ORIGINAL RESEARCH

Uncovering Antibiotic Resistance Signatures in Endodontic *Enterococcus faecalis*

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Abstract

Aims and background: Due to its resilience and resistance to common intracanal disinfectants and medicines, *Enterococcus faecalis*, also known as *E. faecalis*, is a notorious pathogen in endodontics that is frequently isolated from persistent and secondary root canal infections. Its growing resistance to antibiotics that target the cell wall is concerning, and its clinical impact on endodontic failure is widely acknowledged. Yet, there remains a lack of comprehensive understanding of the global transcriptional responses that *E. faecalis* employs to survive exposure to widely used β-lactams and glycopeptides.

Materials and methods: The Affymetrix *E. faecalis* OG1RF array microarray dataset, GSE45306, which comprised 15 samples, was examined, and the untreated controls and cultures were exposed to 10× MIC of Ampicillin, Bacitracin, Cephalothin, and Vancomycin for 30 minutes. Gene expression omnibus 2R (GEO2R)/Limma was referred to identify differentially expressed genes (DEGs) after quality control and robust multi-array average (RMA) normalization, using an adjusted $p < 0.05$ and $|\log_2 \text{fold change}| > 1.5$. Heatmaps, Venn diagrams, and volcano plots were used to visualize both treatment-specific and shared transcriptional responses.

Results: With 552 distinct DEGs, Vancomycin caused the biggest transcriptional shift, whereas Cephalothin and Bacitracin affected 77 and 10 genes, respectively. A core set of 55 stress-responsive genes, many of which code for proteins with unclear or poorly understood activities, was shown to be consistently changed across all antibiotic treatments. Common themes revealed by pathway, and expression analysis included increased expression of efflux transporters, metabolic changes, especially involving Adenosine triphosphate (ATP) synthase and pyruvate dehydrogenase, and cell wall remodeling. Furthermore, a number of regulators particular to antibiotics, such as membrane-associated sensors and members of the cell envelope-associated transcriptional attenuator LytR-CpsA-Psr family, indicated specific adaptation mechanisms.

Conclusion: *Enterococcus faecalis* has a sophisticated, adaptable transcriptional response to antibiotic stress, as well as drug-specific and transparently preserved survival strategies.

Clinical significance: During endodontic treatment, the antibiotic-specific transcriptional regulators, such as *E. faecalis*-membrane-associated sensors and members of the LytR-CpsA-Psr family, undergo adaptive responses. Thus, to enhance root canal disinfection and fight antibiotic resistance, these molecular targets are considered the most promising aspects of acumen.

Keywords: Affymetrix, Boxplot analysis, *Enterococcus faecalis*, Gene expression omnibus 2R, Venn diagram, Volcano plot.

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Introduction

As one of the most common nosocomial pathogens in the world, *Enterococcus faecalis* (*E. faecalis)* has become a major healthcare concern in recent decades. About 20,000 people are thought to be affected by *E. faecalis* each year in the United States alone, accounting for about 80% of all enterococcal infections, according to recent worldwide surveillance statistics.[1] Due to the bacterium's great tolerance to harsh conditions and growing resistance to conventional antibiotics, it has become a major public health concern, particularly in healthcare settings where it is the cause of many potentially fatal infections.[2] The gastrointestinal tracts of both humans and animals naturally contain *E. faecalis*, which typically coexists there as a commensal organism. However, it frequently changes from commensal to pathogen in medical environments, particularly in immunocompromised people.[3]

According to the Centers for Disease Control and Prevention (CDC), 14% of hospital-acquired illnesses, including surgical site infections, bacteremia, endocarditis, and urinary tract infections, are brought on by *E. faecalis*.[4] Up to 30% of patients die from bacteremia due to *E. faecalis* infections, according to recent epidemiological studies conducted in hospitals throughout Europe.[5] Complex

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molecular mechanisms are involved in the pathophysiology of *E. faecalis* infections. In order to cling to host tissue and facilitate infection, the bacterium possesses a variety of virulence factors, including cytolysin, extracellular surface protein (ESP), surface adhesins, and aggregation substance (AS).[6]

According to recent genomic studies, *E. faecalis* strains obtained from clinical settings exhibit higher levels of virulence gene expression than commensal strains.[7] Because biofilms give the bacteria immunity from both host immune responses and antimicrobial medicines, the pathogen's propensity to form biofilms on tissue surfaces and medical devices further complicates treatment choices.[8] Treatment options have become significantly more complex due to *E. faecalis's* ability to acquire and spread genes that cause antibiotic resistance.[9] Drug target changes, increased efflux pump activity, and enzymatic drug degradation are only a few of the many known resistance mechanisms.[10] According to recent molecular studies, *E. faecalis* possesses chromosomally encoded genes that confer inherent resistance to certain antibiotics, such as low-level aminoglycosides and cephalosporins.[11]

The incidence of vancomycin-resistant *Enterococcus faecalis* (VREf) has risen by 20% in the last 10 years, which is a very serious problem.[12] Because the gene clusters responsible for high-level Vancomycin resistance, vanA and vanB, are typically found on movable genetic elements, they can spread horizontally between bacterial populations.[13] Nearly 30% of *E. faecalis* isolates recovered in intensive care units in 2023 were Vancomycin-resistant, according to surveillance data, which is a significant rise over prior years.[14] Depending on the patient's condition and the site of infection, *E. faecalis* infections can present with a wide range of symptoms. About 40% of individuals with *E. faecalis* infections develop urinary tract infections, the most common kind.[15] Usually, these infections cause lower abdominal pain, frequency, and dysuria, especially in individuals who are catheterized.[16]

Endocarditis is one of the more significant complications; it affects 5–15% of patients and has a 20% fatality rate even with treatment.[17] The intricate relationship between *E. faecalis* and the immune system of the body has been demonstrated by recent data. The bacterium produces extracellular proteases and superoxide dismutase, which neutralize host immunological molecules, among other tactics to get past the host's defenses.[18]

To further suppress the initial immunological response, *E. faecalis* can alter its cell surface components to evade detection by pattern recognition receptors.[19] Antibiotics are typically used in combination to actively treat *E. faecalis* infections, especially those that are severe.[20] For susceptible organisms, Ampicillin is the preferred antibiotic; for maximum action, it is sometimes coupled with gentamicin.[21] However, in most cases, the effectiveness of this regimen has been diminished due to high-level aminoglycoside resistance.[22] Vancomycin-resistant organisms have been successfully treated with newer medications such as daptomycin, linezolid, and tigecycline; nevertheless, resistance to these medications has also been noted.[23]

