

# Differences of protein expression in enterococcus faecalis biofilm during resistance to environmental pressures

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## Abstract.

**BACKGROUND:** Enterococcus faecalis biofilm was frequently found on the failed treated root canal wall, which survived by resisting disinfectant during endodontic treatment. Many researches have been conducted to explore the mechanisms of persistence of this pathogen in unfavorable conditions. However, no comprehensive proteomics studies have been conducted to investigate stress response in Enterococcus faecalis caused by alkali and NaOCl.

**OBJECTIVE:** Enterococcus faecalis (E.f) has been recognized as a main pathogen of refractory apical periodontitis, its ability to withstand environmental pressure is the key to grow in the environment of high alkaline and anti-bacterial drug that causes chronic infection in the root canal. This study aims to focus on the protein expression patterns of E.f biofilm under extreme pressure environment”.

**METHODS:** Enterococcus faecalis biofilm model was established in vitro. Liquid Chromatograph-Mass Spectrometer (LC-MS/MS)-based label free quantitative proteomics approach was applied to compare differential protein expression under different environmental pressures (pH 10 and 5% sodium hypochlorite (NaOCl)). And then qPCR and Parallel Reaction Monitoring Verification (PRM) were utilized to verify the consequence of proteomics.

**RESULTS:** The number of taxa in this study was higher than those in previous studies, demonstrating the presence of a remarkable number of proteins in the groups of high alkaline and NaOCl. Proteins involved in ATP-binding cassette (ABC) transporter were significantly enriched in experimental samples. We identified a total of 15 highly expressed ABC transporters in the high alkaline environment pressure group, with 7 proteins greater than 1.5 times.

**CONCLUSIONS:** This study revealed considerable changes in expression of proteins in E.f biofilm during resistance to environmental pressures. The findings enriched our understanding of association between the differential expression proteins and environmental pressures.

Keywords: Enterococcus faecalis, biofilm, proteomics, alkaline stress, sodium hypochlorite

## 1. Introduction

Enterococcus are recognized common bacteria that causes infections in various body parts such as

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urinary tract, intra-abdomen and pelvic regions, surgical sites and oral cavity [1]. *Enterococcus faecalis* is one of the most usual species that can be detected in the root canals of teeth where root canal treatment failed [2]. It is an important microorganism for root canal persistent infection and reinfection. In previously treated cases, the prevalence of *Enterococcus faecalis* ranged from 45.8% to 79.5% [3,4,5].

About 80% microbial infections were based on the format of biofilm. Biofilm was resistant to antibiotics and disinfectant which may depend on its property of physical barrier [6]. *Enterococcus faecalis* biofilm was frequently found on the failed treated root canal wall, which survived by resisting disinfectant during endodontic treatment [7]. Calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) and sodium hypochlorite (NaOCl) are common disinfection medications during root canal treatment. Calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) is the most frequently used intracanal medication due to its strong alkaline property. The destruction of phospholipids, structural components of the cellular membrane, can be caused by the induction of lipid peroxidation by hydroxide ions. Genes are destroyed when hydroxide ions react with bacterial DNA and cause the denaturation of the strands [8]. Sodium hypochlorite (NaOCl) is the most widely used irrigation fluid, usually up to 5% concentration, during endodontic treatment because of its effective antimicrobial and tissue-dissolving properties [9]. NaOCl of 5.25% could kill *Enterococcus faecalis* in planktonic state effectively, but *Enterococcus faecalis* biofilm could still grow when concentration of NaOCl was low, such as 1%, even during the alkaline dressing [10].

Many researches have been conducted to explore the mechanisms of persistence of this pathogen in unfavorable conditions. Although some of the previous genetic screening studies have suggested the gene regulation responsible for the adaption process of these bacteria to alkaline stress conditions [11, 12], the actual expression of functional proteins is unclear. Moreover, previous proteomics studies on *Enterococcus faecalis* have been mainly focused on the differential protein expression patterns in planktonic *Enterococcus faecalis* cells exposed to antibiotics or to some stressful conditions [13,14,15, 16,17].

However, no comprehensive proteomics studies have been conducted to investigate stress response in *Enterococcus faecalis* caused by alkali and NaOCl. In the current study, we examine the protein expression profiles of *Enterococcus faecalis* biofilms in these stresses by employing a tandem-mass-tag(TMT)-based quantitative proteomics analysis, which may ultimately contribute to exploring the possible mechanisms governing biofilm formation of *Enterococcus faecalis* under extreme pressure environment and obtaining the evidence on development of strategies for the prevention of *Enterococcus faecalis* infections and improve root canal treatment strategies.

## 2. Materials and method

### 2.1. Bacterial strains and growth conditions

*Enterococcus faecalis* standard strain ATCC33186 was inoculated into tryptic soy broth (TSB) containing 1.7% tryptone, 0.3% polypeptone, 0.1% yeast extract (Sangon, Shanghai, China), 0.5% NaCl, 0.25% glucose, and 0.25% dipotassium phosphate at 37°C overnight. We identified the biofilm after the bacterial strain inoculated from standard bacterial and we are sure the bacterial colony was not contaminated by other species of bacterial or microorganisms.

The bacterial biofilms were performed in three environments including 5% NaOCl, pH 10 and normal condition and labeled as NaOCl group, ALK group and N group, with three samples in each group.

## 2.2. Samples preparation and extraction

After the pellets of *Enterococcus faecalis* biofilm proteins were extracted, all protein samples were taken about 20  $\mu\text{g}$  and added 5 times buffer (250 mM Tris-HCl (pH6.8), 10% (W/V) SDS, 0.5% (W/V) BPB, 50% (V/V) glycerol, 5% DTT). After mixing well, the mixture was boiled for 5 min, and centrifuged to take the supernatant, and performed 12% SDS-PAGE electrophoresis (150 V, 45 min).

## 2.3. Protein digestion and TMT 10-plex labeling

Protein digestion was performed according to the FASP procedure, as previously [18]. After protein digestion with Trypsin in FASP method, TMT labeling was performed according to the manufacturer's instructions (Thermo Scientific).

Peptide Fractionation with High-pH reversed-phase chromatography separation.

