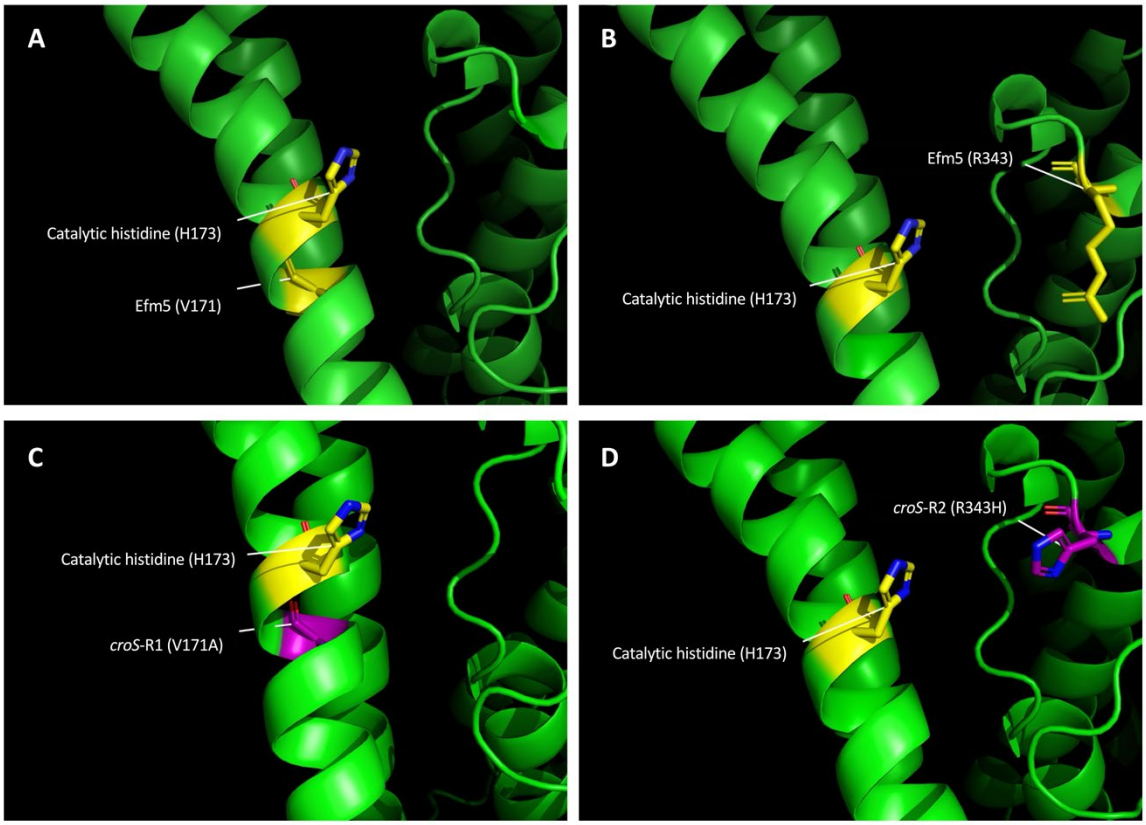


Figure 32. Representation of the CroS protein 3D-structure generated by AlphaFold (Q3XYJ6) and visualized with PyMOL. Important residues are labelled as follows: **(A)** phosphoryl-accepting H173 plus V171 (in yellow), **(B)** phosphoryl-accepting H173 plus R343 (in yellow), **(C)** V171A substitution found in the *croS*-R1 variant (in purple), and **(D)** R343H substitution found in the *croS*-R2 variant (in purple).

The R3 and R4 variants presented mutations in the *nusG* gene, leading to A29P (*nusG*-R3 variant strain) and N84D (*nusG*-R4 variant strain) amino acid substitutions in NusG **[Figure 31; Figure 33]**. NusG is a universally conserved intrinsic transcription termination factor critical for transcriptional regulation in bacteria. It functions as an anti-pausing factor in *Escherichia coli* and as a sequence-specific pausing factor in *Bacillus subtilis* **(237)**. To our knowledge, this protein has not yet been linked to cephalosporin resistance in enterococci.

Lastly, the *rpoB*-R5 variant exhibited the substitution T798K in the RpoB protein **[Figure 31**, **Figure 34]**, which encodes the β-subunit of RNA polymerase. Single point mutations in the *rpoB* gene have been previously shown to alter intrinsic cephalosporin resistance in *Enterococcus* and *Neisseria gonorrhoeae* **[Figure 35] (238,239).**

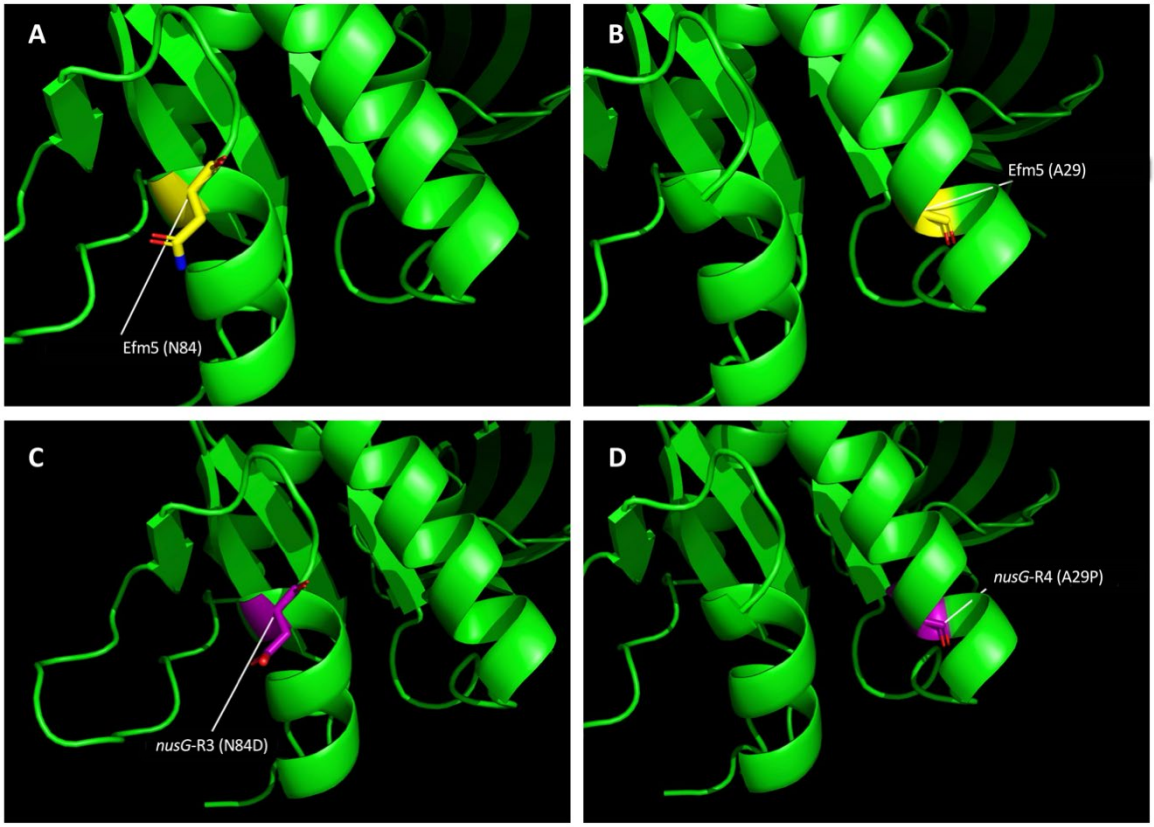


Figure 33. Representation of the NusG protein 3D-structure generated by AlphaFold (A0A133CS68) and visualized with PyMOL. Important residues are labelled as follows: **(A)** N84 (in yellow), **(B)** A29 (in yellow), **(C)** N84D substitution found in the *nusG*-R3 variant (in purple), and **(D)** A29P substitution found in the *nusG*-R4 variant (in purple).

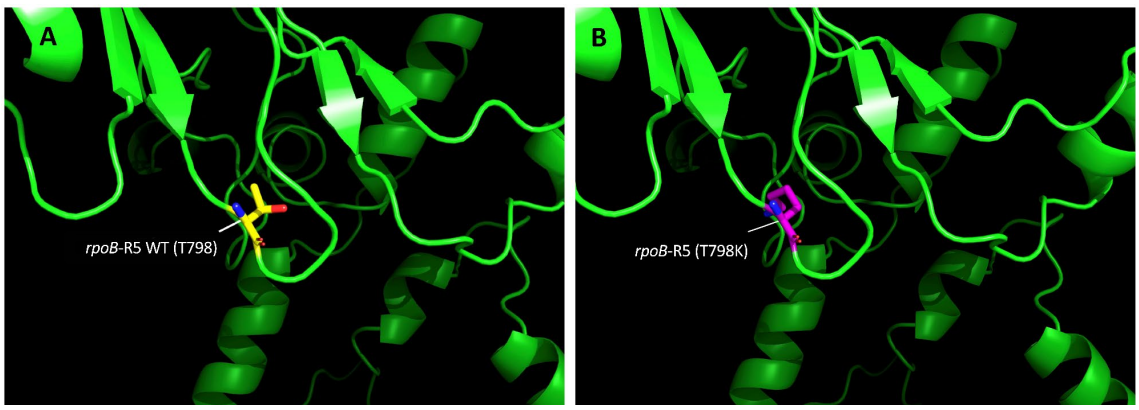


Figure 34. Representation of the RpoB protein 3D-structure generated by AlphaFold (A0A133CS68) and visualized with PyMOL. Important residues are labelled as follows: **(A)** T798 (in yellow) and **(B)** T798K substitution found in *rpoB*-R5 variant (in purple).

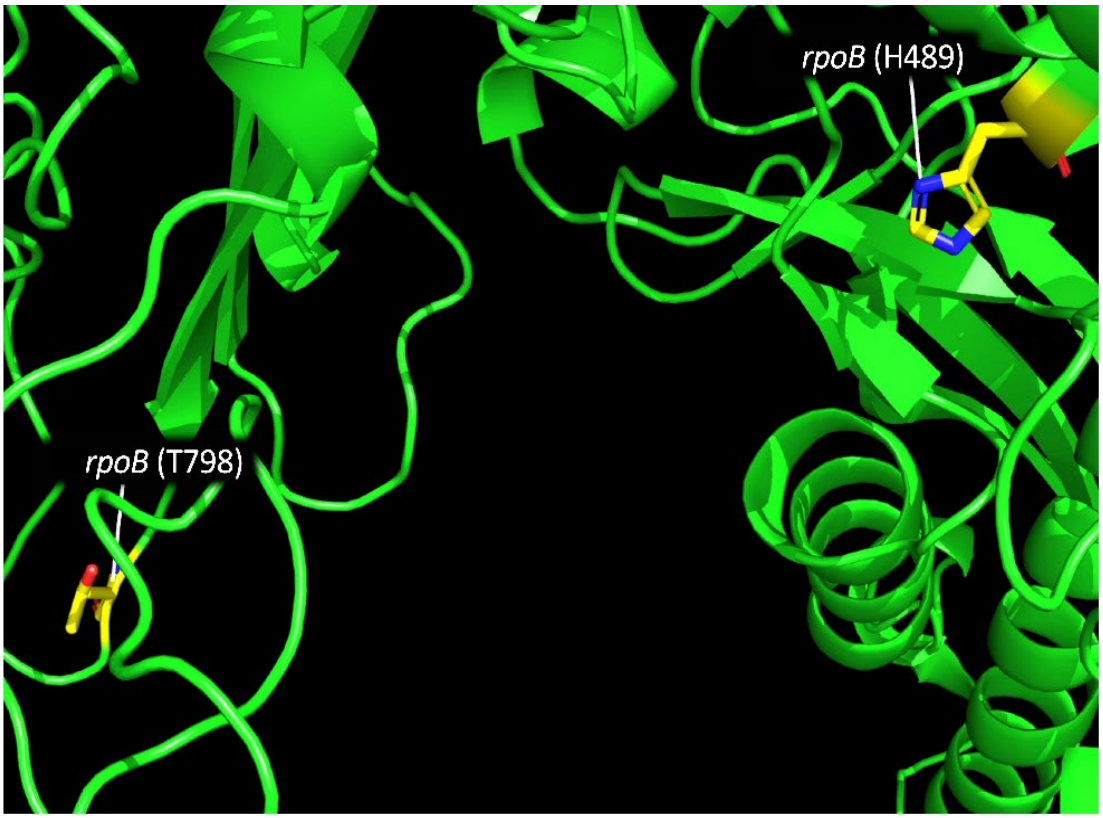


Figure 35. Representation of the RpoB protein 3D-structure generated by AlphaFold (A0A133CS68) and visualized with PyMOL. The picture depicts the location of residues T798 and H489 (in yellow). The latter was identified by Kristich and Little, 2012 **(238)** as important for cephalosporin resistance.

To explore the distribution of these variants across the *Enterococcus* genus, we searched for CroS, NusG and RpoB homologs in all complete *Enterococcus* genome assemblies available at the NCBI database (n=767). Our analysis revealed that over 99.0% of genomes encoded *croS*, *nusG* and *rpoB* genes. Identical NusG, RpoB, and CroS protein sequences from Efm5 were identified in 48.2%, 36.1%, and 40.4% of the genomes analysed in the NCBI database, respectively. However, gene variants predicted to confer high-MIC values to CPHs in our isolates were not detected in any of the analysed NCBI genomes.

6.4.5. Time-kill curves

Time-kill curves in the presence of 10 mg/L of ceftriaxone confirmed the predicted susceptibility of Efm5 to this antibiotic **[Figure 36]**. In contrast, the *croS*-R1, *nusG*-R3, and *rpoB*-R5 variants were all able to grow in the presence of ceftriaxone.