According to recent statistics, the annual cost of medical care for *E. faecalis* infections in the United States alone exceeds $500 million.[24] Additionally, hospital stays are lengthened, with an average of 12 extra days per patient, which significantly raises costs.[25] Furthermore, up to 30% of survivors experience long-term difficulties, indicating a significant influence on patient health and quality of life.[26] Recent developments in molecular diagnostics technology have improved *E. faecalis* infection detection and typing. In order to support tailored therapeutic approaches, next-generation sequencing tools have made it possible to quickly identify virulence factors and resistance profiles.[27]

Additionally, it has been revealed that new diagnostic systems based on machine learning algorithms may predict antibiotic resistance patterns with 90% accuracy.[28] Numerous innovative strategies are being investigated, including immunomodulatory substances designed to stimulate host immunity, bacteriophage therapy, and anti-virulence drugs that specifically block specific pathogenicity factors.[29–31] These novel treatments have shown promising results in preliminary clinical studies; phase II trials showed efficacy rates of 65–75%.[32]

Preventive measures have also progressed, with antibiotic stewardship programs and infection control practices significantly reducing the spread of *E. faecalis* in healthcare facilities, and their implementation has been linked to a 25% reduction in infection rates in the participating facilities.[33,34] Nevertheless, the challenge of maintaining efficient infection control in the face of increasing resistant strains continues to require creative solutions.[35]

Given these difficulties, an explanation of the transcriptional-level molecular mechanisms underlying *E. faecalis* antibiotic resistance and pathogenicity is desperately needed. Analysis of gene expression provides important insights into the development of bacterial adaptation and resistance. Our study intends to examine differential gene expression patterns in *E. faecalis* under different antibiotic stress settings using gene expression omnibus 2R (GEO2R), a potent gene expression analysis tool.

Our goal was to find important regulatory networks and resistance-associated genes that exhibit differential expression in response to antibiotic exposure by examining publicly accessible gene expression datasets in the Gene Expression Omnibus (GEO) database.[36] The investigation of the transcriptional profiles of cultures of *E. faecalis* exposed to Vancomycin, Ampicillin, and Bacitracin—all of which correspond to different antibiotic classes frequently used in endodontic therapy to treat root canal infections was the specific focus of our study. With a fold change threshold of ≥2.0 and a p-value < 0.05, the GEO2R analysis assisted in identifying genes that were strongly upregulated and downregulated, offering important information for additional analysis and in-depth research into the mechanisms underlying antibiotic resistance in endodontic pathogens.[37]

Methods

Enterococcus faecalis Expression Data Retrieval from GEO for Endodontic Studies

The gene expression dataset GSE45306 was first obtained from the NCBI GEO database. The expression profiles of *E. faecalis* OG1RF included in this dataset were produced using the Affymetrix GeneChip *E. faecalis* Genome Array (GPL13991) platform. This provides insight into the molecular reasons behind *E. faecalis's* ability to withstand antibiotic stratagems frequently used in

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endodontic therapy and its resilience in the root canal system. Four distinct antibiotics were used in the experimental setup to treat *E. faecalis* OG1RF cells, a major pathogen in endodontic infections, at doses 10× their respective MICs: Ampicillin, Bacitracin, Cephalothin, and Vancomycin. After being exposed to antibiotics for 30 minutes, samples were taken at the mid-exponential growth phase.

There were 15 samples in total because the study design included three biological replicates for each antibiotic treatment condition and untreated controls. Robust statistical analysis of patterns of differential gene expression was made possible by this design. Understanding the pathogen's reaction to antibiotic therapy in the setting of endodontic infections requires thorough coverage of the bacterial transcriptome, which is provided by the probe sets on the Affymetrix array platform that target over 3,200 *E. faecalis* genes.

Evaluation of Quality and Categorization of Samples

In order to examine the transcriptional behavior of *E. faecalis*, a major pathogen in chronic root canal infections, we next downloaded the microarray expression data (GSE45306) from the GEO database. To guarantee the dataset's dependability for gene expression analysis relevant to endodontics, preliminary quality checks were carried out. Ribonucleic acid (RNA) degradation patterns, array intensity distribution, and probe set performance were all evaluated using Affymetrix standard parameters as part of the quality assessment process.

The robust multi-array average (RMA) algorithm, which carries out background correction, quantile normalization, and summarization of probe sets, was used to reduce technical variation across arrays and guarantee accurate comparison of antibiotic-treated and control samples pertinent to endodontic disinfection strategies. Based on the GEO metadata, 15 samples were included in the analysis and divided into five experimental groups. Three biological replicates of *E. faecalis* OG1RF that were not given antibiotics were included in the control set. Three biological replicates of *E. faecalis* OG1RF were in each of the four treatment groups, which were subjected to various antibiotics: Ampicillin (2 µg/mL), Bacitracin (32 µg/mL), Cephalothin (16 µg/mL), and Vancomycin (4 µg/mL).

Before administering antibiotics, bacterial cultures were already grown to the mid-exponential phase in clinical data, and RNA was extracted after 30 minutes of antibiotic treatment. We were able to investigate how the gene expression patterns of antibiotic-treated samples and untreated controls differed, thanks to our experimental approach.

Differential Expression of *E. faecalis* in Endodontic Antibiotic Subgroups

Additionally, we investigated the transcriptional responses of *E. faecalis*, a significant cause of chronic endodontic infections, by performing differential gene expression analysis on the GSE45306 dataset using GEO2R, the interactive web tool made available by NCBI. In order to replicate antimicrobial exposure circumstances pertinent to root canal disinfection techniques, the analysis compared the expression profiles of *E. faecalis* OG1RF samples treated with antibiotics to untreated controls.

Four comparison groups, Ampicillin vs control, Bacitracin vs control, Cephalothin vs control, and Vancomycin vs control, were established to represent agents that are frequently encountered or clinically significant in endodontic treatment. The Limma R package, which employed empirical Bayes statistics and linear modeling, was integrated into the analysis pipeline. The threshold values of $|\log_2$ fold change$| > 1.5$ and adjusted p-value < 0.05 (Benjamini-Hochberg correction) were used to identify significantly differentially expressed genes (DEGs). To find particular transcriptional responses, each antibiotic treatment group was examined independently in comparison with the control group. This method made it possible to identify both typical and distinct gene expression patterns brought on by several antibiotics that operate on the cell wall.

We specifically looked at genes related to stress response pathways and cell wall production for Ampicillin therapy. The investigation of Bacitracin concentrated on genes related to cell envelope stress and membrane integrity. We examined genes linked to peptidoglycan production and cell wall remodeling processes using Cephalothin and Vancomycin treatments.

Statistical Analysis and Visualization with Plots and Venn Diagrams

We used R version 4.1.0 to analyze the microarray data from GSE45306 using a variety of visualization techniques. The statistical parameters and expression data acquired from GEO2R were stored as tab-separated files. Volcano plots were created using the ggplot2 R tool as part of the analysis workflow.