The TMT-labelled peptides were subjected to High-pH Reversed-Phase Fractionation in 1100 Series HPLC Value System (Agilent) equipped with a Gemini-NX (Phenomenex, 00F-4453-E0) column ( $4.6 \times 150$  mm, 3  $\mu\text{m}$ , 110Å) [19]. All samples were stored at  $-80^\circ\text{C}$  until LC-MS/MS analysis.

## 2.4. LC-MS/MS analysis

The TMT-labeled samples were analyzed using Easy-nLC nanoflow HPLC system connected to Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), The sequential separation of peptides on Thermo Scientific EASY trap column ( $100 \mu\text{m} \times 2$  cm, 5  $\mu\text{m}$ , 100 Å, C18) and analytical column ( $75 \mu\text{m} \times 25$  cm, 5  $\mu\text{m}$ , 100 Å, C18) was achieved with a segmented 2 h gradient from Solvent A (0.1% formic acid in water) to 35% Solvent B (0.1% formic acid in 100% ACN) for 100 min, followed by 35–90% Solvent B for 12 min and then 90% Solvent B for 8 min. The mass spectrometer was operated in positive ion mode, For the Orbitrap Fusion mass spectrometer, the automatic gain control (AGC) targets were  $4e5$  for full scan and  $1e5$  for MS/MS scan, respectively. Full MS spectra with an  $m/z$  range of 375–1800 were acquired with a resolution of 120,000 at  $m/z = 200$  in profile mode. MS/MS acquisition was performed in top speed mode with 3 s cycle time occurred at the high-energy collision dissociation (HCD) collision cell with a normalized collision energy of 32%. The maximum ion injection time was set at 50 ms for the full scan, and 105 ms for the MS/MS scans, The isolation window was 1  $m/z$ .

## 2.5. Protein identification and quantitation

The raw files were analyzed using the Proteome Discoverer 2.4 software (Thermo Fisher Scientific). Search for the fragmentation spectra was performed using the MASCOT search engine embedded in Proteome Discoverer against *Enterococcus faecalis* protein sequence file (uniprot\_E\_Faecalis\_166900\_20200814.fasta, downloaded at 2020/08/14, included 166,900 sequences).

## 2.6. Bioinformatics analysis

### 2.6.1. Selection of significantly differential expressed protein

Student  $t$  test was performed for ALK vs. N, NaOCl vs.N, and NaOCl vs ALK comparisons, respectively. In addition, Fold change of each comparison was calculated in R (version: 3.6.3). Using  $p < 0.05$  that calculated with Student  $t$  test and  $> 1.5$ -fold and  $< 0.67$  (1/1.5)-fold as threshold values for screening statistically significantly differential expressed proteins.

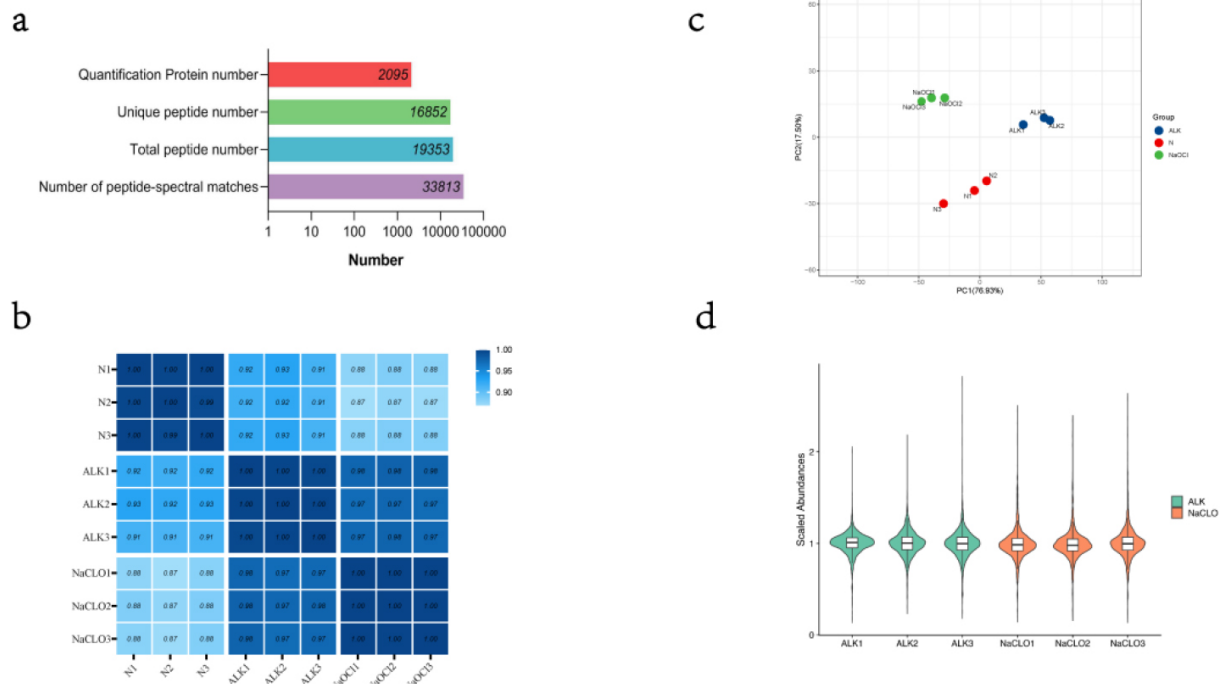


Fig. 1. (a) Summary of mass information. (b) A heatmap of Pearson correlation coefficient. (c) A scatter graph of PC1 and PC2 through principal component analysis. (d) A violin plot of abundances with median normalization. This plot shows the kernel density estimation for distribution of scale abundance of protein and can be superimposed on a box plot. Using the same filling colors indicate technical replicated sample.

### 2.6.2. COG functional analysis

All identified protein sequences were searched against the database of Clusters of Orthologous Groups of proteins (COGs) including 1,781,653 sequences via a standalone blast (version: 2.10.1+) with expect value  $1e-8$ .

### 2.6.3. InterPro annotation and gene ontology functional analysis

InterProscan software (version: 5.45-80.0, <http://www.ebi.ac.uk/Tools/pfa/iprscan/>) with default parameters was used to perform protein sequences searches against InterPro member database for signature identification.