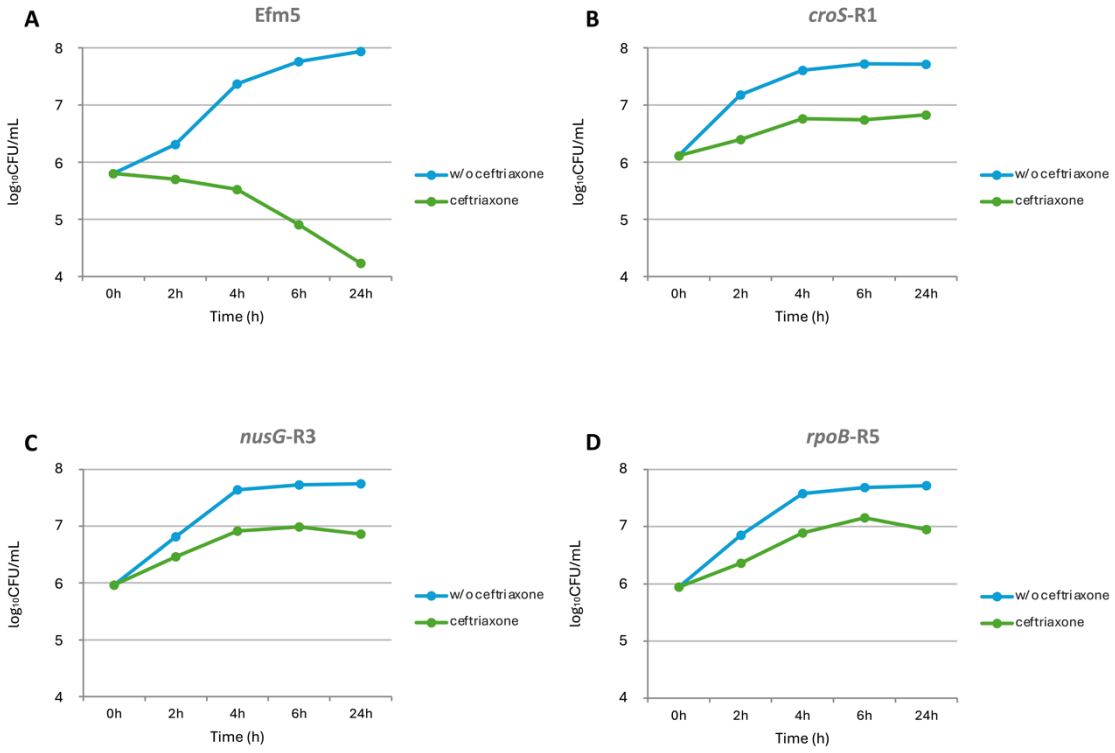
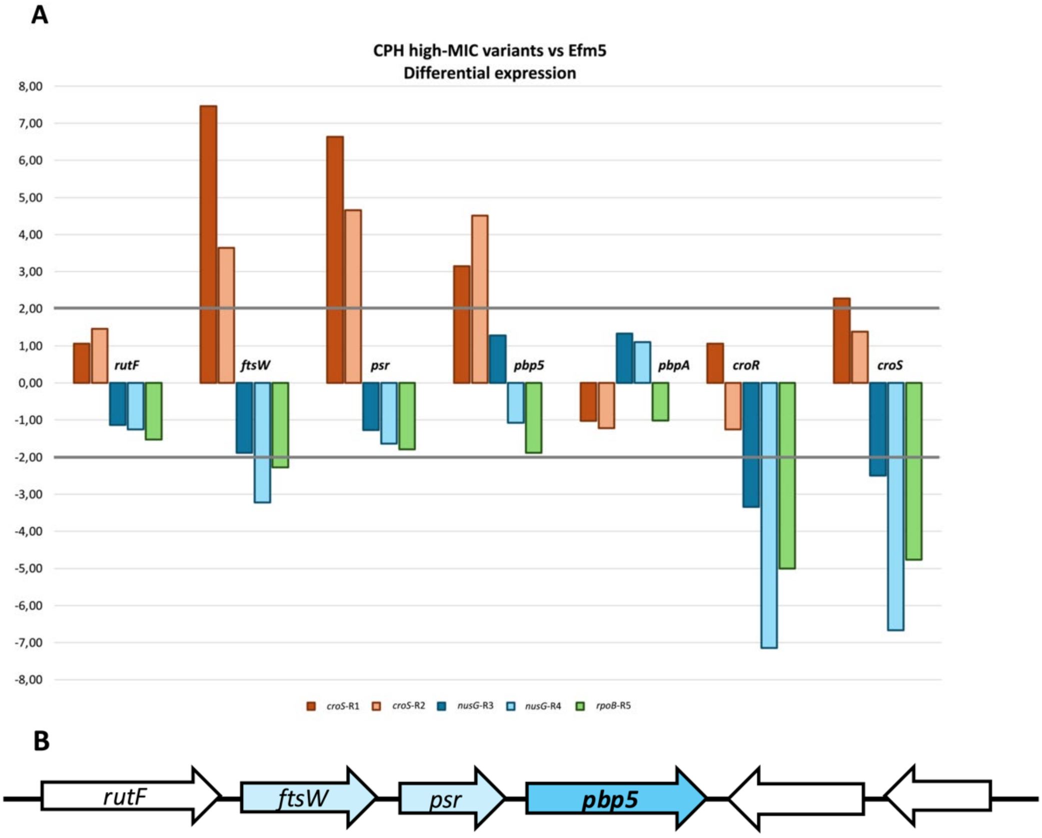


Figure 36. Growth curves of the **(A)** CPH low-MIC Efm5 and the CPH high-MIC variants **(B)** *croS*-R1, **(C)** *nusG*-R3, and **(D)** *rpoB*-R5, both in absence and presence of ceftriaxone (10 mg/L).

6.4.6. Transcriptional analysis

As CroS, NusG, and RpoB function has an impact on gene transcription, we assessed the existence of transcriptional changes in CPH high-MIC variants. Recent reports have identified the whole regulon of the CroRS system in *E. faecalis* **(98,101,226)**. As expression of *pbp5*, a main player in cephalosporin resistance in enterococci, was found to be under the control of CroRS system **(98)**, we evaluated the transcriptional status of the *pbp5* operon in Efm5 and *croS*-R1 and R2 variants by qRT-PCR. Our results indicated that the entire *pbp5* operon, comprising *ftsW*, *psr,* and *pbp5* genes, was markedly activated in the *croS* variants **[Figure 37, panel A and B]**. In contrast, the expression of the *rutF* gene, located immediately upstream of the operon, remained unchanged. Similarly, no changes were observed in the mRNA levels of the *pbpA* or

croR genes. Regarding *croS*, a slight increase was observed in the R1 variant. Activation of the *pbp5* operon in the *croS* variants likely justifies the increased MIC to ceftriaxone.



C

```
>UPR-ftsW_Efm5

TGATGCTAGGTCAGAATTCTTGTTCGAAAAGGTTTCTGACCTTTTCTTTATATTTTTGTGACTGACTTATTT
GATGAGACAAACTCGCTCCTCACCTGACAAGTGGATTGCTGTGTGAGGAGGAGATGCGGTG

>UPR-ftsW_Efm5

TGATGCTAGGTCAGAATTCTTGTTCGAAAAGGTTTCTGACCTTTTCTTTATATTTTTGTGACTGACTTATTT
GATGAGACAAACTCGCTCCTCACCTGACAAGTGGATTGCTGTGTGAGGAGGAGATGCGGTG

>UPR-ftsW_Efm5

TGATGCTAGGTCAGAATTCTTGTTCGAAAAGGTTTCTGACCTTTTCTTTATATTTTTGTGACTGACTTATTT
GATGAGACAAACTCGCTCCTCACCTGACAAGTGGATTGCTGTGTGAGGAGGAGATGCGGTG
```

Figure 37. Transcriptional analysis of the *pbp5* operon in the CPH high-MIC variants with *croS* mutation by RT-qPCR. In panel **(A)**, fold change values (log_{10}) for the three genes comprising the *pbp5* operon (*ftsW*, *psr*, and *pbp5*), *rutF*, located immediately upstream of the operon, *pbpA*, *croR,* and *croS* are shown. The grey line indicates the standard 2-fold cutoff for biological relevance. The results shown represent the average of 5 biological replicates. In panel **(B)**, the *pbp5* operon organization is represented, indicating genes within the operon (marked in blue), and *rutF*, located immediately upstream. Panel **(C)** illustrates the inverted repeats (red boxes) found in the upstream region of the *ftsW* gene. The -35 and -10 promoter elements are highlighted in light blue, the ribosome binding site (RBS) in yellow, and the translational start codon in light green. UPR; upstream region.

The *nusG*-R3 and R4 mutants, together with *rpoB*-R5, exhibit repressed *croS* and *croR* expression, whereas *nusG*-R4 and *rpoB*-R5 exhibit slightly repressed expression of *ftsW.*

This finding prompted us to inspect the upstream region of *ftsW*, the first gene of the *pbp5* operon, to identify candidate regulatory sequences. The predicted -35 (TCGAAA) and -10 (TATATT) regions were pinpointed at 101 and 76 nucleotides upstream from the *ftsW* translational start codon **[Figure 37, panel C]**. As in canonical promoters under the control of the vegetative sigma factor, the -35 and -10 regions were located 19 bp apart. Additionally, several putative regulatory sequences, including direct and inverted repeats, were recognized within the *ftsW* upstream region. Some of these putative regulatory sequences were found in the vicinity of the -35 and -10 promoter elements, reinforcing its possible role in modulating RNA polymerase binding and in turn, transcription of the *pbp5* operon. Also, some putative regulatory sequences were found close to or even overlapping the ribosome binding site (RBS), suggesting a potential role in post-transcriptional regulation.

As transcriptome analyses of *nusG* and *rpoB* mutants are not available in enterococci, we conducted a genome-wide transcriptional analysis of the *nusG*-R3 and *rpoB*-R5 variants by RNAseq. In both cases, we found extensive transcriptional changes consistent with a profound functional and metabolic reprogramming **[Figure 38]**. Specifically, transcriptional analysis of the *nusG* variant identified 98 differentially transcribed genes, with 52 genes upregulated and 46 downregulated **[Figure 39; Table S2; Table S3]**. Notably, regulated genes felt primarily into functional categories associated with carbohydrate transport and metabolism (21%) as well as transcriptional processes (13%).

On the other hand, analysis of the *rpoB* variant revealed a global impact on gene expression. Specifically, among the 180 genes differentially expressed with respect to strain Efm5, 96 were upregulated and 84 downregulated **[Figure 39; Table S4; Table S5]**. In this case, the predominant categories of regulated genes were also dominated by carbohydrate transport and metabolism (18%) and transcription (14%), along with translation, ribosomal structure, and biogenesis (13%).

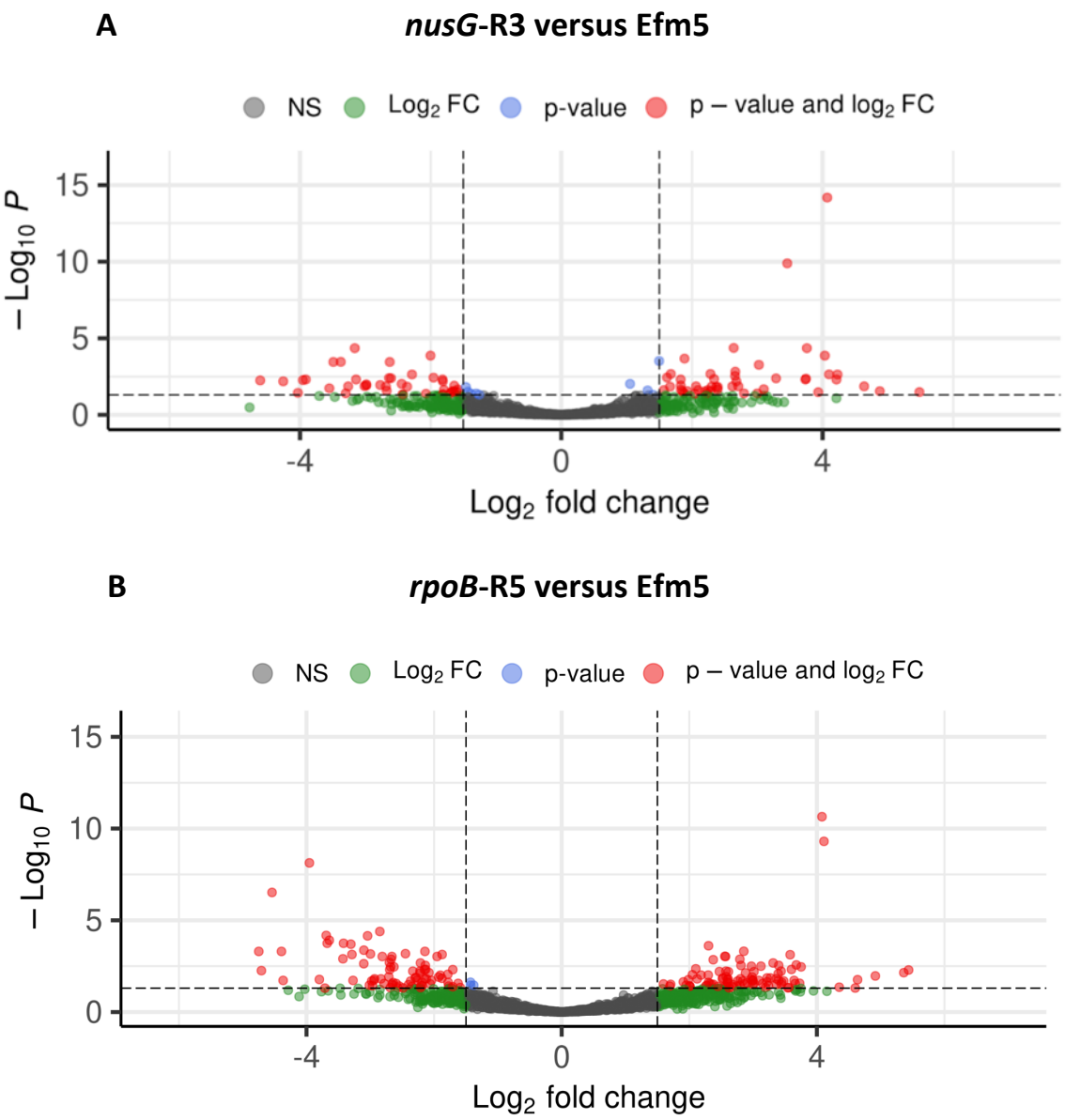


Figure 38. Differentially expressed genes in the **(A)** *nusG*-R3 and **(B)** *rpoB*-R5 variants with respect to Efm5. The x-axis represents the Fold Change (log_2) in gene expression between the two strains, with genes exhibiting increased expression positioned to the right and those with decreased expression to the left. The y-axis denotes the statistical significance of these expression changes, with genes showing a higher significance located towards the top. Each point in the plot represents a single gene, where genes with substantial expression changes and high statistical significance are positioned in the upper right or upper left quadrants.

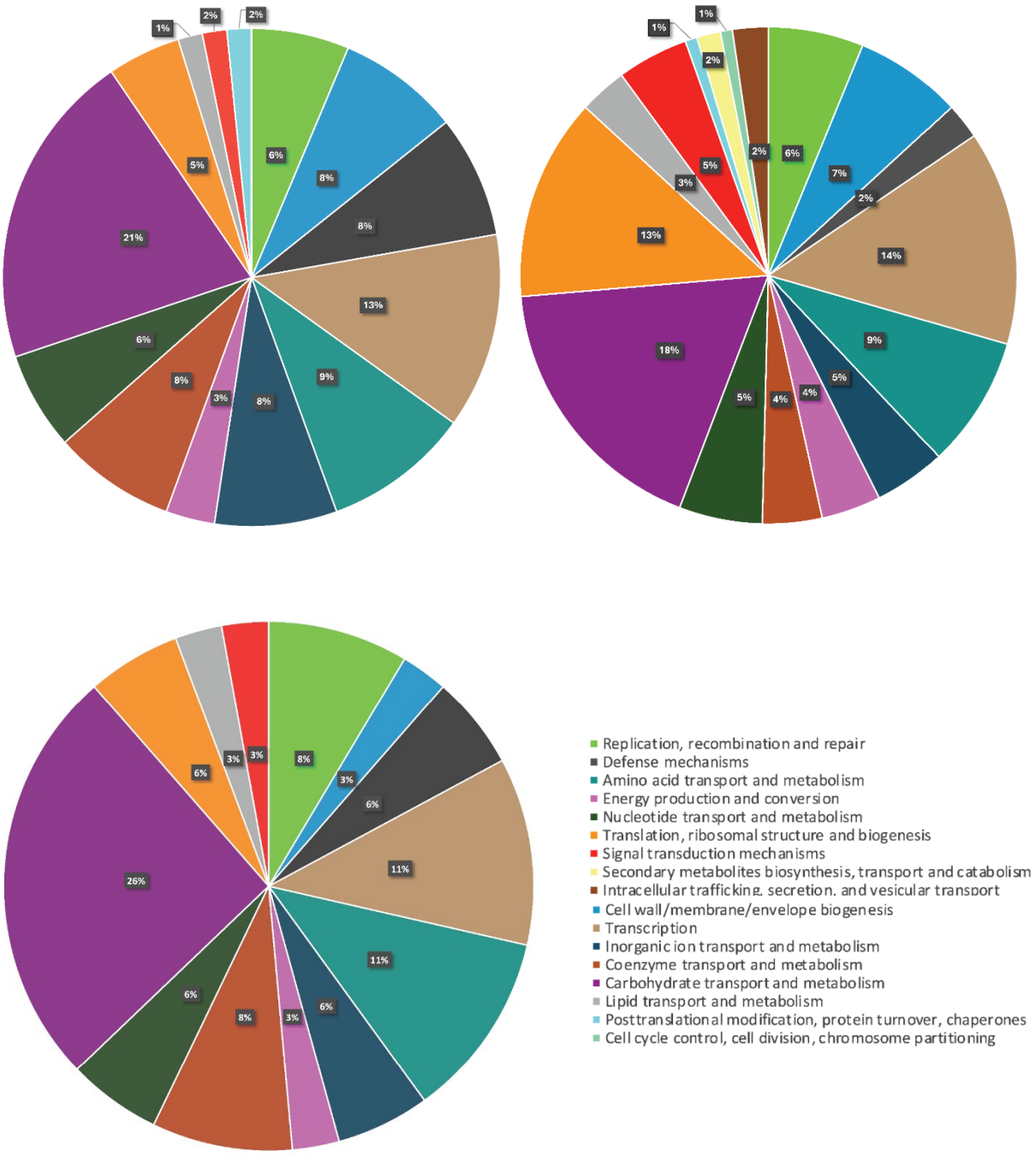


Figure 39. Functional classification and gene abundance within each category among the regulated genes identified in the **(A)** *nusG*, **(B)** *rpoB*, and **(C)** the overlap between *nusG* and *rpoB* variants, with "unknown" and "other" categories omitted.