Plotting $\log_2$ fold changes vs −log10 (adjusted p-value) allowed these graphs to display changes in gene expression. To distinguish between genes with higher and lower expression, we indicated those that met statistical cutoffs (adjusted p-value < 0.05, $|\log_2$ fold change$| > 1.5$). Heat maps created using the R pheatmap package were used to visualize expression patterns and were applied to the Ward. D2 hierarchical clustering algorithm using Euclidean distance.

Using box plots created with the ggplot2 program, expression level discrepancies for carefully selected genes were displayed. Using gene lists from each treatment group, we created four-way Venn diagrams using the Venny 2.1.0 web tool to examine the overlap in gene expression during antibiotic behaviors.

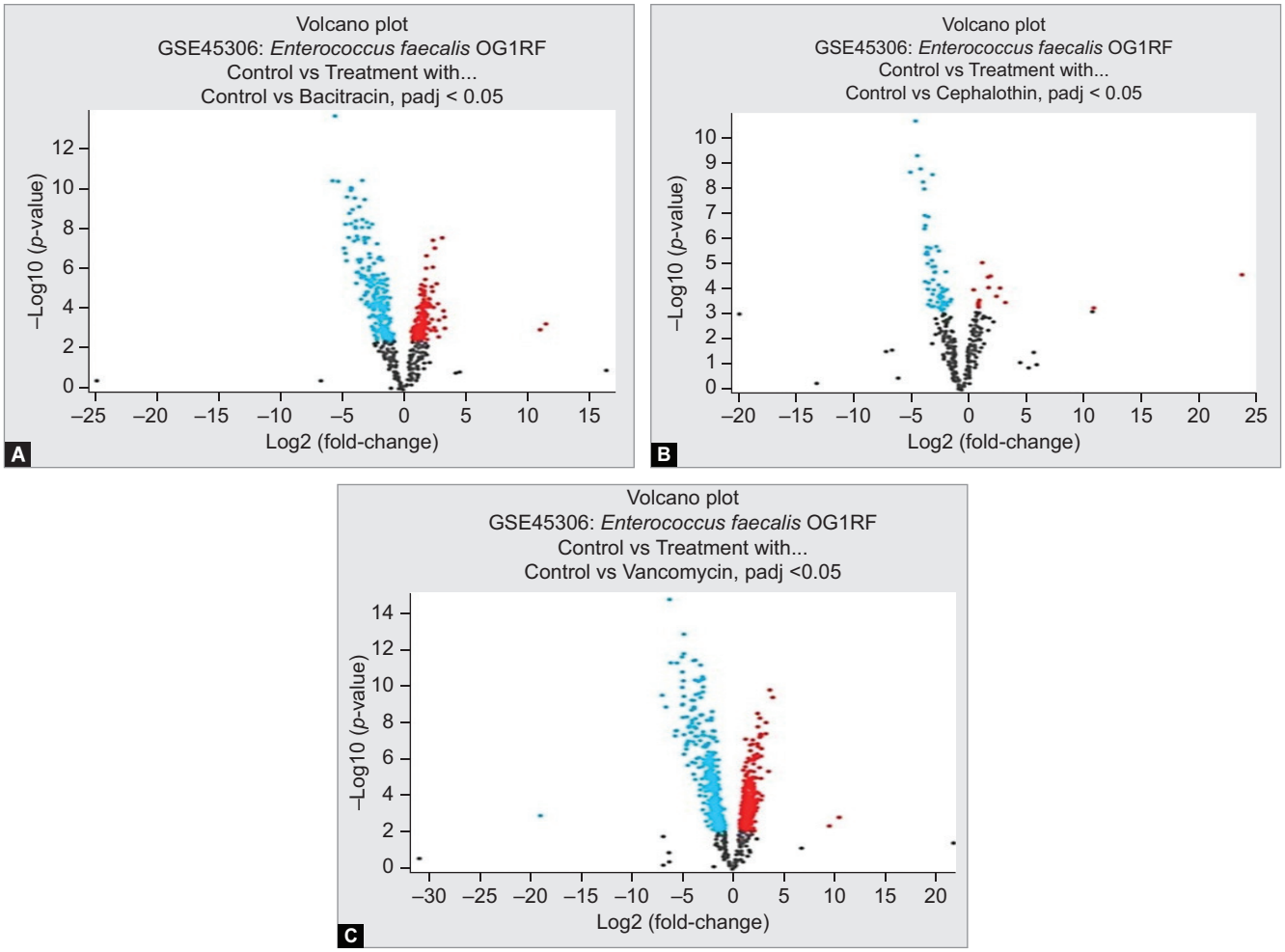
The hypergeometric distribution was used to determine the numerical impact of gene set overlaps. We were able to investigate the transcriptional responses of *E. faecalis* to numerous cell wall-active antibiotics at the gene expression level thanks to our integrative analysis.

Results

Data Selection and Parameterization of the GSE45306 Dataset

Enterococcus faecalis OG1RF, a major pathogen in endodontic infections, responded transcriptionally to cell wall-active antibiotics frequently used in root canal therapy, according to the GEO2R analysis of the GSE45306 dataset. Benjamini and Hochberg correction for false discovery rate control in multiple testing was a statistical processing step. To provide robustness in detecting antibiotic-induced alterations, Limma precision weights were applied to account for variability in expression across samples. When the adjusted p-value was less than 0.05 and the $|\log_2$ fold change$|$ was greater than 1.5, the expression changes were deemed significant.

Quality assessments on the microarray data validated correct normalization through analysis of probe intensity patterns.



Figs 1A to C: Volcano plot analysis displaying discrete groups of genes with differential expression

Standard degradation analysis was part of the RNA quality evaluation process to guarantee sample integrity, which is essential for accurately reflecting endodontic stress responses. By looking at expression distributions and checking for possible batch effects, sample quality control was carried out.

In order to evaluate sample grouping patterns and validate the clustering of antibiotic-treated vs control samples, which represented various treatment conditions pertinent to endodontic antimicrobial methods, principal component analysis (PCA) was conducted. We compared the gene expression of each antibiotic treatment group to that of untreated controls. This involved measuring fold changes, identifying important genes, and determining the direction of expression. Genes associated with cell wall functions, stress reactions, and membrane alterations were the main focus of the analysis. By using this method, we were able to determine the gene-level responses of *E. faecalis* to several cell wall antibiotics.