### 2.6.4. Pathway analysis

The protein sequences were submitted to KOBAS (version 3.0) with expect value:  $1e-8$ , database: **efa** (*Enterococcus faecalis* V583), and method: hypergeometric test. The experimental workflow for TMT labeling and analysis was illustrated in Fig. 1.

### 2.6.5. RNA Extraction and Real-time PCR

Total RNA was extracted from 48 h *Enterococcus faecalis* biofilms using Qiagen RNeasy Protect Bacteria Mini Kit, (Magen, Guangzhou, China), according to the manufacturer's instructions, and stored at  $-80^{\circ}\text{C}$  until use. The first strand cDNA was synthesized from  $1\ \mu\text{g}$  total RNA per  $20\ \mu\text{L}$  reaction volume using a First Strand cDNA Synthesis Kit (Toyobo, Kita-ku, Osaka, Japan), according to the

manufacturer's instructions, and as previously described [19]. The real-time quantitative PCR (RT-qPCR) assay was conducted on an ABI7900HT Sequence Detection System (Applied Biosystems QuantStudio 5, Foster City, CA, USA) using the Hieff qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). At least 3 biological replicates, with 3 technical replicates, were used for each treatment. The relative gene expression levels were calculated according to the  $2^{-\Delta\Delta C(t)}$  method [20]. In each reaction, the 23sRNA gene was used as an internal control. All primers used in RT-qPCR are listed in Supplementary material Table S1.

### 3. Result

#### 3.1. TMT-based Quantitative Analysis of the Proteome of *Enterococcus faecalis* Biofilm

After LC-MS/MS and searching database with Mascot, we identified a total of 19,353 peptides (included 16,852 unique peptides), corresponding to 33,813 peptide-spectral matches in an assembly of 2,095 protein groups with a protein-level FDR (false discovery rate)  $< 0.01$  (Fig. 1a).

We used original abundances of identified proteins to calculate the Pearson correlation coefficient among samples and visualized by a heatmap (Fig. 1b). The results showed that there was a very high correlation between the samples. Especially, the correlations between samples belonging to the same technical repeat group were close to 1. In addition, the scatter graph of the first principal component and second principal component through principal component analysis (PCA) also showed that repeatability of the technical replicated group was satisfactory (Fig. 1c).

For the following differentially expressed protein screening, protein quantitative abundances were normalized with median normalization, and visualized by a violin plot (Fig. 1d).

#### 3.2. Identification of significantly differentially expressed proteins

Differential expressed analyses of the proteome were respectively performed by comparing ALK with N, comparing NaOCl with ALK, and comparing NaOCl with N. Differentially Expressed Proteins (DEPs) were determined by  $> 1.5$ -fold change and  $p < 0.05$  using student  $t$  test in each comparison. The profile of differentially expressed proteins in each comparison were represented using volcano plots by applying the fold change and  $p$ -value cutoff values (Fig. 2a). The proteins with  $> 1.5$ -fold change and  $p$ -value  $< 0.05$  were considered as the most significantly affected proteins in each comparison.

In total, 80, 71, and 122 up-regulated proteins were determined by thresholds cutoff  $> 1.5$ -fold change and  $p$ -value  $< 0.05$ , while 116, 41, and 135 down-regulated proteins were determined by thresholds cutoff  $> 0.67$ -fold change and  $p$ -value  $< 0.05$  in the comparisons ALK versus N, NaOCl versus ALK, NaOCl versus N, respectively (Fig. 2b). To load protein accessions to the 'VennDiagram' package, a Venn diagram of DEPs acquired by the previous three comparisons was plotted (Fig. 2c). As Fig. 2c showed 31 common proteins were obtained (Table 1). After removing the duplicated gene names, finally 29 common proteins were determined, and displayed with a heatmap (Fig. 2d).

#### 3.3. COG functional analyzes

A total of 1,919 (91.6%) proteins were annotated to COG protein sequence databases via blastp with expect value  $1e-8$ . These proteins were assigned to at least one COG functional categories. Five top COG categories that included 21 sub COG categories were mapped and counted (Fig. 3a). As Fig. 3a showed

Table 1  
The common DEPs screened from comparisons ALK versus N, NaOCl versus ALK, and NaOCl versus N

Accession #	Gene Names	Description	Biological process	ALK vs. N		NaOCl vs. ALK		NaOCl vs. N	
				Log2FC	p-value	Log2 FC	p-value	Log2 FC	p-value
A0A2Z6BJM6	KUB3007_C13910	2-dehydropanoate 2-reductase	Pantothenate biosynthetic process	-1.48	6.87E-05	-0.79	2.15E-04	-2.27	2.53E-05
E2YC69	HMPREF9493_01162	ATP synthase, subunit F	Ion transmembrane transport	1.14	4.32E-04	0.81	9.06E-04	1.94	9.34E-05
A0A449EHT9	FKY84_00230	ATPase V		1.06	2.75E-05	0.78	2.82E-05	1.84	1.52E-06
A0A1QJFTT7	BZG32_07375	ATPase V		0.93	8.95E-04	1.08	5.47E-05	2.01	4.60E-06
R3KY17	WOU_01483	Branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit		-1.30	2.54E-05	-0.96	6.07E-05	-2.27	6.87E-06
S4FRR3	D351_01409	Cysteine-rich domain protein		-1.12	2.88E-05	-0.67	1.18E-04	-1.78	7.80E-06
A0A4V0DCK7	pdhC_2	Dihydrolipoamide acetyltransferase		-1.34	7.96E-07	-0.82	4.32E-05	-2.16	1.37E-07
A0A4V0CW09	Esp	component of pyruvate dehydrogenase complex		-2.18	2.03E-06	-0.86	4.57E-03	-3.04	3.94E-08
E2YDL9	HMPREF9493_01664	Enterococcal surface protein		-0.63	1.02E-04	-0.71	4.25E-05	-1.33	1.35E-05
		Fructosamine deglycase	Carbohydrate derivative metabolic process; carbohydrate metabolic process						
A0A0M2ARI4	WO5_01154	Iron-sulfur cluster-binding protein	Lactate oxidation	-1.36	5.00E-05	-0.65	1.20E-02	-2.01	1.04E-07

1: Biological process annotation of Gene ontology from UNIPROT KB.