Of note, comparison of the genes differentially transcribed in the *nusG* and *rpoB* variants revealed an overlap of 58 genes, with 31 genes being upregulated and 27 downregulated **[Figure 40]**.

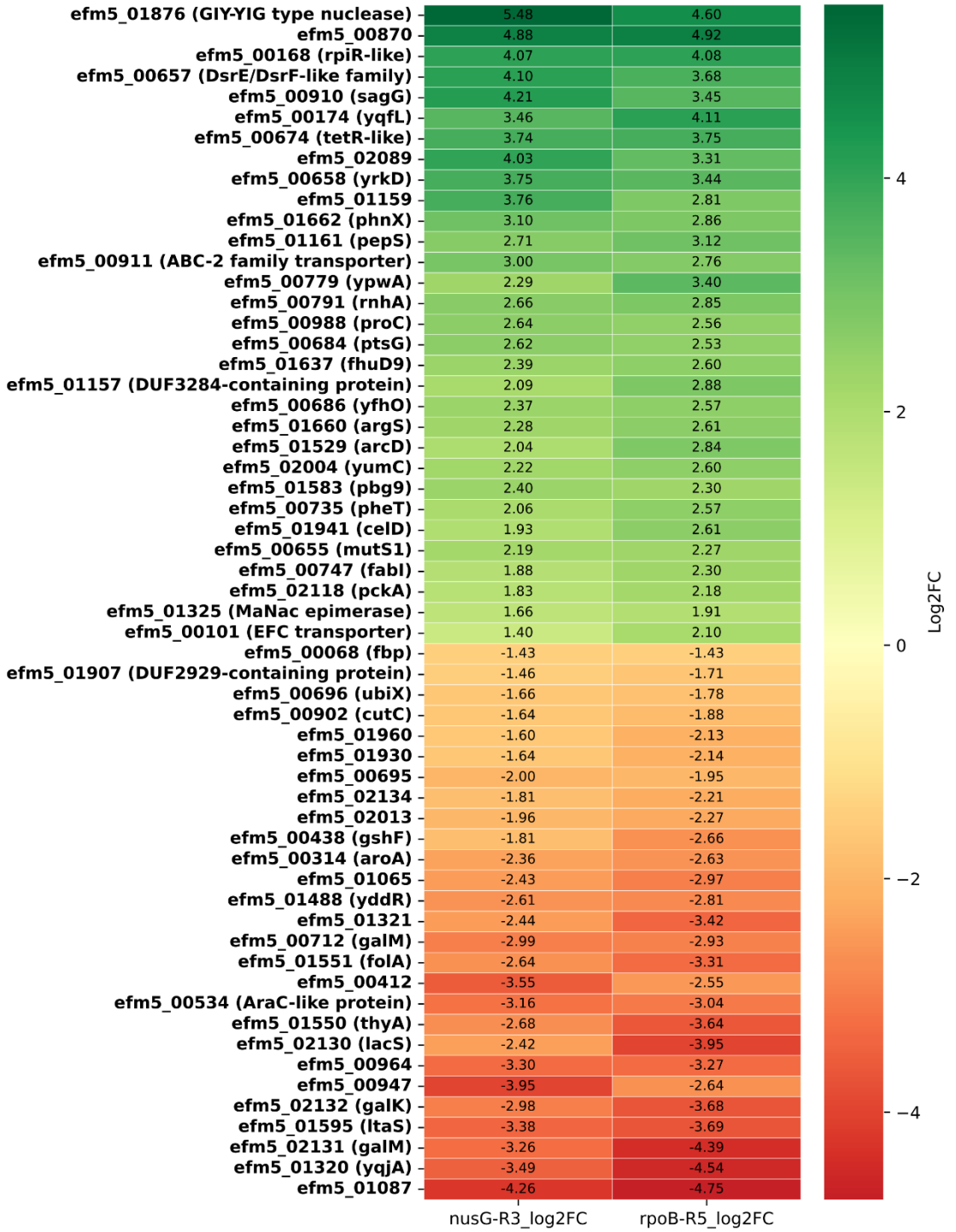


Figure 40. Heatmap of gene expression levels in *nusG*-R3 and *rpoB*-R5 variants. Each row represents a gene, and columns show expression levels in each strain, with values ranging from red (low expression) to green (high expression). Fold-change values are also included.

Genes that were differentially expressed in both variants are more likely to be responsible for the phenotypic switch to ceftriaxone CPH high-MIC. Among the upregulated genes in both strains, they included several involved in carbohydrate transport and metabolism, including *ptsG*, *pbg9*, *celD* and a ManNAc epimerase **[Figure 39]**. We also identified genes related to bacterial gene transcription, such as *rpiR* and *tetR* regulators. Furthermore, we found genes involved in virulence (*sagG* and an ABC-2 family transporter), amino acid metabolism (*pepS, ypwA, proC*), DNA replication and repair (*rnhA*, *mutS1*, a GIY-YIG type nuclease encoding gene), and translation (*argS, pheT*) as overexpressed in *nusG* and *rpoB* variants. Lastly, several genes with unknown functions (DsrE/DsrF-like encoding gene*, yqfL, yrkD, phnX, arcD, fhuD9*) were also predicted. In contrast, among the downregulated genes, we found genes related to carbohydrate metabolism, such as *galM*, *galK*, *lacS*, and *fbp* genes. We also identified genes related to lipoteichoic acid synthesis (*ltaS*), folate metabolism (*folA* and *thyA*), metal homeostasis (*cutC*), and unknown functions (*yqjA, yddR*).

6.4.7. Analysis of cell morphology and ultrastructure

6.4.7.1. Scanning Electron Microscopy (SEM)

SEM analysis was conducted to examine the morphology of the CPH low-MIC Efm5 strain and three representative CPH high-MIC variants: *croS*-R1, *nusG*-R3, and *rpoB*-R5 **[Figure 41]**.

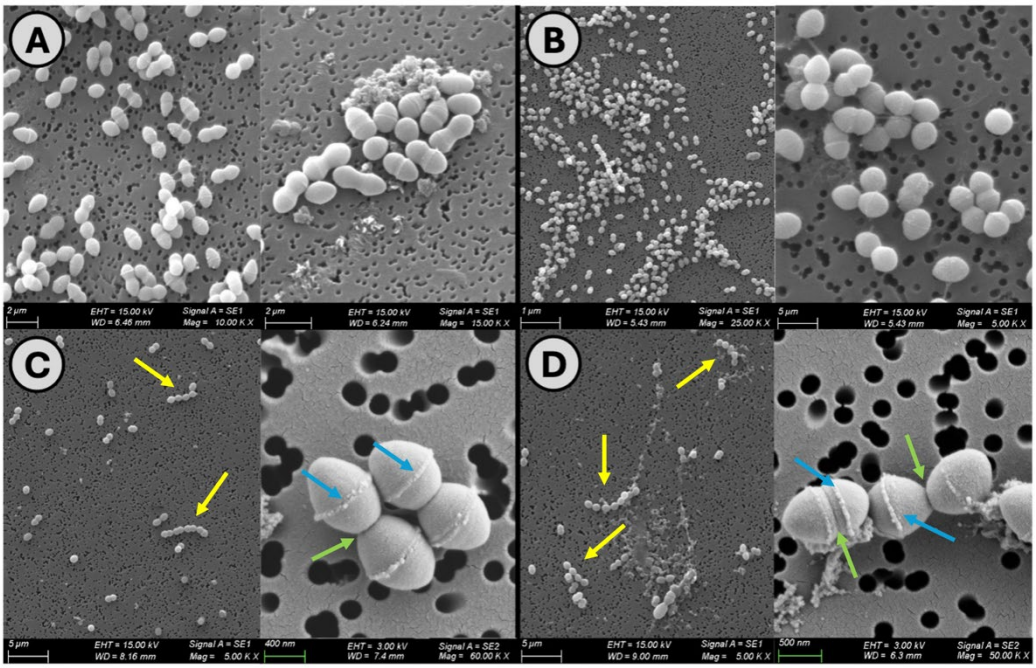


Figure 41. Scanning Electron Microscopy (SEM) analysis of **(A)** Efm5, **(B)** *croS*-R1, **(C)** *nusG*-R3, and **(D)** *rpoB*-R5. Each panel illustrates the distinct morphological characteristics observed in these isolates, highlighting differences in surface structures and cell morphology that may provide insights into their phenotypic variations. Yellow arrows indicate cocci chains, green arrows point to the cell division ring, and blue arrows highlight the rough crest.

As expected, the four strains were characterized by the presence of cell pairs (diplococci). Cocci chains were especially apparent in the *nusG*-R3 and *rpoB*-R5 variants (yellow arrows). The cell division ring (green arrows) and a rough crest (blue arrows) were observed in all strains, but the crests were more prominent in the *nusG*-R3 and *rpoB*-R5 variants. Of note, we did not appreciate the cell elongation previously associated with shortage of peptidoglycan precursors **(240)**.

6.4.7.2. Transmission Electron Microscopy (TEM)

TEM analysis was conducted to evaluate the ultrastructural features of the different strains under study. In contrast to the parental Efm5 CPH low-MIC strain, cells from the *croS*-R1 and *nusG*-R3 variants exhibited cytokinesis defects **[Figure 42, panels A and B]**.

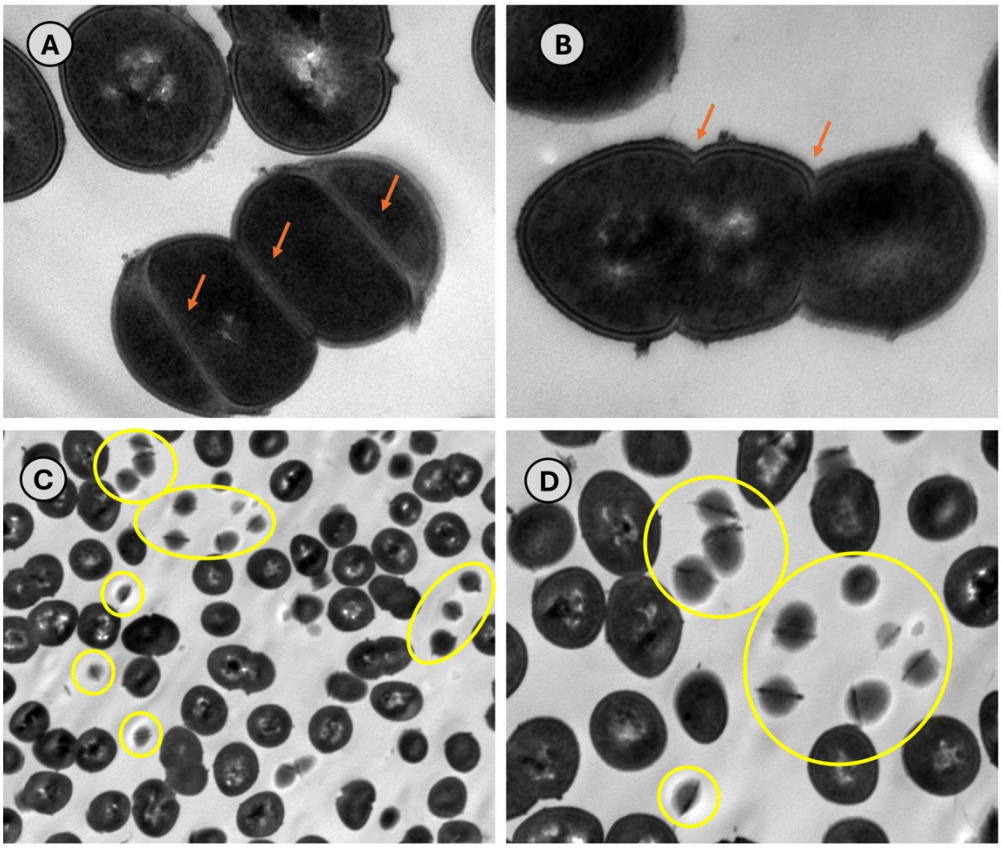


Figure 42. Transmission Electron Microscopy (TEM) analysis illustrates two distinct characteristics exclusive of the CPH high-MIC variants. Panels **(A and B)** illustrates cells with impaired separation following division, with the orange arrows highlighting the regions where cell chaining occurs. Panels **(C and D)** display small, round bodies with an electron-dense band, present in the three mutants, but most notably in *nusG*-R3.

In panel A, what appears as multiple septa likely reflects a situation where cells have failed to separate after division, resulting in cell chains. This phenotype aligns with the chaining pattern observed in the SEM images. Additionally, all three variants showed the presence of small, round bodies with a crossing electron-dense structure, particularly evident in the *nusG*-R3 variant **[Figure 42, panels C and D]**. The misalignment of septa during the second division cycle, where they are not centrally positioned within the cell as shown in panels A and B, may contribute to the formation of these unusual small structures observed in panels C and D. No differences in cell wall thickness were detected among the three variants.

In addition, the *rpoB*-R5 variant exhibited electrolucent cytoplasmic regions, which appear as clear zones **[Figure 43]**. These regions were consistently observed in independent preparations of the *rpoB*-R5 strain, further distinguishing it from the other CPH high-MIC variants.