Bacitracin-responsive Genes in *E. faecalis* Relevant to Endodontics

Significant alterations in gene expression patterns were observed in the transcriptional response of *E. faecalis* OG1RF to Bacitracin therapy. Significant changes were shown by an adjusted *p*-value < 0.05, and the volcano plot analysis revealed clear upregulation and downregulation of particular genes (Fig. 1A). With $\log_2$ fold changes of 11.542 and 11.074, respectively, lpdA and aceF displayed the largest expression changes among the highly elevated genes. The pyruvate dehydrogenase complex components encoded by these genes indicate significant metabolic changes in response to Bacitracin stress. Ade ($\log_2$FC: 3.343) and mtaD1 ($\log_2$FC: 2.803) were two more genes that were noticeably increased, suggesting that nucleotide metabolism pathways were activated.

Several transport-related genes, such as brnQ ($\log_2$FC: 2.563), fhuG ($\log_2$FC: 1.998), and feoB ($\log_2$FC: 1.824), were found to be significantly upregulated, according to the study (Table 1A). This implies increased membrane transport activity when exposed to Bacitracin. With $\log_2$ fold changes ranging from 1.5 to 1.7, the purine metabolism genes (*purR, purN, purD, purM,* and *purF*) consistently showed upregulation, suggesting enhanced nucleotide production.

On the other hand, a number of genes displayed notable downregulation. Strong downregulation was seen in the dlt operon genes (*dltB, dltC,* and *dltD*), with $\log_2$ fold changes ranging from –2.7 to –3.3. These genes regulate teichoic acid D-alanylation, indicating responses to cell wall modification. Modified cell wall stress response mechanisms were indicated by the moderate downregulation of the vanRG-vanSG two-component system genes ($\log_2$FC: –2.412 and –1.63).

Table 1A: Top upregulated genes in *E. faecalis* following bacitracin treatment compared to the control group, identified from the GSE45306 dataset

Sl. No.	*Gene ID*	*Gene symbol*	*Log_2 (fold change)*
1.	HMPREF0345_2366	*lpdA*	11.542
2.	HMPREF0345_2367	*aceF*	11.074
3.	HMPREF0345_2501	*ade*	3.343
4.	HMPREF0345_2500	*mtaD1*	2.803
5.	HMPREF0345_2728	*brnQ*	2.563
6.	HMPREF0345_1673	*xpt*	2.452
7.	HMPREF0345_0149	*ulaB*	2.073
8.	HMPREF0345_2777	*fhuG*	1.998
9.	HMPREF0345_0540	*nhaC-1*	1.865
10.	HMPREF0345_0611	*feoB*	1.824

Table 1B: Top downregulated genes in Control vs Bacitracin antibiotics treated subgroup filtered through GSE45306 dataset

Sl. No.	*Gene ID*	*Gene symbol*	*Log_2 (fold change)*
1.	HMPREF0345_1821	*phnC-2*	−3.864
2.	HMPREF0345_2422	*mgtA-2*	−3.67
3.	HMPREF0345_1350	*dltD*	−3.304
4.	HMPREF0345_1348	*dltB*	−2.926
5.	HMPREF0345_1678	*clpB*	−2.754
6.	HMPREF0345_1349	*dltC*	−2.721
7.	OG1RF_0193	*vanRG*	−2.412
8.	HMPREF0345_2145	*hslU*	−2.371
9.	HMPREF0345_2144	*hslV*	−2.313
10.	HMPREF0345_2146	*codY*	−2.28

Table 1C: Top upregulated (with +logFC values) and downregulated genes (with –logFC values) in Control vs Cephalothin antibiotics treated subgroup filtered through GSE45306 dataset

Sl. No.	*Gene ID*	*Gene symbol*	*Log_2 (fold change)*
1.	HMPREF0345_2366	*lpdA*	11.394
2.	HMPREF0345_1610	*guaD*	2.504
3.	OG1RF_0050	*recN*	2.342
4.	HMPREF0345_2728	*brnQ*	1.54
5.	HMPREF0345_1387	*recX*	1.477
6.	HMPREF0345_0492	*actP1*	−2.496
7.	HMPREF0345_2422	*mgtA-2*	−2.547
8.	HMPREF0345_0491	*copY*	−2.981
9.	HMPREF0349_2977	*copY*	−3.308
10.	HMPREF0348_2338	*copY*	−3.522

Significant downregulation was observed in stress response genes such as *groEL* (log_2FC: −1.321), *dnaK* (log_2FC: −1.927), and *clpB* (log_2FC: −2.754). Genes involved in cell division and cell wall synthesis, such as murG (log_2FC: −1.513) and divIVA (log_2FC: −1.67), also showed decreased expression. Changes in magnesium transport systems are suggested by the significant downregulation of mgtA-2 (log_2FC: −3.67) (Tables 1B and C).

The expression data demonstrated several cellular mechanisms by which *E. faecalis* reacted to Bacitracin. Genes involved in energy metabolism have increased, while genes involved in cell wall modification have decreased. These modifications imply that the bacteria modify their biological functions to cope with stress brought on by Bacitracin. Our knowledge of how *E. faecalis* adjusted to this antibiotic was further enhanced by the changed expression of genes involved in stress response and transport systems. These results suggested that certain genes and pathways may be targeted to increase the efficacy of Bacitracin in treating *E. faecalis* infections.

Differential Gene Expression Analysis of the Control vs Cephalothin Antibiotic-treated Subgroup

Compared to the Bacitracin treatment group, the *E. faecalis* OG1RF transcriptional response to Cephalothin showed fewer but substantial changes in gene expression. With an adjusted *p*-value < 0.05 as the significance criterion, the volcano plot displays discrete groups of genes with differential expression (Fig. 1B).

The *lpdA* gene was the most significantly altered, exhibiting significant overexpression with a log_2 fold change of 11.394. Under Cephalothin stress, this gene, which codes for lipoamide dehydrogenase, indicates significant alterations in cellular energy metabolism. GuaD (log_2FC: 2.504), which is involved in purine metabolism, and recN (log_2FC: 2.342), which is involved in DNA repair pathways, showed the next notable increase. While recX, another DNA repair gene, showed minor upregulation (log_2FC: 1.477), the branched-chain amino acid transport gene *brnQ* showed considerable overexpression (log_2FC: 1.54) (Tables 2 and 3).

A distinct pattern centered on metal transport and copper homeostasis was evident in the downregulated genes. With log_2 fold changes of −2.981, −3.308, and −3.522, three copies of copY, which encode copper-responsive transcriptional regulators, showed significant downregulation. This coordinated reduction points to important modifications in processes that rely on copper. Additionally, there was a decrease in expression of the copper transport gene *copZ* (log_2FC: −1.523) (Table 1C).