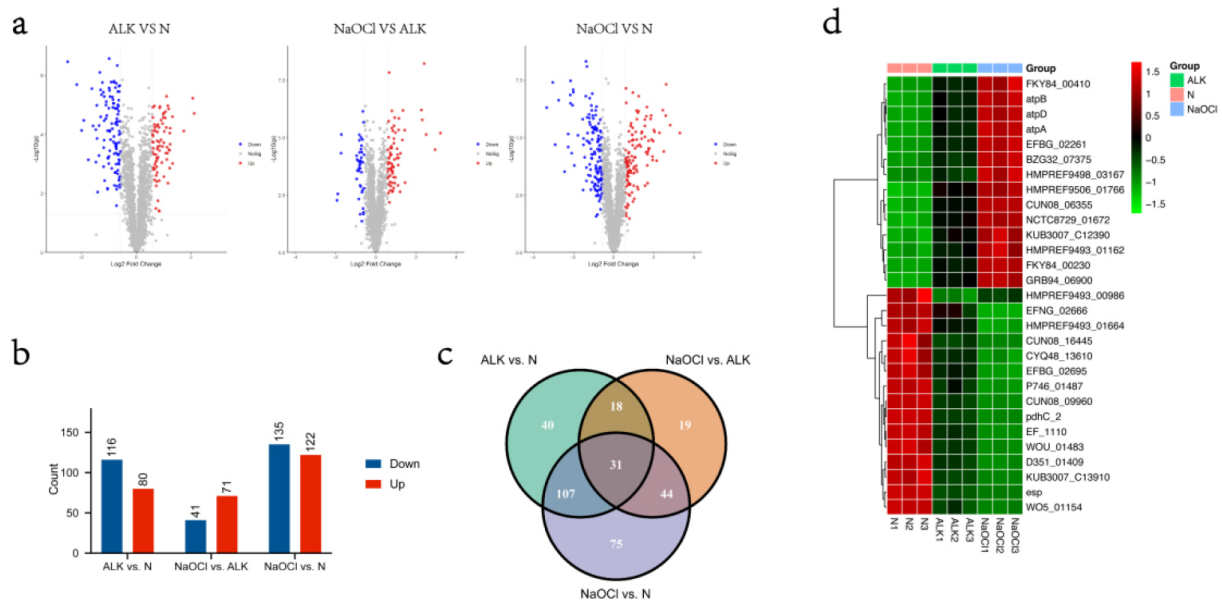


Fig. 2. (a) Volcano plots of differentially expressed proteins reveal significance patterns. Threshold cutoffs determined for log<sub>2</sub> fold change ratios are represented by vertical solid lines, and *p* value cutoff of 0.05 are represented by a horizontal solid line. X-axes are the fold change values (log<sub>2</sub> scaled), and Y-axes are the *p* values (log<sub>10</sub> scaled). Gray, blue, and red dots represent no significantly expressed proteins, down-regulated proteins, and up-regulated proteins, respectively. (b) Bar plot of counts of statistical up-regulated and down-regulated proteins. Dark blue represents down-regulated proteins, and Dark red represents up-regulated proteins. (c) Venn diagram of DEPs that screened from comparisons ALK versus N, NaOCl versus ALK, and NaOCl versus N. (d) A heatmap of common DEPs that is drawn by ‘pheatmap’ package to use Euclidean distance and complete method for protein clustering.

243 proteins was maximumly found in “Translation, ribosomal structure and biogenesis”. Enriched analyzes of COG categories of DEPs were performed with Fisher’s exact test using all identified proteins as a background, and calculated *p*-values were corrected with Benjamini & Hochberg (FDR) method, respectively.

### 3.4. Pathway enrichment analyzes of significantly differentially expressed proteins

For obtaining summary of Kyoto Encyclopedia of Genes and Genome (KEGG) pathways, all identified protein sequences were submitted to BlastKOALA (<https://www.kegg.jp/blastkoala/>) that was KEGG’s internal annotation tool for K number assignment of KEGG GENES using BLAST searches. In total, 1444 (68.9%) sequences were annotated. In these, 226, 220, 197 protein entries were found in “Protein families: genetic information process”, “Carbohydrate metabolism”, and “Genetic Information processing” as the top 3 categories.

KOBAS (version:3.0) were performed to search efa (Enterococcus faecalis V583) protein sequences database for assignment of KEGG gene identifiers to DEPs and all identified proteins. A total of 1,985 (94.7%) identified proteins was successfully mapped to gene identifiers of Enterococcus faecalis V583, while 184 (93.8%) DEPs of ALK versus N, 105 (93.8%) DEPs of NaOCl versus ALK, 244 (94.9%) DEPs of NaOCl versus N were succeeded in KOBAS searches.