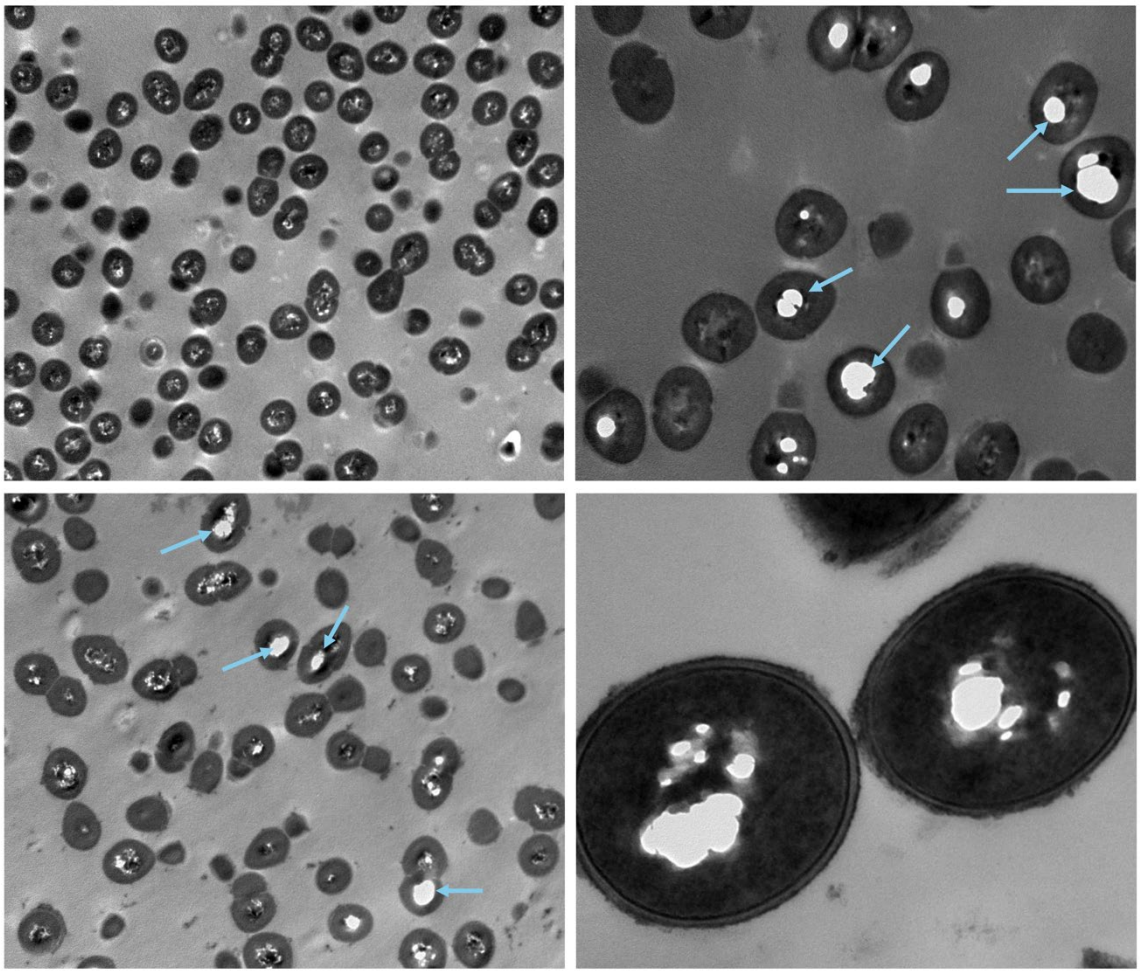


Figure 43. Transmission Electron Microscopy (TEM) analysis illustrates the presence of electrolucent cytoplasmic regions in the *rpoB*-R5 variant (blue arrows).

6.5. Discussion

In this study, we describe the isolation of an *E. faecium* clinical strain showing low MICs to several cephalosporins. To our knowledge, this represents the first documented instance of a clinical *E. faecium* natural isolate lacking inherent resistance to cephalosporins, challenging the current paradigm of intrinsic resistance **(19,136)**. This unique isolate represents an ideal platform to investigate the mechanisms underlying cephalosporin resistance.

Genomic analysis of the CPH high-MIC variants revealed unique SNPs in the *croS*, *nusG*, and *rpoB* genes as the sole genetic differences between the parental Efm5 strain and the CPH high-MIC variants **[Figure 31]**.

The mutations in the *croS* gene identified in our study were to significantly upregulate of the *pbp5* operon, including the *ftsW*, *psr*, and *pbp5* genes **[Figure 31A]**. This result aligns with previous studies that have established the CroRS system as key regulator of cephalosporin resistance in enterococci **(98,100,102,154)**. This upregulation corresponds to the observed increase in MIC values to cephalosporins in the *croS* CPH high-MIC variants, as previously described **(100)**. Notably, the *rutF* gene, located upstream and oriented in the same direction as the operon, showed no transcriptional changes, indicating that the regulatory effect of the mutations is specifically targeted within the *pbp5* operon itself. Our data reveal activation of the CroRS system, as evidenced by the upregulation of the *pbp5* operon. This statement is based on the previous observation that the inactivation of the CroRS system leads to the *pbp5* downregulation **(98,101)**. Structurally, the mutations identified in this study are located adjacent to (V171A) and just in front of (R343H) the catalytic histidine (H172), a residue essential for CroS autophosphorylation **(102) [Figure 32]**. The proximity of these mutations to the active site, combined with the fact that one of the mutations results in a substitution for an histidine, suggests that these changes may interfere with the normal function of the CroRS system.

RNA sequencing of the *nusG*-R3 and *rpoB*-R5 CPH high-MIC variants revealed significant alterations in gene expression **[Figure 38]**, particularly in categories related to carbohydrate transport and metabolism, as well as transcriptional processes **[Figure 39AB]**. Products encoded by *nusG* and *rpoB* genes are critical in bacterial transcription dynamics. While the *rpoB* gene encodes the RNA polymerase (RNAP) β subunit, NusG is a transcriptional regulator essential for coupling transcription and translation in prokaryotes **(241,242)**. By tethering RNAP to the lead ribosome during transcription, NusG supports RNAP's forward progression along the DNA template through interactions with the RNAP β and β' subunits and the small ribosomal subunit.

Mutations in both *nusG* and *rpoB* genes alter the activity of RNAP, leading to changes in the expression of multiple genes **(241,243)**. This shared regulation of RNAP activity by *nusG* and *rpoB*, likely explains the remarkable overlap observed in the differentially expressed genes between the *nusG*-R3 and *rpoB*-R5 **[Figure 39C, Figure 40]**.

Transcriptional changes in genes involved in carbohydrate transport are particularly important because these molecules are critical for cell wall biosynthesis. In this sense, a gene encoding a RpiR-like regulator was overexpressed in both transcriptomes. RpiR/MurR regulators are a family of transcriptional regulators found in various bacteria, where they primarily control genes involved in carbohydrate metabolism **(244)**. While RpiR proteins are primarily associated with sugar metabolism, recent studies suggest that they might also play an indirect role in regulating bacterial cell wall components. Changes in carbohydrate metabolism can influence the synthesis of cell wall precursors **(245)**. In this way, a ManNAc epimerase-encoding gene was upregulated in *nusG*-R3 and *rpoB*-R5 variants. This gene is predicted to be involved in the conversion of ManNAc to N-acetylglucosamine (GlcNAc), a glucose-derived monosaccharide, which is part of a peptidoglycan **(246)**. On the other hand, the *ltaS* gene, responsible for synthesizing lipoteichoic acid, an essential cell wall polymer in Gram-positive bacteria **(247)**, was found to be downregulated in both *nusG*-R3 and *rpoB*-R5 variants. This gene is also known to be regulated by the CroRS system in *E. faecalis* **(98)**.

To our knowledge, no association between NusG and cephalosporin resistance has been previously documented. However, such transcriptional changes observed in the *nusG*-R3 variant, including the regulation of genes related to carbohydrate metabolism and cell wall synthesis, suggest that *nusG* mutations may play an indirect role in remodelling the bacterial cell wall, potentially contributing to cephalosporin resistance.

Conversely, mutations in the *rpoB* gene have been extensively linked to antibiotic resistance in *Enterococcus* spp., primarily with resistance to rifampicin, but also with daptomycin and cephalosporins **(238,248)**. In this line, the *rpoB* variant exhibited a marked increase in rifampicin resistance, with MIC values rising from 8 mg/L in the parental strain Efm5 to >32 mg/L in the *rpoB*-R5 variant. The downregulation of the *ltaS* gene in this variant further supports a role for *rpoB* mutations in influencing cell wall synthesis and antibiotic susceptibility. Such changes may impact the integrity and structure of the cell, making it less susceptible to cephalosporins and thereby protecting the bacteria from the effects of these antibiotics, which target cell wall synthesis **(249)**.

Regarding the ultrastructural features, the *rpoB*-R5 variant displayed electrolucent regions in the cytoplasm, which is an indication of cell damage and stress **[Figure 43] (250)**. Such structures have been observed in other studies, where they were noted in daptomycin-resistant VREfm strains, contrasting with daptomycin-sensitive strains **(251)**. These structures were also identified in cells treated with fluoride, an antimicrobial agent that inhibits bacterial growth and metabolic activity **(250)**. TEM analysis further provided valuable insights into the cellular division and ultrastructure of the Efm5 strain and the CPH high-MIC variants. Notably, Efm5 exhibited a single, symmetrical septum, indicative of typical binary fission. In contrast, the CPH high-MIC variants, mainly *croS* and *nusG* variants, presented irregular division processes **[Figure 42AB]**. This observation correlated with findings by Arias and colleagues, who noted similar septal irregularities among its daptomycin-resistant and -sensitive vancomycin-resistant *E. faecium* (VREfm) strains **(252)**. The potential disruption of the transcription machinery may lead to replication fork stalling. This can occur when replication encounters transcription complexes, triggering a DNA damage response. Additionally, the *folA* and *thyA* genes, which are involved in folate and thus thymidine synthesis **(253)**, were also downregulated in both the *nusG*-R3 and *rpoB*-R5 variants. A reduction in thymidine synthesis is also reported to induce DNA replication stress due to thymine depletion **(254)**. This potential cellular stress could explain *lexA* induction in *rpoB*-R5 variant, which encodes the key regulator of bacterial SOS DNA damage response **(255)**. Through this pathway, the SOS response can arrest cell division to help prevent the propagation of damaged DNA **(256)**.

A possible contributing factor to the observed differences in cephalosporin resistance among Efm5 strain may be the “seesaw effect”, a phenomenon in which heightened resistance to one type of stressor can result in diminished resistance to another. This phenomenon has been observed in vancomycin-intermediate *S. aureus* (VISA) strains, where resistance to vancomycin correlates with heightened susceptibility to certain β-lactam antibiotics, such as oxacillin and ceftaroline **(257**,**258,259,260)**. Additionally, it has been noted that strains resistant to daptomycin may paradoxically exhibit greater susceptibility to β-lactam antibiotics. This susceptibility is linked to the deletion of the LiaX protein, which is implicated in daptomycin resistance. The loss of LiaX enhances sensitivity to β-lactams like ceftriaxone **(261)**.

To conclude, the isolation of *E. faecium* strains exhibiting susceptibility to cephalosporins, despite their known intrinsic resistance, highlights a need to explore the mechanisms behind this phenotype further. Notably, the mutations in *croS*, *nusG*, and *rpoB* genes in our CPH high-MIC variants represent unique sequences absent in existing *Enterococcus* entries in the NCBI database. This finding suggests that these mutations do not align with standard resistance

mechanisms in *Enterococcus*. Rather, they may represent compensatory mutations—selected under selective pressures—that enhance resistance in strains originally exhibiting low MICs to cephalosporins. These compensatory mutations may provide a survival advantage, balancing out other genetic or metabolic limitations by restoring and potentially enhancing resistance. Understanding these novel mutations and their roles in resistance profiles will offer important insights into adaptive pathways to cephalosporin resistance in *E. faecium*.

7. General discussion

This thesis was built upon clinical and laboratory observations that guided the design of each experiment. Each research question developed naturally from previous observations, creating a sequence of inquiries that connected the three chapters into a cohesive study of β-lactam resistance in *Enterococcus faecium*. Over the past five years, I have been dedicated to advance in understanding the mechanisms in *E. faecium* driving its resistance to β-lactams and study uncovering new therapeutic approaches to deal enterococcal infections. Through combined studies on antibiotic efficacy and genomic analysis of resistance determinants, this research highlights the remarkable adaptability of *Enterococcus* species to diverse environments, the complexity of its resistance mechanisms, and the significant challenges these traits pose for treating *Enterococcus*-related infections.

The results obtained in this thesis highlight the complex and multifactorial nature of antibiotic resistance in *E. faecium*, particularly in the context of β-lactam resistance. The findings of my first chapter, which investigated the potential of combining ampicillin with ceftriaxone or ceftaroline against ASEfm *in vitro*, underscores the variability in *E. faecium*'s response to antibiotic combinations. Previous studies **(159,160)** had demonstrated the efficacy of the ampicillin-ceftriaxone combination in the treatment of difficult-to-treat infections caused by *E. faecalis*, where a significant synergistic effect was consistently observed. However, when applied to ASEfm strains, the data on the efficacy of this combination still remain limited, though some research has suggested the possibility of a synergistic trend in certain strains **(161)**. This variability found in our results suggests that the interplay between the genetic and phenotypic characteristics of *E. faecium* strains influences their response to treatment, indicating that combination therapies may require a more personalized approach based on strain-specific resistance profiles. The therapeutic challenge associated with the treatment of enterococcal infections is compounded by the genetic mechanisms underlying *E. faecium* resistance. Our investigation into these mechanisms aims to complement the development of combination therapies, as a deeper understanding of resistance pathways, which may pave the way for more targeted and effective treatments, ultimately improving outcomes for resistant infections.

Gene redundancy, the presence of multiple copies of the same gene within a genome, plays a significant role in bacterial resistance mechanisms **(188,189)**. This redundancy can provide bacteria with a survival advantage, allowing them to maintain their vital functions even when one gene copy is compromised by mutations or antibiotics. In the context of antibiotic resistance, gene redundancy can make it more challenging to eradicate bacterial populations,

as multiple copies of resistance genes can enhance the bacteria's ability to withstand drug treatments and adapt to selective pressures more effectively. Previous studies have revealed that the redundancy and dissemination of the *pbp5* gene across *Enterococcus* genomes from diverse species occurs more frequently than initially anticipated, with significant findings demonstrating its presence in isolates from various origins **(184,186)**. However, our study marks the first instance of *pbp5* being carried on a plasmid in *E. faecium*. The plasmid-borne nature of this gene introduces a novel dimension to its dissemination, suggesting that horizontal gene transfer may play a pivotal role in the rapid spread of resistance within and across *Enterococcus* species. The mobilization of *pbp5* across different *Enterococcus* species and environments, including clinical and community settings, suggests that plasmid-borne resistance is more dynamic and widespread than previously thought. The presence of redundant *pbp5* genes on plasmids that also carry other resistance determinants, such as those conferring resistance to linezolid or tetracyclines, underscores the risk of co-selection and the evolution of multidrug resistance in diverse ecological niches. Furthermore, it is essential to recognize that environmental factors, such as temperature fluctuations, may further exacerbate the persistence and spread of these resistance genes **(222,223)**. In this thesis, the temperature-dependent effects observed in some ASEfm strains, where lower temperatures enhanced the growth advantage conferred by plasmid-encoded *pbp5*, raise concerns about the potential for resistance phenotypes to be underestimated in standard susceptibility testing. This highlights a key limitation in current surveillance systems, which may fail to account for environmental variability that influences resistance expression. The broader implications of this phenomenon are particularly relevant in the context of climate change, where fluctuating environmental conditions could alter the dynamics of antibiotic resistance across clinical, agricultural, and environmental settings.