Magnesium transport genes *mgtA-1* and *mgtA-2* both exhibited considerable downregulation (log_2FC: −1.956 and −2.547), indicating a notable impact on metal transport systems. The hemolysin III family protein gene *hlyIII* showed a moderate reduction (log_2FC: −1.416), whereas the copper-transporting P-type ATPase actP1 showed significant downregulation (log_2FC: −2.496).

The Bacitracin reaction and the expression pattern under Cephalothin stress are very different. Although lpdA was strongly upregulated by both antibiotics, Cephalothin administration produced a more targeted response that mostly affected genes related to metal homeostasis. Compared to Bacitracin, Cephalothin appears to elicit a more focused cellular response, as seen by the smaller number of significantly changed genes. These results demonstrate how exposure to Cephalothin alters the energy metabolism and metal transport mechanisms of *E. faecalis*. The information suggests that Cephalothin's ability to cure *E. faecalis* infections may be improved by targeting particular cellular functions.

Differential Gene Expression Analysis of the Control vs Vancomycin Antibiotic-treated Subgroup

Significant alterations in several cellular pathways were found in our investigation of the transcriptional response of *E. faecalis* OG1RF to Vancomycin therapy. There were more downregulated genes than upregulated ones, according to the volcano plot analysis, with a *p*-value < 0.05 indicating statistical significance. Changes in expression were grouped around particular cellular processes, particularly those related to stress responses, transport systems, and cell wall formation (Fig. 1C).

Genes involved in metabolic pathways showed the most significant changes in expression. With log_2 fold changes of 10.454

and 9.501, two pyruvate dehydrogenase complex components, lpdA and aceF, demonstrated remarkable overexpression. This implies a significant metabolic shift in response to Vancomycin toxicity.

Notable increases were also seen in transport systems, most notably the branched-chain amino acid transporter brnQ, which increased by 3.652 log_2 fold. Significant overexpression of other transport genes, such as *mscL, treB*, and *ulaB*, indicated active membrane transport changes (Table 2A).

Table 2A: Top upregulated genes in Control vs Vancomycin antibiotics treated subgroup filtered through GSE45306 dataset

Sl. No.	*Gene ID*	*Gene symbol*	*Log_2 (fold change)*
1.	HMPREF0345_2366	*lpdA*	10.454
2.	HMPREF0345_2367	*aceF*	9.501
3.	HMPREF0345_2728	*brnQ*	3.652
4.	HMPREF0345_2860	*purR*	2.685
5.	HMPREF0345_0205	*mscL*	2.455
6.	HMPREF0345_1720	*treB*	2.451
7.	HMPREF0345_0149	*ulaB*	2.233
8.	HMPREF0345_2501	*ade*	2.14
9.	OG1RF_0050	*recN*	2.085
10.	HMPREF0345_1424	*mgtE*	2.069

Table 2B: Top downregulated genes in Control vs Vancomycin antibiotics treated subgroup filtered through GSE45306 dataset

Sl. No.	*Gene ID*	*Gene symbol*	*Log_2 (fold change)*
1.	OG1RF_0191	*vanY*	−4.925
2.	HMPREF0345_2422	*mgtA-2*	−4.776
3.	HMPREF0345_1349	*dltC*	−4.441
4.	HMPREF0345_2144	*hslV*	−4.3
5.	HMPREF0345_2145	*hslU*	−3.976
6.	HMPREF0345_2146	*codY*	−3.797
7.	HMPREF0345_1348	*dltB*	−3.675
8.	HMPREF0345_1350	*dltD*	−3.663
9.	HMPREF0345_0157	*rpmG-4*	−3.634
10.	HMPREF0345_2105	*hlyIII*	−3.352

Genes linked to cell walls displayed an unexpected expression pattern. The components of the mur operon were among the many peptidoglycan manufacturing genes that showed a considerable decline. MurG and murD displayed comparable downregulation patterns; however, MurB displayed a −2.631 log_2 fold drop. Even more pronounced declines were seen in the dlt operon genes, which are involved in teichoic acid modification; dltC reached −4.441 log_2 fold change. These coordinated declines point to significant structural changes in the cell wall. Surprisingly, the Vancomycin resistance gene cluster was downregulated. With a −4.925 log_2 fold change, the *vanY* gene had the most significant decline, but the regulatory genes *vanRG* and *vanSG* both displayed comparable declines. Genes related to cell division also declined; ftsZ, ftsQ, and ftsA all displayed coordinated downregulation, indicating that the division mechanisms were changed by Vancomycin exposure (Table 2B).