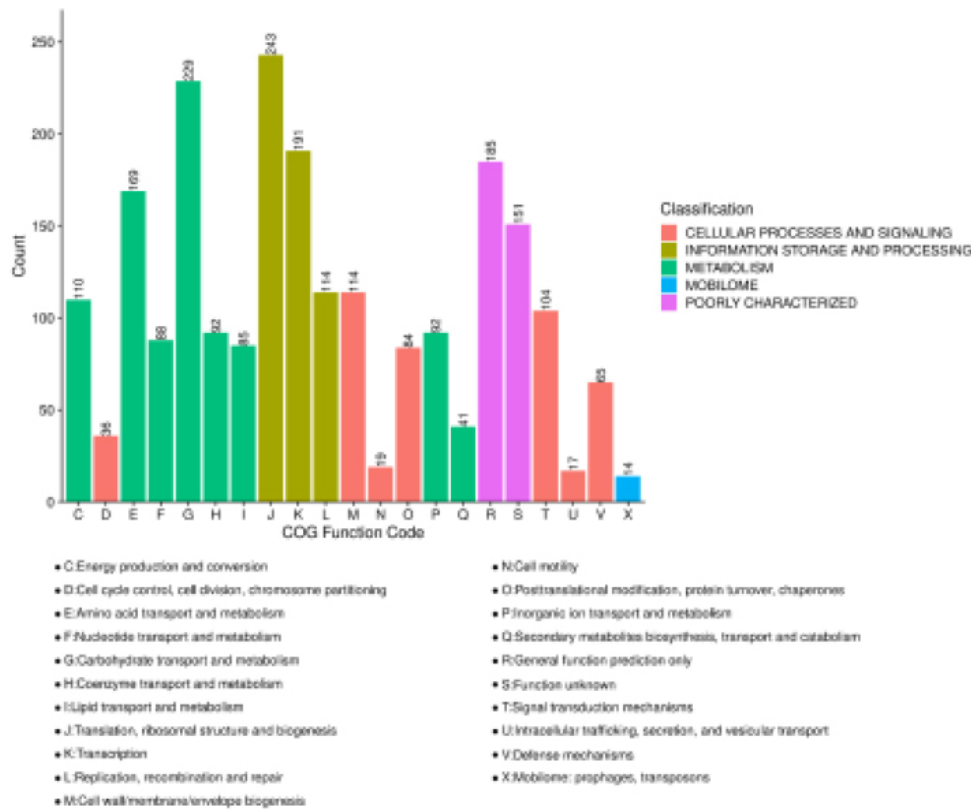


Fig. 3. Functional analyzes of DEPs: Bar plot of COG functional classification. The same top categories are filled by the same color. The letter as a COG function code represents the COG functional categories. The counts of protein in categories are indicated on the bar.

### 3.5. Correlation of TMT data with quantitative real-time PCR (qPCR) and PRM results

The results of qPCR analysis were checked for the correlation with TMT data (Fig. 4a). About half of the genes expression in ALK vs N and NaOCl vs N had the positive correlation between qPCR data and TMT results.

A total of 39 peptides that were significantly up-regulated or down-regulated and involved important pathways were selected and verified by PRM (Table 2). And the results of PRM analysis were checked for the correlation with TMT data (Fig. 4b). The majority of the proteins in ALK vs N and NaOCl vs N had the positive correlation between PRM data and TMT results.

## 4. Discussion

Many studies had detected the existence of *Enterococcus faecalis* biofilm in root canals that had failed to be treated [7], and in vitro studies had confirmed that *Enterococcus faecalis* could survive in the commonly used root canal disinfection drugs such as calcium hydroxide and sodium hypochlorite [10, 11], but the mechanism is still unclear. We studied the changes in protein expression of *Enterococcus faecalis* biofilm in alkaline and sodium hypochlorite environments by means of TMT-based quantitative



Table 2  
The list of DEPs verified by PRM

Accession	Description	GeneName
A0A2S7M560	ATP synthase subunit b	atpF
J5E1D1	ATP synthase subunit c	atpE
C7VAZ6	ATP synthase subunit a	atpB
Q831A3	ATP synthase subunit alpha	atpA
Q831A4	ATP synthase gamma chain	atpG
A0A4V0DH50	ATP synthase subunit beta	atpD
A0A3N3KJK1	ATP synthase epsilon chain	atpC
E2YC69	ATP synthase, subunit F	HMPREF9493_01162
A0A3N3ZC17	V-type ATP synthase beta chain	atpB
Q834X8	V-type ATP synthase beta chain	atpB
S4D5A4	V-type ATP synthase beta chain	atpB
E2YAA6	ABC transporter, permease protein	HMPREF9493_00602
E2YAA7	ABC transporter, permease protein	HMPREF9493_00603
R3JG22	His/Glu/Gln/Arg/opine family amino ABC transporter, permease, 3-TM region	WOK_00999
E2YFG9	ABC transporter, ATP-binding protein	HMPREF9493_02321
J6MXR6	Putative glutamine ABC transporter permease protein GlnP	HMPREF1332_03123
E2YAV8	ABC transporter, ATP-binding protein	HMPREF9493_00680
E2YAV7	Efflux ABC transporter, permease protein	HMPREF9493_00679
A0A1G1S9Y6	PTS mannose transporter subunit IID	BHU49_07340
A0A449DZ27	PTS system mannose/fructose/N-acetylgalactosamine-transporter subunit IIB	FKY84_01780

analysis. The results showed that compared with the alkaline environment, *Enterococcus faecalis* had a greater number of proteins that have changed expression in sodium hypochlorite media, and the degree of change was greater.

For pathogens, many secreted proteins are toxins, adhesins, and enzymes that determine virulence, which may mediate interactions between the pathogen and the host. In this study, for the first time, we used the method of metaproteomics to compare the bacterial secreted proteins of *Enterococcus faecalis* biofilms under different pressure environments. A total of 2850 differentially expressed proteins were obtained, of which 580 had statistical differences. Further bioinformatics analysis revealed that proteins with expression differences of more than 1.5 times were mainly concentrated in ABC transporters, glycolysis/gluconeogenesis, pyruvate metabolism, citric acid cycle, and other aspects.

The ABC transporter superfamily is a group of transmembrane proteins widely present in eukaryotes, prokaryotes, and archaea [20,21]. In the K-12 genome of *Escherichia coli*, approximately 5% of the genome encodes ABC transporters, which constitute one of the largest known gene families in humans [22].

The core structure of a typical ABC transporter generally includes four domains and two highly hydrophobic transmembrane junctions.

The transmembrane domain (TMD) and two hydrophilic nucleotide binding domains (NBD) embedded in the cell membrane but protruding from the cytoplasm that catalyze ATP hydrolysis. ABC transporters typically utilize the energy of hydrolyzing ATP to transport various substances across membranes, including ions, sugars, amino acids, metal ions, vitamins, peptides, hormones, cellular metabolites, and drugs. Through material transport, ABC transporters participate in various cellular processes, such as maintaining osmotic pressure dynamic balance, nutrient uptake, resistance to exogenous toxins, antigen processing, cell division, cancer cell resistance, bacterial immunity, pathogenic mechanisms and spore formation, cholesterol and lipid transport, and developmental stem cell biological processes [23]. The bacterial ABC transporter protein was first discovered as a high affinity nutrient transporter, and later attracted attention due to its involvement in multidrug resistance (MDR) in *Candida albicans*. Research