Building upon the investigation of resistance mechanisms to β-lactam antibiotics, the investigation presented in the third chapter of this thesis has specifically focused on the mechanisms underlying cephalosporin resistance in *E. faecium*. The low MICs observed for ampicillin and ceftaroline in strain Efm5 challenge the established notion of *E. faecium*'s intrinsic resistance to cephalosporins **(136)**, suggesting that specific genetic factors may influence its resistance profile. Mutations identified in the cephalosporin-resistant variants, including alterations in the *croS, nusG* and *rpoB* genes, demonstrate the genetic plasticity of *E. faecium* in response to selective pressures. While previous studies have linked CroRS and RpoB as contributing factors to cephalosporin resistance in both *E. faecalis* and *E. faecium* **(100,238)**, our findings provide new insights into the genetic transitions that lead from susceptibility to

resistance in *E. faecium* clinical isolates. Specifically, we report a novel mutation in the *rpoB* gene associated with cephalosporin resistance, underscoring its role in this process. Additionally, mutations in *nusG*, which have not been previously connected to cephalosporin resistance, open up a new avenue for understanding the molecular basis of cephalosporin resistance in *E. faecium*. Several genes have been identified as key players in conferring resistance through their overexpression in response to antibiotic pressure. Notably, the *pbp5* gene has emerged as a pivotal player in conferring β-lactam resistance in both *E. faecium* and *E. faecalis*. Studies have shown that the upregulation of *pbp5* significantly alters the binding affinities of β-lactams, thereby diminishing their effectiveness against bacterial targets **(90,139)**. Our findings also reflect this extreme, as we observed significant upregulation of the *pbp5* operon (*ftsW*, *psr*, and *pbp5* genes) in the *croS* resistant variants. This indicates that mutations in the *croS* gene of these variants contribute to enhanced resistance mechanisms, further solidifying the role of *pbp5* in the adaptative response to antibiotic pressure. Interestingly, RNA sequencing of the *nusG* and *rpoB* CPH high-MIC variants also revealed significant alterations in gene expression, particularly in categories related to carbohydrate transport and metabolism, as well as transcriptional processes. Products encoded by *nusG* and *rpoB* genes are critical in bacterial transcription dynamics. While *rpoB* gene encodes the RNA polymerase (RNAP) β subunit, NusG is a transcriptional regulator essential for coupling transcription and translation in prokaryotes **(241,242)**. By tethering RNAP to the lead ribosome during transcription, NusG supports RNAP's forward progression along the DNA template through interactions with the RNAP β and β' subunits and the small ribosomal subunit. Mutations in both *nusG* and *rpoB* genes alter the activity of RNAP, leading to changes in the expression of multiple genes **(241,243)**. This shared regulation of RNAP activity of *nusG* and *rpoB*, likely explains the remarkable overlap observed in the differentially expressed genes between the *nusG*-R3 and *rpoB*-R5. Transcriptional changes in genes involved in carbohydrate transport are particularly important because these molecules are involved in cell wall biosynthesis. Such changes may impact the integrity and structure of the cell wall, making it less susceptible to cephalosporins and thereby protecting the bacteria from the effects of these antibiotics, which target cell wall synthesis **(249)**. The isolation of *E. faecium* strains exhibiting susceptibility to cephalosporins, despite their known intrinsic resistance, highlights a need to explore the mechanisms behind this phenotype further. Notably, the mutations in *croS*, *nusG*, and *rpoB* genes in our cephalosporin-resistant variants represent unique sequences absent in existing *Enterococcus* entries in the NCBI database. This finding suggests that these mutations do not align with standard resistance mechanisms in *Enterococcus*. Rather, they may represent compensatory mutations—selected under selective pressures—that enhance resistance in strains originally exhibiting low MICs to cephalosporins.

These compensatory mutations may provide a survival advantage, balancing out other genetic or metabolic limitations by restoring and potentially enhancing resistance. Understanding these novel mutations and their roles in resistance profiles will offer important insights into adaptive pathways in *E. faecium*.

This thesis represents a comprehensive exploration of the molecular mechanisms driving *E. faecium* resistance to β-lactam antibiotics, advancing our understanding of this critical challenge in infectious disease management. By integrating findings from studies on antibiotic synergy, gene redundancy, and cephalosporin susceptibility, I have uncovered the multifaceted nature of resistance in *E. faecium*, emphasizing the genetic plasticity and adaptability of this pathogen. The discovery of *pbp5* gene redundancy, its plasmid-mediated dissemination, and the role of novel mutations in resistance profiles highlights the complexity of resistance evolution in both clinical and environmental settings. Moreover, the variable efficacy of antibiotic combinations, particularly the promising yet incomplete synergy observed with ampicillin and ceftaroline, points to the necessity of refining therapeutic strategies to account for strain-specific responses and resistance mechanisms. Our research underscores the importance of tailoring treatments based on a deeper molecular understanding of resistance, moving towards a more individualized approach to combat multidrug-resistant infections. The implications of this work extend beyond the immediate treatment of *E. faecium* infections, offering valuable insights into the broader fight against antibiotic resistance. By elucidating key genetic factors and resistance pathways, this thesis lays the groundwork for future studies aimed at optimizing therapeutic regimens and improving clinical outcomes for patients facing difficult-to-treat infections. The knowledge gained from these findings may be crucial for informing the development of more effective, targeted interventions that can address the growing global threat of antibiotic resistance in *E. faecium* and beyond.

8. Conclusions

1. Ampicillin plus ceftaroline or ceftriaxone revealed an heterogeneous activity against ampicillin susceptible *E. faecium* isolates. In this regard, we observed synergism, additive activity or no effect, which was not directly associated with the isolate's susceptibility to cephalosporins.

2. Phylogenetic analysis of *Enterococcus* spp. isolates harbouring redundant *pbp5* copies revealed three distinct clades, with unique mobilization patterns and gene arrangements. This result suggests different evolutionary pathways for the dissemination of β-lactam resistance within the *Enterococcus* genus. The observation that redundant *pbp5* genes were associated with various resistance genes on plasmids, especially those isolated from food-chain sources, emphasizes the role of plasmid-mediated gene transfer in the global spread of antibiotic resistance across diverse ecological niches.

3. The identification of a plasmid carrying a redundant *pbp5* copy (pEfm57_1) in a *E. faecium* clinical isolate (Efm57), highlights a potential mechanism for β-lactam resistance evolution and dissemination within clinical and environmental settings. The close genetic relationship between plasmid-borne *pbp5* variants and native chromosomal *pbp5* copies across different *Enterococcus* spp. underscores the evolutionary adaptability to these bacteria in acquiring and mobilizing resistance genes, which may pose challenges for treatment.

4. The higher minimum inhibitory concentrations (MICs) values observed for Efm57 and the transconjugants, both carrying pEfm57_1, compared to the recipient strain lacking the plasmid, indicate that the presence of pEfm57_1 enhances ampicillin resistance. Notably, the significant increase in MICs values at 22ºC, with transconjugants displaying a 4-fold rise and Efm57 showing a 2- to 3-fold increase, highlights the impact of environmental conditions on antibiotic resistance.

5. The unusually low MICs observed for multiple cephalosporins in a *E. faecium* clinical isolate (Efm5) challenged the current paradigm of intrinsic resistance.

6. Comparative genomic analysis of ceftriaxone high-MIC variants revealed unique mutations in three genes: *croS*, *nusG*, and *rpoB*. Our study confirmed the key role of the CroRS two component system (TCS) in cephalosporin resistance in enterococci. Moreover, we identified two amino acid substitutions in the CroS protein that led to the activation of the

pbp5 operon, directly involved in β-lactam resistance. In addition, we uncovered a role for the intrinsic transcriptional terminator NusG in cephalosporin resistance in *E. faecium*. Also, we identified an additional amino acid substitution in the RpoB protein that significantly increases the MIC to cephalosporin antibiotics. The identified mutations in *nusG* and *rpoB* triggered important transcriptional changes, illustrating an extensive bacterial cell reprogramming.

9. Future directions

The findings from this research provide several avenues for future studies aimed at deepening our understanding of *E. faecium* resistance mechanisms and advancing therapeutic strategies.

Building on the exploration of β-lactam combinations, further investigations into combination therapies with ampicillin and other cephalosporins could uncover novel therapeutic options. Translating *in vitro* results to *in vivo* studies using animal models would provide a more comprehensive understanding of the clinical applicability of the antibiotic combinations and would be crucial for determining the efficacy of the proposed treatments in a living system before advancing to human clinical trials.

Understanding the role of the *pbp5* gene in resistance remains a priority. Future work could focus on assessing the impact of an additional plasmid-borne copy of *pbp5* on the resistance of *E. faecium* to ampicillin resistance under diverse stress conditions. Exploring the mechanisms responsible for the observed increase in minimum inhibitory concentration (MIC) of ampicillin at lower temperatures in *E. faecium* and extending this analysis to other enterococcal strains would further elucidate how environmental factors influence resistance.

In terms of cephalosporin resistance, identifying the prevalence and genetic basis of the cephalosporin-susceptible phenotype in *E. faecium* is essential. Investigating the genes highlighted in the transcriptomic analysis of *nusG* and *rpoB* variants and characterizing cephalosporins (CPH) high-MIC variants of other CPH low-MIC strains could provide new insights into the diverse mechanisms driving cephalosporin resistance.

To maximize the impact of this research, fostering collaborations and interdisciplinary approaches will also be crucial to advance in our understanding of *E. faecium* resistance mechanisms and the development of novel therapeutic strategies. For example, collaborations with structural biologists can provide detailed insights into the molecular interactions between antibiotics and key bacterial proteins, such as PBP5, offering more precise understanding of how mutations alter drug binding and efficacy. This knowledge can inform the designs of new antibiotics or antibiotic combinations that better target resistant strains. Additionally, partnering with pharmacologists could prove invaluable for optimizing treatment protocols by refining dosing regimens based on pharmacokinetic and pharmacodynamic properties of antibiotic combinations. Furthermore, engaging with environmental scientists is essential for understanding how external factors, such as temperature fluctuations or the presence of other

microbial species, influence the spread and persistence of resistance genes in both clinical and non-clinical environments.

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11. Annexes

Table S1. List of predicted virulence factors in the Efm57 genome.

Virulence gene*	Identity (%)	Query / Template	Protein function (variant)**	Accession number of template (NCBI)
Surface-exposed cell wall-anchored proteins (*E. faecium* surface proteins)				
acm	99.08	2167 / 2166	Adhesin of collagen (community variant Efm C59)	NZ_WLYR01000013.1
fnm	99.65	1707 / 1707	Fibronectin-binding protein (community variant C59)	NZ_WLYR01000008.1
scm	97.63	1728 / 1728	Second collagen adhesin (community variant C59)	WLYR01000005.1
sgrA	96.92	975 / 975	Serine-glutamate repeat containing protein A (hospital variant Efm DO)	CP003583.1
Phosphotransferase systems				
bepA	99.79	1419 / 1419	Biofilm and endocarditis-associated permease A/PTS transporter subunit EIIC (community variant Efm C5	NZ_WLYR01000001.1
Pili gene cluster (PGC) 2 (fms14-fms17-fms13)				
fms13	94.94	1701 / 1698	Surface protein 13 (variant Efm DO)	CP003583.1
fms14	99.33	4038 / 4038	Surface protein 14 (variant Efm DO)	CP003583.1
fms17	97.95	1300 / 1314	Surface protein 14 (community variant C59)	NZ_WLYR01000016.1
Biofilm and colonization				
sagA	99.27	1512 / 1512	Secreted antigen A (community variant C59)	NZ_WLYR01000005.1
Carbohydrate metabolism and cell growth				
ccpA	99.71	1020 / 1020	Carbon catabolite control protein A (hospital variant Efm TX0082)	AEBU01000039.1
General stress proteins				
gls20	99.28	558 / 558	General stress protein GlsB20 (gls20-glsB1 cluster) (variant Efm DO)	CP003583.1
gls33	96.57	903 / 903	General stress protein Gls33 (gls33-glsB cluster) (variant Efm DO)	CP003583.1
glsB	99.59	243 / 243	General stress protein GlsB (gls33-glsB cluster) (variant Efm DO)	CP003583.1
glsB1	100.0	243 / 243	General stress protein GlsB1 (gls20-glsB1 cluster) (variant Efm_DO)	CP003583.1

* Virulence genes were identified in Center for Genomic Epidemiology using raw reads in the Virulence Finder 2.0 service, option "Enterococcus faecium&Enterococcus lactis" (https://cge.food.dtu.dk/services/VirulenceFinder/). Abbreviations; Efm, *Enterococcus faecium.* ** Roer et al (2024).Microb Spectrum, 12: e03724-23

Table S2. Summary of the upregulated genes identified in the *nusG* variants, with regulation values expressed as log_2FoldChange.

***nusG* variants upregulated genes**			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_01876	5,482604	0,001686	-
efm5_00870	4,875675	0,001344	-
efm5_00515	4,635069	0,000444	-
efm5_01942	4,227466	0,000028	*murE*
efm5_00910	4,211629	0,000103	*sagG*
efm5_00657	4,099638	0,000025	-
efm5_00168	4,070987	0,000000	-
efm5_02089	4,034559	0,000001	-
efm5_00516	3,934480	0,001739	
efm5_01159	3,760122	0,000000	-
efm5_00658	3,747357	0,000072	*yrkD*
efm5_00674	3,737131	0,000091	-
efm5_00174	3,458976	0,000000	*yqfL*
efm5_00784	3,286473	0,000066	-
efm5_01662	3,097349	0,000815	*phnX*
efm5_02290	3,023603	0,000004	-
efm5_00911	2,998272	0,002205	-
efm5_01661	2,797525	0,002196	*phnX*
efm5_01161	2,710396	0,000416	*pepS*
efm5_00912	2,680885	0,000156	-
efm5_00791	2,664733	0,000014	*rnhA*
efm5_00921	2,660139	0,000037	*sdaAA*
efm5_00988	2,640142	0,000000	*proC*
efm5_00684	2,619923	0,000452	*ptsG*
efm5_01583	2,396370	0,000551	*pbg9*
efm5_01637	2,394477	0,000840	*fhuD9*
efm5_00686	2,370141	0,000535	-
efm5_01689	2,339462	0,000079	-
efm5_01911	2,301700	0,000507	*mleR*
efm5_00779	2,285608	0,001277	*ypwA*
efm5_01660	2,283293	0,000021	*argS*
efm5_01427	2,241257	0,001049	*XK27_05385*
efm5_02004	2,224499	0,002345	*yumC*
efm5_00655	2,193591	0,001440	*mutS1*
efm5_01157	2,092199	0,001031	-
efm5_00735	2,062207	0,000447	*pheT*
efm5_01529	2,041818	0,002418	*arcD*
efm5_01941	1,926566	0,001301	*celD*
efm5_00747	1,884779	0,000001	*fabI*
efm5_00673	1,878957	0,002096	*ybhF_1*
efm5_01749	1,853527	0,001422	*nadE*
efm5_01093	1,830232	0,000965	*pmrB*
efm5_02118	1,827458	0,000348	*pckA*
efm5_00948	1,825814	0,000102	-
efm5_00502	1,675531	0,000022	*ubiB*
efm5_01325	1,663107	0,000511	-
efm5_00156	1,617054	0,000049	*pepQ*
efm5_00522	1,567991	0,001020	*queA*
efm5_00729	1,497140	0,000002	*XK27_02070*
efm5_00101	1,404578	0,003099	-
efm5_01016	1,322591	0,001111	*spr7*
efm5_01748	1,053073	0,000242	-

Table S3. Summary of the downregulated genes identified in the *nusG* variants, with regulation values expressed as log_2FoldChange.