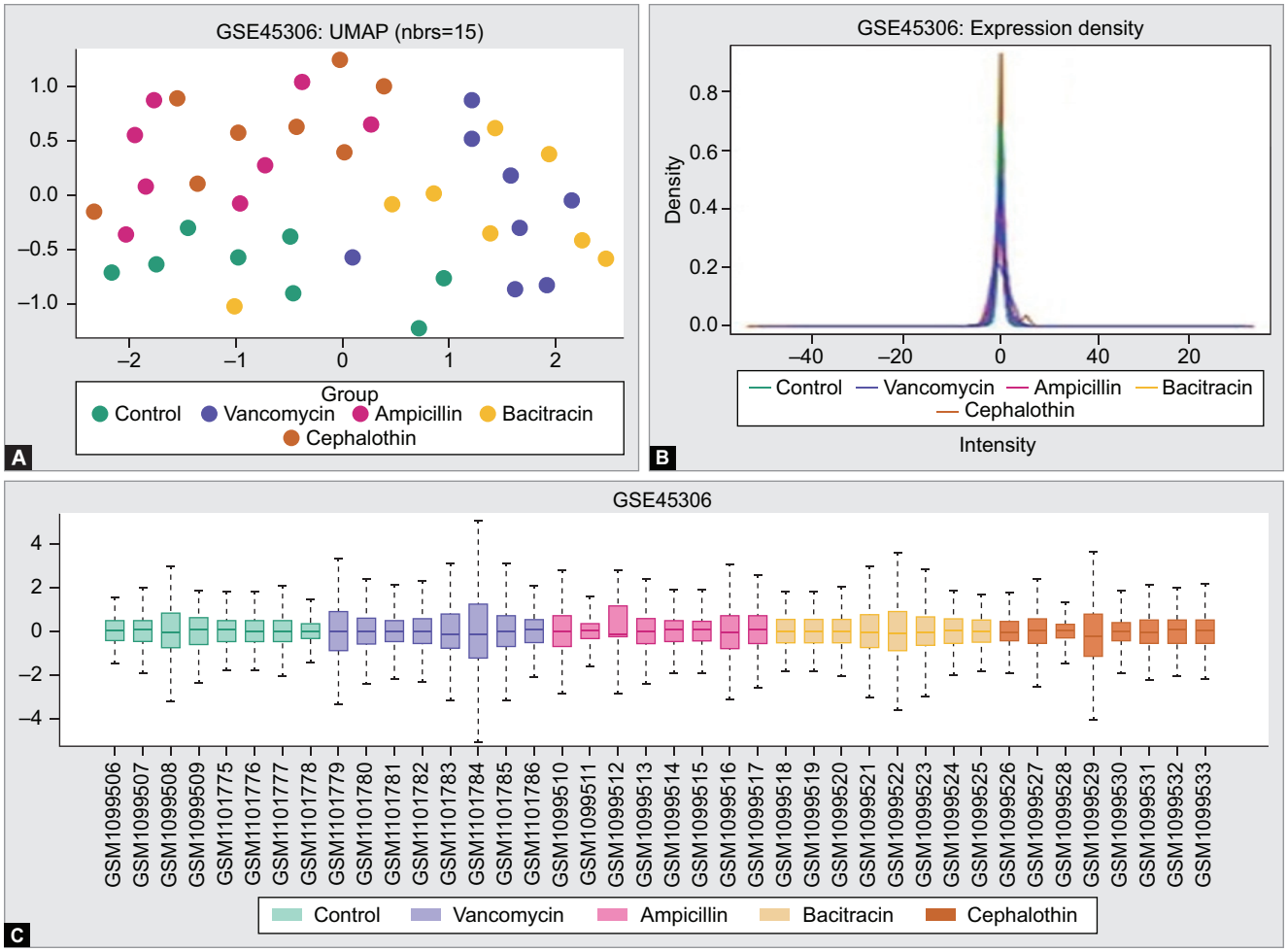
Enterococcus faecalis revealed heterogeneous regulation of stress response genes during Vancomycin stress. While major stress-related proteins, including HslU, HslV, ClpB, and GroEL, were significantly downregulated, the DNA repair gene recN was widely increased. Additionally, the global regulator CodY dropped, indicating significant changes in metabolism. Different gene clusters were revealed by volcano plot analysis: Downregulated stress and cell wall modification genes were found in the upper left quadrant, whereas metabolic and transport genes were elevated in the upper right. These summaries demonstrate *E. faecalis* adaptation through variations in cell wall production, membrane transport, and stress regulation, pointing to coordinated transcriptional alterations. This may help guide future therapeutic approaches and provide insight into how bacteria survive under the influence of antibiotics.

Uniform Manifold Approximation and Projection (UMAP) Plot, Expression Density, and Boxplot Analysis of Differential Gene Expression in *E. faecalis* Control vs Antibiotic-treated Subgroups Relevant to Endodontic Infections

Different transcriptional patterns in response to several cell wall-targeting antibiotics are shown by the gene expression analysis of the GSE45306 dataset. When comparing control samples with treatments of Vancomycin, Ampicillin, Bacitracin, and Cephalothin,

Table 3: Distribution of DEGs with their log_2FC values under control and antibiotic treatments (Vancomycin, Bacitracin, and Cephalothin) in GSE45306 dataset (padj <0.05)

Gene ID	*Gene symbol*	*Log_2 (fold change) (Control vs Vancomycin)*	*Log_2 (fold change) (Control vs Bacitracin)*	*Log_2 (fold change) (Control vs Cephalothin)*
HMPREF0345_0128	Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F2	−6.205	13.689	8.902
HMPREF0345_0132	Hypothetical protein	−1.337	7.48	6.849
HMPREF0345_0300	Hypothetical protein	−1.626	4.26	4.179
HMPREF0345_0951	Hypothetical protein	−2.532	9.586	6.863
HMPREF0345_1080	Hypothetical protein	−2.177	4.721	4.272
HMPREF0345_1241	Integral membrane protein	−3.971	5.405	3.915
HMPREF0345_2255	Hypothetical protein	−3.66	10.451	5.847
HMPREF0345_2888	Hypothetical protein	−2.245	8.308	6.125
HMPREF0346_1644	Hypothetical protein	−1.938	3.75	4.444
HMPREF0346_2076	Hypothetical protein	−2.966	10.106	5.396
HMPREF0348_0317	Hypothetical protein	−2.026	5.125	5.645



Figs 2A to C: Expression density curves and clustering patterns suggesting common transcriptional programs across various treatments

the box plot analysis displayed variations in expression across several genes. While some genes exhibited more drastic changes under particular antibiotic treatments, the majority of genes exhibited modest expression changes between –2 and +2 $\log_2$ fold change. Genes with steady expression levels are represented by a distinctive peak in the expression density distribution that is close to zero intensity. The distribution pattern of control samples differed slightly from that of groups treated with antibiotics. Interestingly, Bacitracin and Cephalothin treatments exhibited minor differences in their distribution patterns, especially at higher expression intensities, whereas Vancomycin and Ampicillin treatments provided expression density curves that were identical (Fig. 2).

Treatment-specific clustering of data is shown by UMAP analysis, demonstrating distinct transcriptional fingerprints for every antibiotic. A distinct baseline expression pattern is established by the tight clustering of control samples. Samples treated with Vancomycin cluster apart from samples treated with other therapies, indicating distinct transcriptional reactions to this antibiotic. A clear yet somewhat overlapping cluster with other antibiotics is produced by Ampicillin therapy, suggesting some common response mechanisms. The clustering pattern of the Bacitracin-treated samples is more dispersed, indicating that the transcriptional responses of the duplicates differ. Treatment with Cephalothin produces a distinct cluster that is distinct from other antibiotics and indicates distinct alterations in gene expression. Both antibiotic-specific reactions and some common transcriptional programs across various treatments are suggested by these clustering patterns (Fig. 2).

The box plot examination of gene-specific responses revealed intriguing trends. While some genes exhibit treatment-specific changes, others retain constant expression throughout treatments. The box plot whiskers, which indicate the expression variability within each treatment group, pointed to dynamic transcriptional responses. Under particular antibiotics, some genes exhibit notably high variance, suggesting varying sensitivity to different cell wall stresses. Although each antibiotic causes notable transcriptional alterations, the overall expression landscape indicates that the response patterns vary depending on the therapy. Reliable expression measurements and appropriate data normalization are shown by the density plot's strong, symmetrical peak. While identifying possible mechanistic commonalities between various antibiotics, UMAP clustering successfully divides treatment groups.