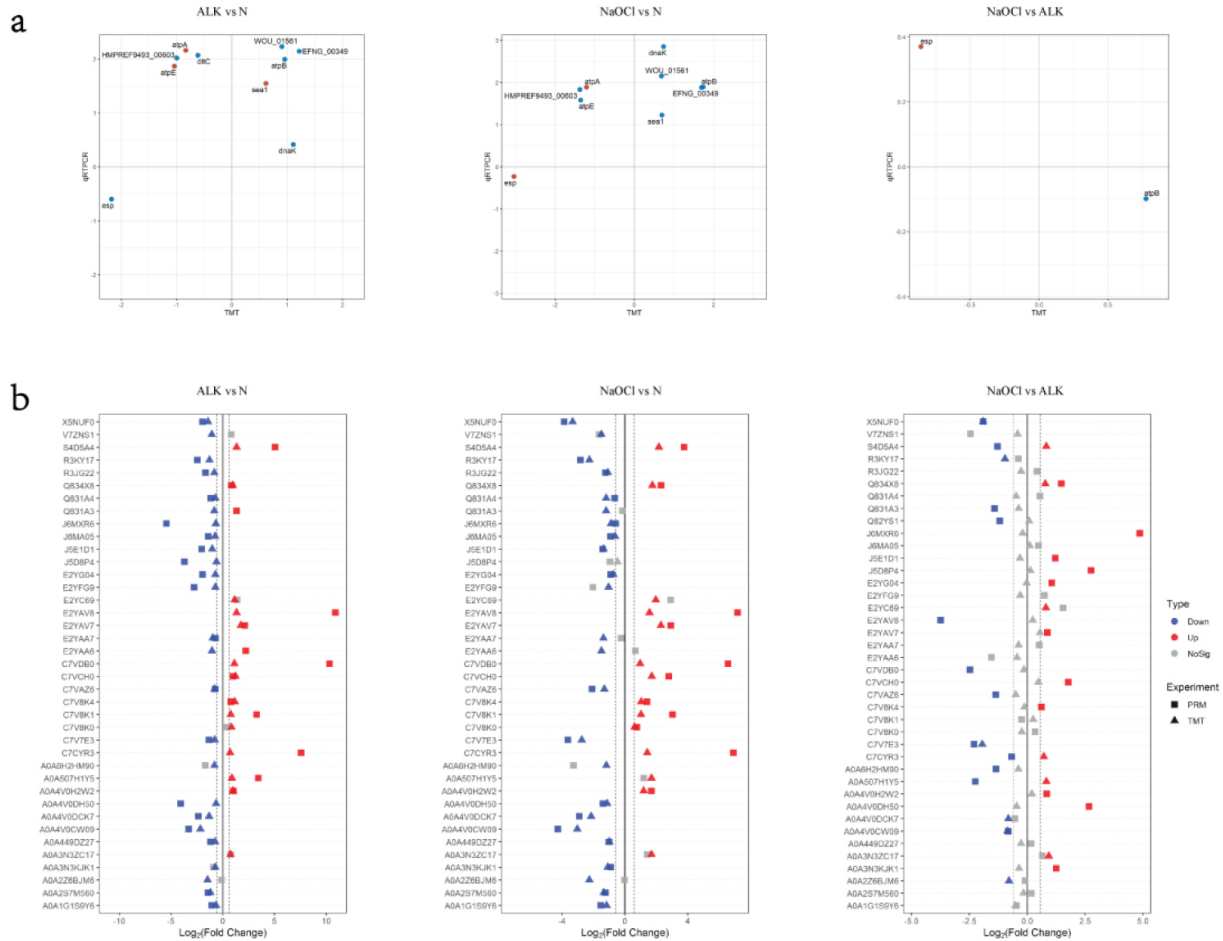


Fig. 4. Correlation of TMT Data with Quantitative Real-time PCR (qPCR) and PRM: (a) Correlation of qPCR and TMT. Log<sub>2</sub> (fold change) is used to draw the scatter plot, and only the significant proteins in the TMT experiment are selected. The blue dot is qPCR  $p$  value < 0.05, and the dark red dot is the smallest qPCR  $p$  value  $\geq$  0.05. X-axes are the fold change values (log<sub>2</sub> scaled) of TMT, and Y-axes are the fold change values (log<sub>2</sub> scaled) of qRT-PCR. (b) Correlation of PRM and TMT. Log<sub>2</sub> (fold change) is used to draw the scatter plot. The blue icons represent significant down-regulation while the red ones represent significant up-regulation. The grey icons represent  $p$  value  $\geq$  0.05 which means no significant difference. The square icons represent PRM, and triangle icons represent TMT.

has shown that overexpression of genes encoding ABC transporters is one of the main reasons for pathogenic fungi to acquire multidrug resistance [24,25]. Overexpressed ABC transporters efficiently pump antifungal drugs that enter fungal cells out of the cell, reducing the concentration of drugs inside the cell and allowing pathogenic fungi to survive in the patient's body, leading to fungal resistance [26]. In recent years, with the efforts of scholars from various countries, the reconstruction of ABC transport systems for species such as *Escherichia coli* [27], *Bacillus subtilis* [28], and *Bacillus subtilis* [29] has been gradually completed.

This study identified over 50 peptide segments corresponding to adenosine triphosphate binding cassette transporter (ABC transporter); Multiple ABC transporters related to the transport of iron, nickel, cobalt ions, iron compounds, metal ions, oligopeptides, dipeptides, amino acids, branched chain amino acids, lactidases, and cleavage nucleases were detected in the plaque fluid of the three groups with no differential

co expression. It can be seen that ABC transporters are involved in various physiological functions of bacteria. Correspondingly, foreign scholars have applied proteomic techniques in *S. oralis* during the formation of biofilms by mutants and *S. oralis*, as well as in the expression profiles of intracellular and extracellular proteins under various environmental pressures, traces of ABC transporters have also been found [30,31], suggesting their involvement in regulating bacterial adaptation to the environment, survival, and virulence. Recently, Alexander further confirmed that two related transport proteins, *S. mutans*, are involved in the uptake of disaccharides and oligosaccharides by cells, and there may be interactions between different transport proteins [32]. At the same time, the ABC transport device system plays an important role in the recognition process of autoinducers (AIs) transport in the density sensing (QS) system, participating in the regulation of intracellular specific gene expression and completing signal exchange between bacteria [33, 34].