***nusG* variants downregulated genes**			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_01869	-1,255222	0,003022	*clpE*
efm5_01043	-1,303473	0,002264	-
efm5_01035	-1,382946	0,002494	-
efm5_00068	-1,427303	0,001555	*fbp*
efm5_01907	-1,460660	0,000565	-
efm5_00933	-1,585760	0,000866	*nrdF*
efm5_01960	-1,599949	0,001485	-
efm5_01930	-1,638880	0,001385	-
efm5_00902	-1,639468	0,000362	*cutC*
efm5_00696	-1,661463	0,001287	*ubiX*
efm5_00042	-1,680306	0,001619	*yrzB*
efm5_00504	-1,709917	0,002401	*murF*
efm5_00934	-1,796890	0,000941	*nrdE*
efm5_00438	-1,811422	0,003027	*gshF*
efm5_02134	-1,813421	0,000156	-
efm5_01280	-1,813463	0,002713	-
efm5_00697	-1,819207	0,000103	*yclC*
efm5_02013	-1,957300	0,000052	-
efm5_00695	-2,003718	0,000001	-
efm5_01572	-2,073710	0,002464	-
efm5_00597	-2,287731	0,000028	*tas3*
efm5_00314	-2,361062	0,000498	*aroA*
efm5_02130	-2,423808	0,002893	*lacS*
efm5_01065	-2,432173	0,002832	-
efm5_01321	-2,443765	0,000244	-
efm5_01488	-2,607074	0,000062	*yddR*
efm5_02048	-2,625601	0,000003	*yheA*
efm5_01551	-2,636582	0,000059	*folA*
efm5_00669	-2,672527	0,001195	*dhaL*
efm5_01550	-2,684125	0,000467	*thyA*
efm5_01796	-2,771503	0,000305	-
efm5_02132	-2,984843	0,000269	*galK*
efm5_00712	-2,985127	0,000348	*galM*
efm5_00668	-3,013192	0,000391	*dhaK*
efm5_01086	-3,143180	0,000099	*ydiA*
efm5_00534	-3,163390	0,000000	-
efm5_02131	-3,263628	0,000441	*galM*
efm5_00964	-3,301967	0,002141	-
efm5_01595	-3,377824	0,000002	*ltaS*
efm5_01320	-3,491436	0,000003	*yqjA*
efm5_00412	-3,552776	0,000700	
efm5_00214	-3,911191	0,000099	-
efm5_00947	-3,954844	0,000117	-
efm5_02246	-4,032253	0,001939	-
efm5_01087	-4,257319	0,000158	-
efm5_01630	-4,611095	0,000126	-

Table S4. Summary of the upregulated genes identified in the *rpoB* variant, with regulation values expressed as log_2FoldChange.

***rpoB* variant upregulated genes**			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_01327	5,437157	0,000150	*yesP*
efm5_01188	5,361486	0,000250	-
efm5_00870	4,916264	0,000434	-
efm5_01676	4,637729	0,000915	*ylxR*
efm5_01876	4,600586	0,005076	-
efm5_01225	4,350278	0,004263	*iscS2*
efm5_00174	4,110680	0,000000	*yqfL*
efm5_00168	4,079439	0,000000	-
efm5_00674	3,752415	0,000087	-
efm5_00645	3,729136	0,001909	*bglP11*
efm5_00657	3,677192	0,000056	-
efm5_01156	3,659379	0,002052	-
efm5_00961	3,642155	0,001082	*padR5*
efm5_00306	3,601682	0,000129	*galR*
efm5_01504	3,580929	0,000010	*mvaS*
efm5_01895	3,561280	0,004563	*ylbM*
efm5_01186	3,543720	0,004869	-
efm5_01474	3,479526	0,001240	*rpmD*
efm5_00910	3,450012	0,001651	*sagG*
efm5_01330	3,441177	0,002458	*yteT*
efm5_00658	3,438670	0,000183	*yrkD*
efm5_01665	3,402120	0,001062	*rpsB*
efm5_00779	3,401500	0,000064	*ypwA*
efm5_01782	3,393430	0,000300	*malA*
efm5_00945	3,367065	0,002443	-
efm5_01450	3,362877	0,000723	*rpiA*
efm5_02089	3,313625	0,000040	-
efm5_00642	3,271444	0,000610	-
efm5_01890	3,238051	0,003130	*yhbY*
efm5_00169	3,219095	0,001469	*celF*
efm5_01894	3,218479	0,000623	*yqeM*
efm5_00458	3,195072	0,004643	*ccl*
efm5_00852	3,149709	0,001989	*hit*
efm5_01161	3,122839	0,000073	*pepS*
efm5_01783	3,089237	0,001125	*cfa*
efm5_01468	3,013152	0,000213	*rplE*
efm5_01297	3,012980	0,002051	*rplU*
efm5_00830	2,998051	0,001197	*ulaA*
efm5_01460	2,996101	0,002444	*rpsS*
efm5_01891	2,992663	0,000989	*nadD*
efm5_01679	2,986682	0,001171	*rbfA*
efm5_01916	2,983976	0,000517	*arlS*
efm5_02006	2,973287	0,000075	-
efm5_01940	2,965520	0,000884	-
efm5_01507	2,906764	0,001707	-
efm5_01157	2,880067	0,000264	-
efm5_01662	2,861332	0,002263	*phnX*
efm5_00791	2,854204	0,000004	*rnhA*
efm5_01529	2,836893	0,001298	*arcD*
efm5_01159	2,810179	0,000062	-
efm5_00759	2,802635	0,000744	*apt*
efm5_00008	2,794375	0,002836	*exoA*
efm5_00758	2,792360	0,000023	*recJ*
efm5_00911	2,757804	0,001277	-
efm5_00761	2,741844	0,003619	*lexA*
efm5_00890	2,688859	0,002612	-
efm5_01926	2,676219	0,004062	*ytqA*
efm5_02023	2,623473	0,004876	*rnjB*
efm5_02005	2,612626	0,000215	*ltrC*
efm5_01660	2,608518	0,000819	*argS*
efm5_01941	2,606780	0,001687	*celD*
efm5_01637	2,598537	0,000935	*fhuD9*

rpoB variant upregulated genes			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_02004	2,597799	0,001563	*yumC*
efm5_00782	2,589772	0,003573	-
efm5_02291	2,578962	0,000484	*msmX*
efm5_00686	2,573929	0,000187	-
efm5_00817	2,570251	0,004294	*XK27_08845*
efm5_00735	2,568539	0,000013	*pheT*
efm5_00136	2,561779	0,003489	*ftsE*
efm5_00988	2,556392	0,000012	*proC*
efm5_00684	2,527139	0,000627	*ptsG*
efm5_01936	2,518051	0,000755	-
efm5_01750	2,471045	0,000204	*pncB*
efm5_02285	2,448832	0,003822	-
efm5_01473	2,377832	0,002379	*rpsE*
efm5_00102	2,375016	0,000025	*iunH3*
efm5_00546	2,344657	0,002400	*mvaK2*
efm5_00488	2,317312	0,002110	*pnp*
efm5_00747	2,301235	0,000002	*fabI*
efm5_01583	2,300280	0,001636	*pbg9*
efm5_01394	2,296395	0,000380	*serS*
efm5_00655	2,274195	0,002903	*mutS1*
efm5_00932	2,258243	0,003415	*yueF*
efm5_02118	2,184524	0,000380	*pckA*
efm5_01454	2,122674	0,000785	*tuf*
efm5_00101	2,101723	0,000114	-
efm5_01508	2,048791	0,000352	*yvgN*
efm5_01675	2,008929	0,003522	*nusA*
efm5_01362	2,007539	0,003659	*typA*
efm5_01575	1,999601	0,003488	-
efm5_00679	1,984843	0,000940	*nhaC*
efm5_00524	1,949834	0,002944	-
efm5_01325	1,906419	0,001455	-
efm5_00812	1,707792	0,002482	*FbpA*
efm5_01733	1,695011	0,003591	-
efm5_01678	1,594538	0,002234	*infB*

Table S5. Summary of the downregulated genes identified in the *rpoB* variant, with regulation values expressed as log_2FoldChange.

***rpoB* variant downregulated genes**			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_02167	-1,379457	0,002952	-
efm5_00068	-1,429239	0,002964	*fbp*
efm5_01574	-1,429382	0,001549	*yqcC*
efm5_00927	-1,590817	0,004512	-
efm5_01614	-1,623869	0,001951	*murQ*
efm5_02261	-1,707173	0,003970	*ydiL*
efm5_01907	-1,707373	0,000141	-
efm5_00485	-1,731627	0,004981	*yrxA*
efm5_01254	-1,774097	0,003467	*rho*
efm5_00696	-1,780803	0,001613	*ubiX*
efm5_01562	-1,786020	0,000330	-
efm5_01266	-1,874915	0,000008	-
efm5_00902	-1,877553	0,000670	*cutC*
efm5_01576	-1,903095	0,000391	-
efm5_01923	-1,929137	0,000528	*XK27_09600*
efm5_00695	-1,954149	0,000014	-
efm5_01531	-2,041808	0,001108	*yndB*
efm5_00878	-2,061779	0,002199	-
efm5_00550	-2,081273	0,000251	-
efm5_00028	-2,121789	0,000719	-
efm5_01960	-2,132279	0,000129	-
efm5_00599	-2,137922	0,000065	*licC*
efm5_01930	-2,143852	0,000004	-
efm5_00318	-2,146609	0,000774	-
efm5_00180	-2,151893	0,000136	*brnQ*
efm5_01241	-2,185191	0,003207	-
efm5_01098	-2,190477	0,001582	*mdt(A)*
efm5_01998	-2,206855	0,003153	-
efm5_02320	-2,212050	0,004614	*gadB*
efm5_02134	-2,214392	0,000041	-
efm5_00041	-2,218955	0,000269	*yrrK*
efm5_01707	-2,221570	0,000186	-
efm5_02013	-2,265531	0,001034	-
efm5_01165	-2,284034	0,000557	*rbn*
efm5_01295	-2,296894	0,000633	*yvdB*
efm5_02272	-2,334191	0,000192	-
efm5_00246	-2,349669	0,003806	-
efm5_01012	-2,368640	0,001298	*rpe*
efm5_01549	-2,401925	0,002611	*yfmR*
efm5_02363	-2,449478	0,000007	*B4168_4126*
efm5_00838	-2,487181	0,004972	-
efm5_00412	-2,546839	0,003485	
efm5_01756	-2,568772	0,004860	*nanE*
efm5_00874	-2,595719	0,002062	*prmA*
efm5_00314	-2,630297	0,000085	*aroA*
efm5_00947	-2,643570	0,001719	-
efm5_01063	-2,648432	0,002936	
efm5_02321	-2,656494	0,002273	*gadC*
efm5_00438	-2,662032	0,000015	*gshF*
efm5_02111	-2,673422	0,000023	*ohrB*
efm5_00568	-2,681311	0,000086	*ytpR*
efm5_01100	-2,686221	0,004645	*iscS*
efm5_01042	-2,695906	0,000141	*sthIR*
efm5_01041	-2,701440	0,000037	-
efm5_00483	-2,710837	0,000497	*cobB*
efm5_00827	-2,784023	0,002055	-
efm5_01488	-2,812644	0,000014	*yddR*
efm5_00563	-2,833479	0,000787	-
efm5_02200	-2,851227	0,000000	-
efm5_00712	-2,933665	0,000752	*galM*
efm5_00247	-2,945271	0,001030	-
efm5_01065	-2,965860	0,003202	-
efm5_01115	-2,978915	0,001252	-
efm5_01314	-2,998455	0,000007	*idhA*
efm5_00288	-3,014572	0,002856	*fruR*
efm5_00534	-3,043595	0,000000	-
efm5_01251	-3,101291	0,000003	-

rpoB variant downregulated genes			
locus_tag	**log_2FoldChange**	**p-value**	**Preferred name**
efm5_01247	-3,102850	0,000047	
efm5_00964	-3,271036	0,001119	-
efm5_00802	-3,288689	0,000009	*pyrC*
efm5_01551	-3,305150	0,000001	*folA*
efm5_01321	-3,420157	0,000001	-
efm5_01594	-3,429199	0,000020	-
efm5_01550	-3,642522	0,000001	*thyA*
efm5_02132	-3,676129	0,000001	*galK*
efm5_01595	-3,691654	0,000000	*ltaS*
efm5_01743	-3,712187	0,005172	-
efm5_02128	-3,799007	0,000871	-
efm5_02130	-3,954777	0,000000	*lacS*
efm5_02127	-4,366789	0,001093	-
efm5_02131	-4,392986	0,000005	*galM*
efm5_01320	-4,540825	0,000000	*yqjA*
efm5_00671	-4,707578	0,000166	*flp*
efm5_01087	-4,748672	0,000005	-

Table S6. Genes identified as upregulated (coloured in green) and downregulated (coloured in red) showing overlapping regulation patterns between the *nusG* and *rpoB* variants. The first column specifies the functional category, the second the gene name (if available), and the third a described function. Gene function information has been primarily sourced from PaperBlast due to its credibility, as it references specific articles based on sequence homology. This was complemented by annotations from UniProt, NCBI, and other relevant literature to provide a throughout functional overview. COG, Clusters of Orthologous Genes.