According to these results, *E. faecalis*, a major cause of endodontic infections, reacts to antibiotics that target the cell wall through different transcriptional processes while retaining some response components in common. In the context of root canal

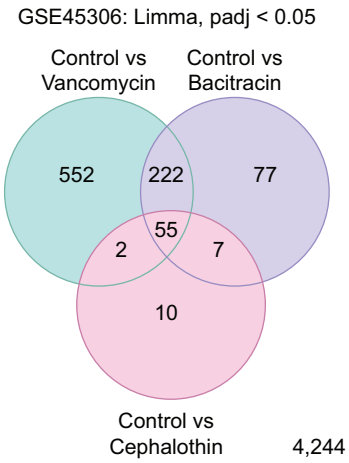


Fig. 3: Expression pattern of the cell envelope-associated transcriptional attenuator

disinfection, the distinct division of treatment groups in UMAP analysis, along with comprehensive gene expression patterns, offers important insights into bacterial responses unique to antibiotics. This information helps us better understand how bacteria adapt to various cell wall stresses, which is essential for overcoming *E. faecalis's* persistence in the root canal. The development of more focused and efficient antibacterial techniques in endodontic therapy may be influenced by these findings.

Comparative Analysis of Upregulated and Downregulated Genes in *E. faecalis* Control vs Antibiotic-treated Subgroups Relevant to Endodontic Infections

Complex transcriptional responses of *E. faecalis*, a pathogen essential to chronic endodontic infections, are shown by comparing the DEGs between the control and antibiotic-treated groups. Vancomycin, Bacitracin, and Cephalothin treatments exhibited different and overlapping gene sets, according to the Venn diagram analysis, with significance at $p < 0.05$. Compared to other antibiotics frequently used in root canal treatments, Vancomycin treatment produced the greatest number of distinct DEGs (552), indicating a wider transcriptional response. Upon analyzing the patterns of gene expression, several genes that are pertinent to endodontic disinfection techniques exhibited significant variations across various antimicrobial treatments.

The expression pattern of the cell envelope-associated transcriptional attenuator LytR-CpsA-Psr (subfamily F2) was striking. This gene exhibited notable upregulation with Bacitracin (log_2FC: 13.689) and Cephalothin (log_2FC: 8.902) but considerable downregulation with Vancomycin (log_2FC: −6.205). For these medicines, the conflicting regulation points to distinct cell wall stress response mechanisms (Fig. 3 and Table 3).

According to the data, 55 genes are shared by all three antibiotic treatments, and 222 genes were shared by Vancomycin and Bacitracin responses. Ten distinct genes were impacted by Cephalothin treatment, while 77 genes were uniquely regulated by Bacitracin treatment. Differential regulation patterns were consistent across a number of putative proteins. For instance, HMPREF0345_0132 exhibits considerable upregulation with Cephalothin (log_2FC: 6.849) and Bacitracin (log_2FC: 7.48), but moderate downregulation with Vancomycin (log_2FC: –1.337).

The expression of an integral membrane protein (HMPREF0345_1241) varied significantly with treatment: It is downregulated under Vancomycin (log_2FC: –3.971) and upregulated with Cephalothin (log_2FC: 3.915) and Bacitracin (log_2FC: 5.405). Important membrane modifications in response to various cell wall stresses are suggested by this pattern. Consistent patterns among hypothetical proteins can be seen in the data. Both HMPREF0345_2255 and HMPREF0345_2888 exhibit significant overexpression with Bacitracin and Cephalothin but considerable downregulation with Vancomycin. Antibiotic stress responses are probably significantly influenced by these unidentified proteins (Fig. 3 and Table 3).

Seven genes responded differently to Vancomycin but similarly to Bacitracin and Cephalothin treatments. Vancomycin and Cephalothin responses shared two genes, indicating that their stress response pathways had little in common. Similar trends were seen in the expression variations of hypothetical proteins HMPREF0346_1644 and HMPREF0346_2076, which exhibited substantial upregulation with other antibiotics and modest downregulation by Vancomycin. This raised the possibility of functional connections between these unidentified proteins in antibiotic stress reactions.

These results showed that each antibiotic also activates distinct transcriptional pathways, even when cell wall-targeting antibiotics, such as those employed in endodontic cleaning, cause certain common stress responses in *E. faecalis*. Vancomycin is frequently used to treat endodontic infections that are resistant to other therapies and the high frequency of Vancomycin-specific responses points to distinct cellular adaptations to this antibiotic. Despite having different molecular targets, *E. faecalis* exhibits common cellular adaptation mechanisms that enable the organism to survive in the hostile root canal environment, as seen by the shared responses between Bacitracin and Cephalothin treatments.

Discussion

Because of their growing patterns of antibiotic resistance, *E. faecalis* infections pose serious therapeutic problems. According to recent surveillance statistics, *E. faecalis* is responsible for around 25% of hospital-acquired infections worldwide, with severe bacteremia cases having a 35% fatality rate.[38,39] By exposing intricate biochemical reactions that differ greatly between various antibiotic treatments, our transcriptional study contributes to our knowledge of how *E. faecalis* adapts to cell wall-targeting drugs. With 552 genes that are specifically regulated, the broad transcriptional response to Vancomycin points to complex adaptive mechanisms.

These genes are involved in a variety of physiological functions, such as stress responses, membrane transport, and cell wall construction.[40] The downregulation of several genes involved in peptidoglycan synthesis was particularly noteworthy, suggesting a deliberate decrease in antibiotic targets. Significant alterations in the genes governing the composition of cell membranes were also a part of the Vancomycin response, indicating adaptive tactics that went beyond straightforward cell wall alterations.[41]

Different cellular tactics against these antibiotics are indicated by the more restricted responses to Cephalothin (10 genes) and Bacitracin (77 genes). Significant alterations in stress response pathways and membrane protein expression were brought on by Bacitracin therapy. Cephalothin, on the other hand, produced a very selective reaction that was primarily directed toward genes involved in cell wall formation.[42] These clear trends imply

that, depending on the particular antibiotic challenge, *E. faecalis* uses specific resistance pathways. Fundamental stress response mechanisms were represented by a core group of 55 genes that exhibited consistent regulation across all antibiotic treatments.[43]

Proteins involved in cellular metabolism, stress response, and cell wall maintenance are encoded by these genes. Their crucial involvement in bacterial survival under antibiotic stress is shown by the steady pattern of regulation. These numerous genes encode proteins that have not yet been identified, suggesting the possibility of new resistance mechanisms.[44] The behavior of the transcriptional attenuator belonging to the LytR-CpsA-Psr family was especially intriguing. Its function as a molecular switch in stress response pathways is suggested by its differential regulation, which is downregulated with Vancomycin but upregulated with Bacitracin and Cephalothin.[45] This discovery sheds light on how *E. faecalis* regulates its reaction to various forms of cell wall stress.