It is worth noting that we identified a total of 15 highly expressed ABC transporters in the high alkaline environment pressure group, with 7 proteins greater than 1.5 times. However, in the control group, only 6 ABC transporters were highly expressed, all lower than 1.5 times, suggesting that they may be potential target molecules for testing the resistance of fecal enterococci biofilms to alkaline environments.

## 5. Conclusion

Through comprehensive analysis, we obtained a total of 2850 peptide segments with quantitative information in this experiment. Resulting in a dataset of 580 differentially expressed peptide segments with statistical significance. In addition, proteins undergo glycosylation, phosphorylation, and acetylation modifications from synthesis to secretion, and complex interactions occur between proteins. It is worth noting that we identified a total of 15 highly expressed ABC transporters in the high alkaline environment pressure group, with 7 proteins greater than 1.5 times. Nevertheless, macroproteomics, secretory proteomics, and bioinformatics analysis still provide a very good platform for our research. This experiment obtained the largest known dataset of differentially secreted proteins in the biofilm of *Enterococcus faecalis* under different environmental pressure states, providing experimental data for further clarification of the pathogenesis of refractory periapical periodontitis, research directions, and possible ecological interventions. In the future, we will do more study about functions of meaningful proteins so that we can recognize more detail about the mechanisms of how *E.f* resistant to pressure environment.

## Acknowledgments

This study was funded by the Shanghai Natural Science Foundation of China (18zr1422300) and Natural Science Foundation of Tibet Autonomous Region (XZ2019ZR-ZY43(Z)). We acknowledge Shanghai ShengZi Biotechnology Co., Ltd and Dr. Yu Hong for their great effort on the LC-MS/MS experiments.

## Conflict of interest

None to declare.

## References

- [1] Mohamed JA, Huang DB. Biofilm formation by enterococci. *Journal of Medical Microbiology*. 2007; 56: 1581-1588. doi: 10.1099/jmm.0.47331-0.

- [2] Hancock HH, Jr., Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics*. 2001; 91: 579-586. doi: 10.1067/moe.2001.113587.
- [3] Pinheiro ET, Gomes BP, Ferraz CC, Teixeira FB, Zaia AA, Souza Filho FJ. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. *Oral Microbiology Immunology*. 2003; 18: 100-103. doi: 10.1034/j.1399-302x.2003.00058.x.
- [4] Siqueira JF, Jr., Rocas IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics*. 2004; 97: 85-94. doi: 10.1016/s1079-2104(03)00353-6.
- [5] Sedgley C, Nagel A, Dahlen G, Reit C, Molander A. Real-time quantitative polymerase chain reaction and culture analyses of *Enterococcus faecalis* in root canals. *Journal of Endodontics*. 2006; 32: 173-177. doi: 10.1016/j.joen.2005.10.037.
- [6] Muras A, Mallo N, Otero-Casal P, Pose-Rodríguez JM, Otero A. Quorum sensing systems as a new target to prevent biofilm-related oral diseases. *Oral Diseases*. 2020; 1-7. doi: 10.1111/odi.13689.
- [7] Swimerbergh RCD, Coenye T, De Moor RJG, Meire MA. Biofilm model systems for root canal disinfection: a literature review. *International Endodontic Journal*. 2019; 52: 604-628. doi: 10.1111/iej.13050.
- [8] Mohammadi Z, Dummer PM. Properties and applications of calcium hydroxide in endodontics and dental traumatology. *International Endodontic Journal*. 2011; 44: 697-730. doi: 10.1111/j.1365-2591.2011.01886.x.
- [9] Gonçalves LS, Rodrigues RC, Andrade Junior CV, Soares RG, Vettore MV. The Effect of Sodium Hypochlorite and Chlorhexidine as Irrigant Solutions for Root Canal Disinfection: A Systematic Review of Clinical Trials. *Journal of Endodontics*. 2016; 42: 527-532. doi: 10.1016/j.joen.2015.12.021.
- [10] Ma J, Tong Z, Ling J, Liu H, Wei X. The effects of sodium hypochlorite and chlorhexidine irrigants on the antibacterial activities of alkaline media against *Enterococcus faecalis*. *Archives of Oral Biology*. 2015; 60: 1075-1081. doi: 10.1016/j.archoralbio.2015.04.008.
- [11] Appelbe OK, Sedgley CM. Effects of prolonged exposure to alkaline pH on *Enterococcus faecalis* survival and specific gene transcripts. *Oral Microbiology and Immunology*. 2007; 22: 169-174. doi: 10.1111/j.1399-302X.2007.00340.x.
- [12] Ran S, Liu B, Jiang W, Sun Z, Liang J. Transcriptome analysis of *Enterococcus faecalis* in response to alkaline stress. *Frontiers in Microbiology*. 2015; 6: 795. doi: 10.3389/fmicb.2015.00795.
- [13] Arntzen MØ, Karlsk IL, Skaugen M, Eijsink VG, Mathiesen G. Proteomic investigation of the response of *Enterococcus faecalis* V583 when cultivated in urine. *PLoS One*. 2015; 10: 1-17. doi: 10.1371/journal.pone.0126694.
- [14] Giard JC, Laplace JM, Rince A, Pichereau V, Benachour A, Leboeuf C, Hartke A. The stress proteome of *Enterococcus faecalis*. *Electrophoresis*. 2001; 22: 2947-2954. doi: 10.1002/1522-2683(200108)22:14<2947::AID-ELPS2947>3.0.CO;2-K.
- [15] Wang X, He X, Jiang Z, Wang J, Chen X, Liu D, Yuan J. Proteomic analysis of the *Enterococcus faecalis* V583 strain and clinical isolate V309 under vancomycin treatment. *Journal of Proteome Research*. 2010; 9: 1772-1785. doi: 10.1021/pr901216e.
- [16] Pessione A, Lamberti C, Cocolin L, Campolongo S, Grunau A, Giu-bergia, S, ... Pessione E. Different protein expression profiles in cheese and clinical isolates of *Enterococcus faecalis* revealed by proteomic analysis. *Proteomics*. 2012; 12: 431-447. doi: 10.1002/pmic.201100468.
- [17] Bøhle LA, Færgestad EM, Veiseth-Kent E, Steinmoen H, Nes IF, Eijsink VG, Mathiesen G. Identification of proteins related to the stress response in *Enterococcus faecalis* V583 caused by bovine bile. *Proteome Science*. 2010; 8: 37. doi: 10.1186/1477-5956-8-37.
- [18] Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature Methods*. 2009; 6: 359-362. doi: 10.1038/nmeth.1322.
- [19] Hogrebe A, von Stechow L, Bekker-Jensen DB, Weinert BT, Kelstrup CD, Olsen JV. Benchmarking common quantification strategies for large-scale phosphoproteomics. *Nature Communications*. 2018; 9: 1045. doi: 10.1038/s41467-018-03309-6.
- [20] Fan W, Huang Z, Fan B. Effects of prolonged exposure to moderate static magnetic field and its synergistic effects with alkaline pH on *Enterococcus faecalis*. *Microbial Pathogenesis*. 2018; 115: 117-122. doi: 10.1016/j.micpath.2017.12.028. Epub 2017 Dec 11.
- [21] Suriyanarayanan T, Qingsong L, Kwang LT, Mun LY, Truong T, Seneviratne CJ. Quantitative Proteomics of Strong and Weak Biofilm Formers of *Enterococcus faecalis* Reveals Novel Regulators of Biofilm Formation. *Molecular and Cellular Proteomics*. 2018; 17: 643-654. doi: 10.1074/mcp.RA117.000461.
- [22] Kane PM. The where, when, and how of organelle acidification by the yeast vacuolar H<sup>+</sup>-ATPase. *Microbiology and Molecular Biology Reviews*. 2006; 70: 177-191. doi: 10.1128/MMBR.70.1.177-191.2006.
- [23] Zhu X, Liu W, Lametsch R, Aarestrup F, Shi C, She Q, ... Knöchel S. Phenotypic, proteomic, and genomic characterization of a putative ABC-transporter permease involved in *Listeria monocytogenes* biofilm formation. *Foodborne Pathogens and Disease*. 2011; 8: 495-501. doi: 10.1089/fpd.2010.0697.