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
Replication, recombination and repair (L)	-	**UniProt and NCBI**: Nucleotide excision repair (NER) endonuclease, a process by which the cell removes damaged segments of DNA. DNA repair and cutting of specific DNA sequences **PaperBlast (PMID:20738841):** ORF: **EF1750**. gene name: endo/excinuclease amino terminal domain protein.
	rnhA	**UniProt**: RNase HI (*E. coli*). Remove the RNA from the RNA-DNA hybrids (R-loops), facilitating the synthesis of new DNA avoiding genomic instability (DNA replication at uncontrolled sites). **1. PaperBlast (PMID:15659682):** TIGR designation: **EF1728.** Gene: *ebsB*. Function: LTA biosynthesis. **2. PaperBlast (PMID:34867878):** gene ID: ***rnhA LMOf2365_1909***. gene product: Ribonuclease HI. Transcript levels increased for select genes in response to bile in anaerobic conditions at pH5.5 (FC=4.9).
	mutS1	**UniProt**: DNA mismatch repair protein MutS2. It is a key enzyme of DNA mismatch repair (MMR), which corrects mismatched bases produced during DNA replication. The endonuclease plays a critical role in maintaining genetic stability within bacteria by limiting the exchange of genetic material, which in turn affects how diverse bacterial populations can be in terms of their genetic makeup. This can have implications for bacterial adaptation and evolution. **NCBI (PMID:17215294/PMID: 32471288**): DNA repair **PaperBlast (PMID: 24400070): CDR20291_2052**. DNA mismatch repair protein. Differential protein expression of ***Clostridium difficile*** CD26A54_R (Met-resistant) and CD26A54_S (reduced- susceptibility) compared against reference strain VLOO13 (Met-susceptible), in the absence of metronidazole: metS:2.3±0.57metR:2.4±0.3
Cell wall/membrane/envelope biogenesis (M)	***ltaS***	**UniProt and NCBI (PMID:19168632)**: Sulfatase. Responsible for the synthesis of lipoteichoic acid (LTA) polyglycerol phosphate (a polymer that is part of the cell wall) in *Staphylococcus aureus*. It has a functional relationship with the cell wall and cell division. It features a potential active site for binding Mn^{2+}, with key residues that, through directed mutagenesis, have been shown to be crucial for its enzymatic function. **PaperBlast (PMID:37475106**): Paper Susanne. Lipoteichoic acid (LTA) synthase **EF1813**. Pathway: Teichoic acids biosynthesis. ***Enterococcus faecalis*** WT versus Δ*croRS* strain in the presence of TXB: FC of 3.4. A key enzyme for the cell wall biosynthesis of Gram-positive bacteria.
Defense mechanisms (V)	***sagG***	**UniProt**: ABC transporter ATP-binding protein. ABC transporters are essential in importing vital molecules, such as metal ions, amino acids, peptides, and vitamins, which contribute to bacterial survival and virulence. They also export virulence factors, facilitating infection processes. Additionally, these transporters help bacteria persist by exporting toxic substances, aiding in the development of antimicrobial resistance. **PaperBlast (PMID:32234818)**: targocil treatment of ***B. anthracis*** activates a previously unstudied TCS, EdsRS. Upon activation, EdsRS upregulates self-expression and expression of an additional operon, consisting of ***BAS1661***-*BAS1663clsT*. ***BAS1661***-*BAS1663* operon in *B. anthracis* contains putative ABC transporter genes. *BAS1661-BAS1663* are predicted to contain domains from the ABC-2 subfamily of transporters associated with the

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		export of drugs and carbohydrates. Upregulation of *BAS1661-BAS1663clsT* with targocil. ***BAS1661*** to BAS1663clsT encode an ABC transporter ATP binding protein (***BAS1661***), an ABC transporter permease (*BAS1662*), an ABC transporter permease (*BAS1663*), and a cardiolipin synthase (*clsT*).
	-	**UniProt and InterProt:** ABC transporter permease. The ABC-2 transporters are a specialized subgroup of exporters characterized by having their transmembrane domain encoded on a separate polypeptide from the ATP-binding domain, unlike most other ABC transporters where these regions are often fused in the same protein. Involved in transporting specific substances, often related to the export of toxins or other secreted products. **PaperBlast (PMID:32611694)**: gene: *altF* "**DS891_00385**". Function: ABC transporter permease. It belongs to the bromoalterochromide biosynthetic operon.
Transcription (K)	-	**NCBI (PMID:24006471):** TetR family transcriptional regulator. The TetR family of regulators (TFRs) consists of two domains: an N-terminal DNA-binding domain and a C-terminal ligand-binding domain. In its native conformation, TFRs bind to DNA to repress gene expression in the absence of ligands. However, when a ligand binds to the C-terminal domain, the protein undergoes a conformational change that disrupts its ability to bind DNA, thereby allowing gene expression to proceed. Ligands can include antibiotics or small molecules. Approximately 25% of TFRs are involved in regulating antibiotic resistance, while the remainder control various processes such as sugar metabolism, virulence, and stress responses. **PaperBlast (PMID:34683359)**: **LSL_0723**, transcriptional regulator. Whole genomes of bile salts tolerant strains were compared with the core genes of the non-tolerant group to determine the redundant genes under bile salt solutions. The results showed seven different genes between the two groups, including one TetR/AcrR family transcriptional regulator-encoding gene.
	-	**InterProt:** MurR/RpiR family transcriptional regulator. MurR is a transcriptional repressor that regulates the expression of *murP* and *murQ*, which encode for proteins involved in the metabolism of N-acetylmuramic acid (MurNAc), a component of the bacterial cell wall. When MurNAc is metabolized, the resulting metabolites weaken MurR's binding to DNA, allowing the expression of these two genes to occur. On the other hand, RpiR is also a transcriptional repressor involved in sugar metabolism. **PaperBlast (PMID:19175920)**: ***Streptococcus uberis***. Regulators in the accessory genome that are associated with sugar detection and metabolism, including an RpiR family regulator (**SUB1582**) that contain SIS phospho-sugar binding domain.
	-	**PaperBlast (PMID:20579342): *E. faecalis***. **EF0432**. Functional class: Transcription and translation. Putative function: Transcriptional regulator, AraC family. In response to bile, 20min FC=0.91, 60min FC=2.64, 120min FC=0.18.
	-	**UniProt:** Integron-associated effector binding protein. Transcriptional regulator **PaperBlast (PMID:36759782)**: ***Bacillus cereus***. BC3433. Hypothetical protein. COG: S. Is a SigB and Bc1009-dependent-induced protein upon heat shock in *B. cereus.*
Amino acid transport and metabolism (E)	***pepS***	**NCBI (PMID:10406960):** Aminopeptidase. *pepS* form *Streptococcus thermophilus*, is an aminopeptidase T family found in thermophilic and extremophilic bacteria. Involved in bacterial growth by hydrolyzing peptides (supplying essential amino acids), which are essential for protein synthesis and cellular metabolism. Characterized as a monomeric metallopeptidase with optimal activity at a pH range of 7.5 to 8.5 and at a temperature of 55 °C. **PaperBlast (PMID:37628007): *E. faecium* (*E. faecium* DMEA09)**. Gene

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		locus: **PAN98_RS08995**. Product: Aminopeptidase. Protease gene. COG: U. *E. faecium* DMEA09 strain showed growth in the presence of 6% salt and protease activity in the presence of 3% salt.
	proC	**UniProt**: Pyrroline-5-carboxylate reductase. Catalyzes the reduction of 1-pyrroline-5-carboxylate (PCA) to L-proline. It does not catalyze the reverse reaction. The conversion of PCA to L-proline supports cellular function, protein synthesis, and adaptation to environmental stressors. **PaperBlast (PMID:37758814): *E. faecalis***. Protein ID: Q836Y3. Protein description: Pyrroline-5-carboxylate reductase. *E. faecalis* when grown in the presence of gliotoxin (5 µg/ml), compared to identical cultures grown without gliotoxin (fungic metabolite with antibacterial activity), FC absent (>-1.5).
	ypwA	**UniProt:** Metal-dependent carboxypeptidase. Broad specificity carboxypetidase that releases amino acids sequentially from the C-terminus, including neutral, aromatic, polar and basic residues. **PaperBlast (PMID: 37628007): *E. faecium (E. faecium* DMEA09*)*** Gene locus: PAN98_RS06880. Product: Carboxypeptidase M32. Protease gene. COG: J. *E. faecium* DMEA09 strain showed growth in the presence of 6% salt and protease activity in the presence of 3% salt.
	aroA	**UniProt:** 3-phosphoshikimate 1-carboxyvinyltransferase. Catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) to produce enolpyruvyl shikimate-3-phosphate and inorganic phosphate. **PaperBlast (PMID:21296946)**: *E. faecalis*. EF1566. Gene: *aroA*. Functional category: 3-phosphoshikimate 1-carboxyvinyltransferase. Function: Amino acid biosynthesis. FC=-1.25 when a mutant lacking *ldh* genes (lactate deshidrogenase) is compared to the wild-type strain. It belongs to a gene cluster involved in the biosynthesis of aromatic amino acids (EF1561 to EF1568).
Inorganic ion transport and metabolism (P)	***fhuD9***	**InterProt**: ABC transporter substrate-binding protein. Periplasmic binding proteins are essential components of ATP-binding cassette (ABC) transport systems. In bacterial import systems, periplasmic binding proteins bind substrates (such as nutrients or molecules) with high specificity and deliver them to the transmembrane domains of the ABC transporter for further movement into the cell. This process ensures the correct substance is transported into the cell. **PaperBlast (PMID: 26067254)**: ***E. faecalis***. **OG1RF_10136**. Virulence-related gene of *E. faecalis* OG1RF. Definition: iron (Fe3+) ABC superfamily ATP binding cassette transporter, binding protein
	cutC	**InterProt:** PF03932 family protein CutC. Participates in the control of copper homeostasis. **PaperBlast (PMID:21362400*): E. faecalis***. In bacteria, two independent systems are responsible for maintaining the balance of copper within the cells (Cop operon and Cut family proteins). Previous studies describe **CutC** as a member of the Cut family that is probably involved in copper homeostasis. In this work, a homolog of CutC was studied in *E. faecalis*, a bacterial model for copper homeostasis. Through quantitative real-time PCR (qPCR), it was demonstrated that efCutC expression is induced late by copper stimulus. Our results describe efCutC as a protein able to respond transcriptionally to copper and to participate in the control of copper homeostasis in *E. faecalis*. This bacterium is the first reported organism containing a *cop* operon and an active member of the Cut protein family.
Energy production and conversion (C)	***yumC***	**NCBI (PMID:15252706):** Ferredoxin--NADP reductase (FNR). The YumC protein in *Bacillus subtilis* functions as an FNR. It catalyzes the reduction of NADP+ to NADPH using electrons from ferredoxin (Fd). YumC efficiently supports the reduction of cytochrome c with NADPH as the electron donor, though it has a much lower activity with NADH. It also exhibits diaphorase activity (electron transfer from NADPH to artificial electron acceptors like DPIP) with a higher affinity for NADPH than NADH. YumC's role as an NADPH-cytochrome c reductase highlights its involvement in electron

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		transfer processes, essential for maintaining the cell's redox balance and energy metabolism. **PaperBlast (PMID:39156124): *E. faecalis***. Gene name: ***OG1RF_12200***. Description: NAD(P)/FAD-dependent oxidoreductase. Increased prevalence of multidrug-resistant bacterial infections has sparked interest in alternative antimicrobials, including bacteriophages (phages). Limited understanding of the phage infection process hampers our ability to utilize phages to their full therapeutic potential. To understand phage infection dynamics, we performed proteomics on *E. faecalis* infected with the phage VPE25. *OG1RF_12200* it is within the 20 highest differential abundance ratio (DAR) values at 40 min bacteriophage postinfection. DAR40=21.05
Coenzyme transport and metabolism (H)	***pckA***	**InterProt**: phosphoenolpyruvate carboxykinase (ATP). Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed (rate-limiting) step in hepatic gluconeogenesis, namely the reversible decarboxylation of oxaloacetate to phosphoenolpyruvate (PEP) and carbon dioxide, using either ATP or GTP as a source of phosphate. **PaperBlast (PMID:34093453):** Pyruvate metabolism. Gene ID: **LSEI_1820**. Phosphoenolpyruvate carboxykinase (ATP). ***Lacticaseibacillus paracasei*** production of organic acids. It is a gene involved in the TCA cycle and related to amino acids (the precursor of taste substances). Testing of a single inoculation with *L. paracasei* and mix inoculation (*L. paracasei* + *K. marxianus*) during rice-acid fermentation process FC in the 1st day post inoculation FC=-1.83.
	folA	**UniProt:** Dihydrofolate reductase. This enzyme plays a key role in folate metabolism within the cell. It catalyzes an essential reaction required for the *de novo* synthesis of glycine and purines, which are crucial for DNA precursor synthesis, making it vital for cell growth and division. **PaperBlast (PMID:38570789): *E. faecalis***. UniProt ID: **Q834R2_ENTFA.** Protein function: Dihydrofolate reductase. Down-expressed protein in *E. faecalis* in the presence of 0.05% DCA, CDCA or CA and 0.01% DCA (**the most prominent bile acids**) under aerobic as well as microaerophilic conditions. Involved in biosynthesis of folic acid and amino acids.
	ubiX	**UniProt**: Flavin prenyltransferase UbiX. Flavin prenyltransferase enzyme facilitates the synthesis of a cofactor called prenyl-FMN. This cofactor is required for the UbiD enzyme, which is involved in the synthesis of ubiquinone (coenzyme Q), a key component in cellular respiration. The enzyme is metal-independent and transfers a dimethylallyl group from dimethylallyl monophosphate (DMAP) to two specific atoms on the FMN molecule, thereby creating the modified cofactor. **PaperBlast (PMID:28966611): *E. faecium* DO. Q3Y2U4.** Flavoprotein. *Arxula adeninivorans* LS3 gallic acid decarboxylase (Agdc1p) plays an essential role in the tannic acid catabolism and could be useful in the production of catechol and cis,cis-muconic acid. Homologous to subunit B of different LAB, including *E. faecium*.
Nucleotide transport and metabolism (F)	***gshF***	**UniProt:** Glutathione biosynthesis bifunctional protein GshAB. *gshAB*: synthesizes glutathione from L-glutamate and L-cysteine via gamma-L-glutamyl-L-cysteine, a crucial antioxidant in cells which helps protect the cell from oxidative stress and maintain overall cellular health **PaperBlast (PMID:15901709)**: Organism: ***E. faecalis* V583**. Locus name: EF3089. Gene: *gshF*. A multimodular fusion protein that specifies both γ-glutamylcysteine ligase and glutathione synthetase catalytic activities and enables GSH (glutathione) synthesis. Relatively few bacteria have evolved a multimodular enzyme to make GSH.
	thyA	**UniProt:** Thymidylate synthase. This reaction provides a crucial de novo source of dTMP, an essential precursor for DNA biosynthesis, which is vital for cell growth and proliferation. By ensuring an adequate supply of dTMP, this enzymatic activity supports cellular functions necessary for DNA replication and repair, highlighting the significant role of folate derivatives in nucleotide synthesis and overall metabolism. **PaperBlast (PMID:31027295): *E. faecalis*.** UniProt KB: **Q834R3.** It is a folate-