Several putative proteins exhibiting significant differential expression were found by our investigation. Across treatments, three proteins (HMPREF0345_0132, HMPREF0345_0300, and HMPREF0345_0541) showed consistent patterns of regulation.[46] These proteins most likely stand for hitherto unidentified elements of stress response pathways. Finding them creates fresh opportunities for therapeutic intervention. Significant metabolic adjustments were demonstrated by the transcriptional responses. Modified energy generation supporting stress reactions is indicated by changes in electron transport chain proteins and Adenosine triphosphate (ATP) synthase components.[47] Gaining insight into these metabolic alterations may help identify new treatment targets. Strategic changes in bacterial growth patterns under antibiotic stress are suggested by the coordinated regulation of growth regulatory genes.

Improved cellular efflux capabilities were revealed by analysis of membrane transport systems, especially in cells treated with Vancomycin.[48] Increased antibiotic export pathways are suggested by the upregulation of particular ABC transporters. By lowering intracellular antibiotic concentrations, these modifications in membrane transport most likely contribute to antibiotic resistance. Despite having different antibiotic modes of action, the same response between Cephalothin and Bacitracin treatments, which involves seven genes, indicate common stress response pathways.[49] Stress response factors and membrane-associated proteins are the main products of these common genes. Gaining knowledge of these typical response pathways may aid in the creation of more successful combination treatments.

Current combination therapy techniques are supported by the small overlap between Cephalothin responses (two genes) and Vancomycin.[50] According to this research, focusing on several cell wall production routes may help prevent the emergence of resistance. Additionally, the different response patterns suggest that successive antibiotic treatment approaches may be beneficial.

Our research showed that the synthesis and modification pathways of cell wall precursors are intricately regulated. Changes in peptidoglycan cross-linking mechanisms are suggested by notable variations in D-alanine-D-alanine ligase activity.[51] These changes probably improve bacterial survival and decrease antibiotic binding. Particularly intriguing behavior was displayed by the integral membrane protein HMPREF0345_1241, which exhibited conflicting patterns of regulation between Vancomycin and other treatments.[51] Its function as a molecular switch in cellular stress responses is suggested by this differential regulation. These membrane proteins frequently act as stress sensors, coordinating subsequent adaptive mechanisms, according to recent structural investigations. Under antibiotic stress, *E. faecalis* exhibited a complex adaptive response, according to analysis of growth control genes.

Coordinated shifts in the expression of genes linked to cell division imply that the bacterium actively modifies its rate of proliferation to strike a balance between energy requirements and survival. This compromise probably aids in resource conservation for necessary stress reactions. Notably, genes related to peptidoglycan production and cell wall remodeling showed modulation unique to antibiotics. These changes suggest that *E. faecalis* adapts the structure of its cell envelope to the kind of antibiotic it comes into contact with, probably in order to preserve structural integrity and lessen harm.

At the same time, we saw a notable change in the genes involved in energy metabolism. The bacteria reallocate energy resources during stress, perhaps to support resistance mechanisms like efflux pumps or protective protein synthesis, as evidenced by the differential expression of ATP synthase subunits and electron transport chain components. Interestingly, the expression of a number of membrane-associated proteins varied depending on the treatment condition, suggesting that these proteins may play a part in antibiotic resistance or sensing.

In order to decrease bacterial defenses, adjuvant medicines may find these proteins to be interesting targets. A multi-layered adaptation strategy is suggested by the transcriptional landscape. The resilience of the organism is influenced by changes in gene expression that seem to integrate across pathways involved in stress signaling, membrane transport, and cell wall formation. Clinically speaking, knowing how *E. faecalis* reacts to various antibiotics at the transcriptional level could help create more specialized and efficient treatment plans. Finding shared pathways that are triggered by several medications, in particular, may assist in avoiding or lessening cross-resistance.

Going forward, more research is needed in a few areas. First, the previously unidentified resistance factors might be revealed by characterizing the putative proteins that were highlighted in our investigation. Second, further identification of master regulators with superior therapeutic potential may be aided by an analysis of the regulatory networks controlling these transcriptional changes. Lastly, investigating how various stress response systems interact may also assist in identifying the weaknesses that combination medicines may be able to better exploit. Our results further highlight how important membrane transport mechanisms are. *Enterococcus faecalis* appears to employ sophisticated methods to regulate intracellular drug concentrations, as evidenced by the observed modulation of efflux and permeability-related genes.

Targeting these particular systems, especially when combined with already available antibiotics, may improve treatment efficacy and lessen *E. faecalis* resistance mechanisms.

Conclusion

In conclusion, our thorough transcriptional study showed that *E. faecalis*, a significant pathogen in endodontic infections, endures and survives cell wall-targeting antibiotics frequently employed in root canal therapy by using extremely adaptable and antibiotic-specific molecular mechanisms. In contrast to the more restricted adaptations to Bacitracin and Cephalothin, the organism's extensive and widespread response to Vancomycin

underscores its developing resilience and potential for antibiotic resistance in the root canal setting. The identification of multiple previously unidentified proteins and a universal collection of stress response genes points to new targets for therapeutic intervention in endodontic treatment.

Further evidence that *E. faecalis* may dynamically rearrange its cellular structure and metabolism to withstand various antibiotic pressures encountered during root canal disinfection comes from our study's differential regulation of crucial genes involved in membrane and cell wall production. In the context of endodontic care, these observations offer crucial insights into the mechanisms underlying antibiotic resistance in this significant endodontic pathogen and could direct the creation of more focused, efficient therapeutic approaches to counteract the emergence of multidrug resistance in *E. faecalis*.

Clinical Significance

During endodontic treatment, the antibiotic-specific transcriptional regulators, such as *E. faecalis*-membrane-associated sensors and members of the LytR-CpsA-Psr family, undergo adaptive responses. Thus, to enhance root canal disinfection and fight antibiotic resistance, these molecular targets are considered the most promising aspects of acumen.

Authors' Contributions

Conceptualization, Methodology, Formal Analysis, and Resources: Nezar Boreak, Wafa Faqehi, Raghad Essa Ageeli; Investigations, Data Curation, and Software: Hassan Abdulaziz Sumayli, Taym Hadi Ahmed Khormi, Aeshah Mohammed Abuhashem, Abrar Tairy, Ali Yehia Jurdi Mobarki; Visualization and Validation: Tariq Mohammed Qassadi, Calvin Bennion and Shilpa Bhandi; Project Administration: Nezar Boreak, Wafa Faqehi and Shilpa Bhandi. Writing – Original Draft: Nezar Boreak, Wafa Faqehi, Raghad Essa Ageeli, Hassan Abdulaziz Sumayli, Taym Hadi Ahmed Khormi, Aeshah Mohammed Abuhashem and Abrar Tairy; Writing – Review and Editing: Ali Yehia Jurdi Mobarki; Tariq Mohammed Qassadi, Calvin Bennion and Shilpa Bhandi.

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