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		dependent thymidylate synthase (TS). TS is important because it catalyzes an essential reaction for DNA synthesis. Specifically, it converts the nucleotide dUMP into dTMP, which is necessary to form thymidine, one of the basic building blocks of DNA. Without dTMP, cells cannot effectively synthesize DNA, which prevents cell division and replication. Connected with the resistance to p-aminosalicylic acid in *M. tuberculosis*.
Carbohydrate transport and metabolism (G)	***ptsG***	**InterProt:** PTS glucose/maltose transporter subunit IIBCA. This entry represents a family of PTS enzyme II fused B and C components including and most closely related to the MalX maltose and glucose-specific transporter of *Escherichia coli* **PaperBlast (PMID:23951303): *E. faecium E1162*. EfmE1162_1485**. Maltose uptake and utilization. Belongs to a gene cluster (EfmE1162_1485 - EfmE1162_1489; here named malRMBPT), which is predicted to encode five proteins with amino acid identities ranging from 59% to 87% to their homologs in *E. faecalis V583*, and this gene cluster is conserved among all the available genomes of *E. faecium* (amino acid identities >96%).
	pbg9	**UniProt**: 6-phospho-β-glucosidase. Degradation of carbohydrates, specifically glucosides **Paper Blast (PMID:33201910): *Enterococcus mundtii* QU25. EMQU_2185**. It is a glycoside hydrolase family 1 protein. Involved in cellobiose utilization. Upregulated in *E. mundtii QU25* grown in cellobiose.
	celD	**UniProt:** Permease IIC component. The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) transports sugars across the cell membrane and simultaneously phosphorylates them. **PaperBlast (PMID:36639757): *Tetragenococcus halophilus* (LAB),** commonly isolated from fermented foods containing high amounts of NaCl. Locus tag study strain TMW2.2254. **HV360_08980**. NCBI annotation: PTS cellobiose transporter subunit IIC. Within a cellobiose operon. The protein has a premature stopcodon (not functional).
	-	Converts N-acetylmannosamine-6-phosphate (ManNAc-6-P) to N-acetylglucosamine-6-phosphate (GlcNAc-6-P). Hydrolase **PaperBlast (PMID:28771188): *Enterococcus caccae***. Gene: **UC7_RS13110**. Description: hypothetical protein. Description of function: Unknown. Upregulated gene (FC=1.7) in response to apigenin (dietary flavonoid found in many plants and vegetables. It possesses strong antioxidant, anti-inflammatory, antimicrobial and anticancer properties).
	lacS	Glycoside-pentoside-hexuronide (GPH): cation symporter. Catalyze uptake of sugars (mostly, but not exclusively, glycosides). cells can effectively absorb and utilize them for energy production, metabolism, and the synthesis of important biomolecules, such as nucleotides, amino acids, and polysaccharides. This activity is especially important in organisms that rely on sugars for energy, growth, and maintenance of cellular functions. **PaperBlast (PMID:38172710): *E. faecium*.** Gene: *galP*. Protein annotation (UniProt): **Q3Y292.** Protein description: MFS transporter, SP family galactose:H + symporter. Ciprofloxacin as a persister-inducible stress in *E. faecium.* Comparative proteomic analysis revealed that 56 proteins have significantly different abundances in persisters compared to cells harvested before the addition of stressing agent. Most of them were related to energetic metabolisms, including Q3Y292, FC=-4.35 (less abundant in persisters than in growing cells).
	galK	**UniProt:** Galactokinase. Catalyzes the transfer of the gamma-phosphate of ATP to D-galactose to form alpha-D-galactose-1-phosphate (Gal-1-P). Galactose metabolism (energy source). Gal-1-P is essential for the synthesis of important biomolecules, including glycoproteins and glycolipids, which are vital for cell structure and function. **PaperBlast (PMID:21702908): *Lactobacillus sakei* (LAB)**. Specific carbohydrate metabolic pathway. Gene locus: **LSA0764.** Gene: *galK*. Description: Galactokinase. Upregulation of this gene in 2 out of 3 *L. sakei* strains grown on ribose compared with glucose (FC=1.1; 0.7; 1.8). Within the operon *galK-galE1-galT-galM*. Galactokinase is a key enzyme in

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		galactose metabolism, and its overexpression suggests that these bacteria could efficiently use this sugar for growth and persistence in the GIT, where galactose may be available due to carbohydrate digestion and other metabolic processes.
	galM	**UniProt:** Aldose 1-epimerase. In *E. coli, galM* converts alpha-aldose to the β-anomer. It is active on D-glucose, L-arabinose, D-xylose, D-galactose, maltose and lactose (energy production and physiological balance). **PaperBlast (PMID:17220255): *E. faecalis*. EF1068**. Carbohydrate: trehalose. gene: *galM*. Within the operon *galK-galE1-galT-galM*.
	fbp	Fructose-1,6-bisphosphatase class 3. Is a ubiquitous cytosolic enzyme that catalyzes the fourth step of glycolysis. **PaperBlast (PMID:33250881): *Streptococcus sanguinis*. SSA_1056**. Fbp, fructose-1,6-bisphosphatase, responsible for metabolizing FBP (fructose-1,6-bisphosphate). Manganese acquisition is believed to be a key factor for *S. sanguinis*' virulence in streptococcal endocarditis infection. Manganese deprivation alters the expression of various cellular systems, including the repression of carbohydrate metabolism.
	galM	**InterProt:** Aldose 1-epimerase. Also known as mutarotase, is essential for carbohydrate metabolism, specifically in the interconversion of galactose and glucose **PaperBlast (PMID:17220255): *E. faecalis*. EF1068**. Carbohydrate: trehalose. gene: galM. Within the operon *galK-galE1-galT-galM*.
Translation, ribosomal structure and biogenesis (J)	***argS***	**NCBI:** Arginine--tRNA ligase. Attachment of arginine to its corresponding transfer RNA (tRNA) in a process known as arginine-tRNA ligase activity. This is involved in the aminoacylation of arginine tRNA, which is the process by which arginine is loaded onto tRNA for incorporation during protein synthesis. **PaperBlast (PMID:29099858): *Lactobacillus sakei* (LAB).** Locus tac *L. sakei* LS25: **LS25_1495.** Protein: Arginyl-tRNA synthetase. Downregulation in glucose-limited media.
	pheT	**InterProt:** Phenylalanine--tRNA ligase β subunit. It helps link amino acids to their specific transfer RNA (tRNA) molecules. **PaperBlast (PMID:23417491): *E. faecalis*. EF1116.** Strain: DV87-4. Relevant genotype: DV87-4 (**EF1116**-EF1117)2::EF2638-3. Source: this work. While the specific functions of EF1116 are not directly addressed in this work, its presence in the study alongside the Rex factor EF2638 suggests that it may have significant implications for the metabolic responses of *E. faecalis*, particularly in terms of how the bacterium adapts to oxygen availability and manages oxidative stress.
Lipid transport and metabolism (I)	***fabI***	Enoyl-[acyl-carrier-protein] reductase [NADH]. Is a key enzyme involved in fatty acid synthesis mainly mycolic acid biosynthesis **PaperBlast (PMID:31134041): *E. faecium***. Operon: 3824507. Locus tag: **HMPREF0351_11295**. Product: Enoyl- acyl-carrier-protein reductase NADH. COG: I. Fatty acid biosynthesis. In this study, the target genes of BsrR from functional annotation are involved in amino acid metabolism (HMPREF0351_11989 and 10965), fatty acid biosynthesis (**HMPREF0351_11295**), secondary metabolites biosynthesis (HMPREF0351_10954), inorganic ion transport and metabolism (HMPREF0351_10955), cell wall maintenance (HMPREF0351_10944), and DNA mismatch repair (HMPREF0351_11678). These target genes may contribute to **bile salt resistance in *E. faecium*.**
Signal transduction mechanisms (T)	-	**NCBI (PMID:20972419):** ECF transporter, substrate-specific component. ECF transporter S component. Membrane transport proteins responsible for the uptake of vitamins. **PaperBlast (PMID:21702908): *Lactobacillus sakei*. LSA0829.** Hypothetical integral membrane protein.
Unknown function (S)	***phnX***	**UniProt**: Encoding the phosphonoacetaldehyde hydrolase (phosphonatase). Involved in phosphonate degradation, cleaving the carbon–phosphorus bond.

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		NCBI: *P. aeruginosa*. https://www.ncbi.nlm.nih.gov/gene/61416624 **PaperBlast (PMID:38491038): Q183T0**. Bifunctional phosphonoacetaldehyde hydrolase
	-	**UniProt and NCBI:** Cell division protein (YfhO). A typical Gram-positive bacterial cell wall is composed of peptidoglycan and the secondary cell wall polymers, wall teichoic acid (WTA) and lipoteichoic acid (LTA). *yfhO* in *Bacillus subtilis* is essential for LTA glycosylation. **Paper Blast (PMID:17483264)**: Unknown function. Gene: ***ssu1792.*** Putative function (organism): Conserved hypothetical protein (***Streptococcus suis*** 89/1591)
	-	**UniProt and NCBI (PMID:9695921):** DsrE family protein. Involved in the sulfur metabolism (sulfur reduction for energy production or detoxification) **PaperBlast (PMID:23396346):** QS and bacteriocin-related protein "**lp_2254**"
	yrkD	**UniProt**: Metal-sensitive transcriptional regulator. Responsible for adjusting gene expression in response to metal levels, which likely helps the bacterium manage metal toxicity or use metals effectively for metabolic processes. **PaperBlast (PMID:29712426):** CstR protein from ***E. faecalis,*** is an RSS-sensing repressor that transcriptionally regulates a cst-like operon in response to both exogenous sulfide stress and Angeli's salt, a precursor of nitroxyl (HNO). A Δ*cstR* strain exhibits deficiency in catheter colonization in a catheter-associated urinary tract infection mouse model, suggesting sulfide regulation and homeostasis is critical for pathogenicity. **OG1RF_RS11640** (*cstR*) belongs to the cst-like operon.
	arcD	**UniProt** (*E. coli*): YfcC family protein. C4-dicarboxylate anaerobic carrier. Metabolomic profiling of strains with over-expression and deletion of the yfcC gene indicates that it may regulate the glyoxylate shunt, a pathway crucial for utilizing specific carbon sources. **PaperBlast (PMID:18359813): *Streptococcus gordonii***. ORF: **SGO_1590**, gene: *arcD*, predicted function: Arginine-ornithine antiporter. Encodes a membrane transporter (uptake of arginine). Downregulated in response to coaggregation with *Actinomyces naeslundii:* FC=-4.2 (microarray); FC=-2.1 (qRT-PCR). The capacity of *S. gordonii* to synthesize arginine was assessed using a chemically defined growth medium. In monoculture, streptococcal arginine biosynthesis was inefficient, and streptococci could not grow aerobically at low arginine concentrations. In dual-species cultures containing coaggregates, however, *S. gordonii* grew to high cell density at low arginine concentrations.
	-	DUF3284 domain-containing protein **PaperBlast (PMID:24610841): *Listeria monocytogenes*. Lmo2705.** Annotation: unknown.
	yqfL	**UniProt:** Putative pyruvate, phosphate kinase regulatory protein. The specific target of YqfL's activity is pyruvate, phosphate kinase (PPDK). PPDK is an enzyme that plays a crucial role in metabolic pathways, particularly in the conversion of pyruvate to phosphoenolpyruvate (PEP) in certain organisms. By phosphorylating or dephosphorylating PPDK, YqfL helps regulate the activity of this enzyme, influencing the flow of metabolites through the glycolytic and gluconeogenic pathways. - Implications: Metabolic Regulation: By regulating PPDK, YqfL plays a significant role in controlling energy metabolism and the balance between energy production and consumption within the cell. Adaptation to Conditions: The activity of YqfL might be crucial in helping organisms adapt to different environmental conditions by modulating metabolic pathways based on the availability of nutrients or energy sources. - Cellular Signaling: The phosphorylation/dephosphorylation mechanism is a key component of cellular signaling, allowing cells to respond rapidly to changes in their environment. **PaperBlast (PMID:21989394): *E. faecalis*. EF2419**. This regulator is involved

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
		in catabolite control protein A (CcpA)-independent carbon catabolite repression (CCR) in *B. subtilis*. Recent publications using transcriptome analysis suggested that the *cit* operons might be regulated by Rex (a regulator responding to NAD/NADH ratio). CcpA is the major transcriptional regulator in CCR in several gram-positive bacteria.
	yqjA	**NCBI (PMID:25917916)**: Aromatic acid exporter family protein. *E. coli* can grow at pH 5.5 to 9.5 while maintaining a constant cytoplasmic pH of about 7.6. Under alkaline conditions, bacteria rely upon proton-dependent transporters to maintain a constant cytoplasmic pH. The DedA/Tvp38 protein family is a highly conserved but poorly characterized family of membrane proteins. It is descrived that YqjA is critical for *E. coli* to survive at pH 8.5 to 9.5. YqjA requires sodium and potassium for this function and that it plays a significant role in the survival of *E. coli* at alkaline pH. **PaperBlast (https://doi.org/10.1101/2022.08.17.504265): *E. faecalis***. Gene: ***yqjA* EF1910**. Strain 1 has the gene mutation: M267I vs an ancestral strain. And strain 4 has the mutation E150* vs an encestral strain.
	yddR	**NCBI (PMID:33219126):** Metallo- β-lactamase domain-containing protein. β-lactamase activity (zinc in their active side instead of serine) **PaperBlast (PMID:28656172): *S. aureus***. Locus tag (N315): **SA2259** / Locus tag (Newman): NWMN_2368. Function: Hypothetical. **Angeli's salt** stress FC=8.9.
	-	Exo-alpha-sialidase. Hydrolysis of the terminal residues of sialic acid (SA). By doing this, bacteria can access and utilize these sugars, gaining energy and resources. This activity contributes to their survival, colonization, and persistence in environments like the human gastrointestinal tract, aiding their ability to thrive and potentially cause infections or interact with the host's immune system.
	-	**InterProt:** Oxidoreductase. Oxidoreductase activity. Crucial role in key cell processes like respiration and energetic metabolism. These enzymes use NAD or NADP to facilitate redox reactions, with the Rossmann fold helping them bind to these cofactors. **PaperBlast (PMID:22761597): *E. faecium***. Locus tag: **EfmE1162_2490**. annotation: oxidoreductase, Gfo/Idh/MocA family. *E. faecium* gene involved in **ampicillin resistance** (predicted to function as an NAD- or NADP-dependent oxidoreductase). FC=11.8 in the presence of ampicillin. Transposon mutants in this gene appear to exhibit a loss of fitness in the presence of ampicillin.
	-	DUF2929 domain-containing protein **PaperBlast (PMID:33234689): *E. faecalis*.** *E. faecalis* V583 gene IDEF1043: **EF1043**. It encodes unknown functions, but it appears to be extracellular or associated with the cell envelope. Fitness critical gene for growth in *E. faecalis*.
	-	Putative sulfate exporter family transporter. Conserved hypothetical protein 698. Uncharacterized multi-pass membrane protein **PaperBlast (PMID:37167201): *Clostridium difficile***. Locus tag: **CDR20291_0191.** Putative membrane protein. Downregulation when *C. difficile* was grown with *Desulfovibrio piger* (FC=-5.26) or even in a culture medium that *D. piger* had previously grown in (FC=-4.21)..
Others	-	**UniProt:** DUF998 domain-containing protein. Pfam (that is a biological data repository) domain of unknown function (DUF). **PaperBlast:** No hits.
	-	**UniProt:** DUF3941 domain-containing protein. DUF **PaperBlast:** No hits.
	-	Uncharacterized protein **PaperBlast (PMID:37758814): *E. faecalis*.** Protein ID: **Q833H3**. Uncharacterized protein. This protein is absent in *E. faecalis* when grown in the presence of gliotoxin (5 µg/ml), compared to identical cultures grown without gliotoxin (5 µg/ml).
	-	**PaperBlast:** No hits.

***nusG*-R3 and *rpoB*-R5 regulated genes overlapping**		
COG	**Gene**	**Function**
	-	**PaperBlast (PMID:28771188): *Enterococcus caccae*.** Gene: **UC7_RS13265.** Description: hypothetical protein. Description of function: Unknown. *E. caccae* genes upregulated in response to apigenin (FC=1.5).
	-	**PaperBlast (PMID:23645198): *Lactobacillus plantarum*. lp_0272**. Gene: *lpdD*. Lp_2945 (labeled as UbiD), suggested it was part of a three-gene system encoding the three subunits necessary for nonoxidative aromatic acid decarboxylation. *L. plantarum* has a unique gene arrangement on its chromosome, with its genes for gallate decarboxylase subunits—*lpdC* (lp_2945), *lpdB* (lp_0271), and ***lpdD*** **(lp_0272**)—separated from one another.
	-	**PaperBlast (PMID:30631364): *E. faecium E1162*.** Locus tag in *E1162*: **EFME1162_RS19605.** Name: hp3. It is. Agene present in the genetic region up- or downstream of the *tirE* locus in the *E. faecium E1162* genome. The *tirE*-gene cluster promotes bacterial proliferation in human blood, indicating that TirE may contribute to the pathogenesis of bacteremia.
	-	**PaperBlast:** No hits.
	-	**PaperBlast:** No hits.
	-	**PaperBlast (PMID:19218379): *E. faecalis*. EF1081.** Annotation: Conserved hypothetical protein. Biofilm gene.