- [24] Fabretti F, Theilacker C, Baldassarri L, Kaczynski Z, Kropec Holst O, Huebner J. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. *Infection and Immunity*. 2006; 74: 4164-4171. doi: 10.1128/IAI.00111-06.
- [25] Jeckelmann JM, Erni B. The mannose phosphotransferase system (Man-PTS) – Mannose transporter and receptor for bacteriocins and bacteriophages. *Biochimica et Biophysica Acta-Biomembranes*. 2020; 1862: 183412. doi: 10.1016/j.bbamem.2020.183412.
- [26] Peng Z, Ehrmann MA, Waldhuber A, Niemeyer C, Miethke T, Frick JS, . . . Vogel RF. Phosphotransferase systems in *Enterococcus faecalis* OG1RF enhance anti-stress capacity in vitro and in vivo. *Research in Microbiology*. 2017; 168: 558-566. doi: 10.1016/j.resmic.2017.03.003.
- [27] Wei L, Li M, Xia F, Wang J, Ran S, Huang Z, Liang J. Phosphate transport system mediates the resistance of *Enterococcus faecalis* to multidrug. *Microbiological Research*. 2021; 249: 126772. doi: 10.1016/j.micres.2021.126772.
- [28] Cicchillo RM, Baker MA, Schnitzer EJ, Newman EB, Krebs C, Booker SJ. *Escherichia coli* L-serine deaminase requires a [4Fe-4S] cluster in catalysis. *The Journal of Biological Chemistry*. 2004; 279: 32418-32425. doi: 10.1074/jbc.M404381200.
- [29] Lindenstrauß AG, Behr J, Ehrmann MA, Haller D, Vogel RF. Identification of fitness determinants in *Enterococcus faecalis* by differential proteomics. *Archives of Microbiol*. 2013; 195: 121-130. doi: 10.1007/s00203-012-0857-3.
- [30] Großholz R, Koh CC, Veith N, Fiedler T, Strauss M, Olivier B, . . . Kummer U. Integrating highly quantitative proteomics and genome-scale metabolic modeling to study pH adaptation in the human pathogen *Enterococcus faecalis*. *NPJ Systems Biology and Applications*. 2016; 2: 16017. doi: 10.1038/npjbsa.2016.17.
- [31] Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. *Infection and Immunity*. 2004; 72: 6032-6039. doi: 10.1128/IAI.72.10.6032-6039.2004.
- [32] Tendolkar PM, Baghdayan AS, Shankar N. The N-terminal domain of enterococcal surface protein, Esp, is sufficient for Esp-mediated biofilm enhancement in *Enterococcus faecalis*. *Journal of Bacteriology*. 2005; 187: 6213-6222. doi: 10.1128/JB.187.17.6213-6222.2005.
- [33] Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, . . . Lasa I. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Applied and Environmental Microbiology*. 2001; 67: 4538-4545. doi: 10.1128/AEM.67.10.4538-4545.2001.
- [34] Dworniczek E, Wojciech Ł, Sobieszcańska B, Seniuk A. Virulence of *Enterococcus* isolates collected in Lower Silesia (Poland). *Scandinavian Journal of Infectious Diseases*. 2005; 37: 630-636. doi: 10.1080/00365540510031421.

## Supplementary data

Table S1  
List of primers used for RT-PCR analysis

Accession	Gene name	Primer sequence	
		Forward	Reverse
J5E1D1	atpE	TATCGGAGCCGCTATCGGTAA	CTTCGATTAAGCGACCCCGA
A0A4V0CW09	esp	GGGCAAGGCTCTCAATGACT	GTAGGCTCCATGCTGGTTGT
Q82YS1	sea1	CAGGGGTAGCAGGTATTGGG	GCTTGCTCCGTTGTTTGTGT
S4D0N0	dnaK	GTATCGAAACAATGGGCGGC	CAGCCATTGGACGTTCTCCT
J5D8P4	dltC	ACAGGAACAGACGAAGTTGTGA	TGGTGTTCCCCATACTTCACG
Q831A3	atpA	TTCTATGCAGGCACTCGTCC	GCAGTGGCGCATGTAGTTTT
Q834X8	atpB	GAGCGGTATCCAACCACCAA	GTTGGGCAAAAGCTGCGTAA
C7VCH0	EFNG_00349	GCCAAAGGGGTTGGCTTTAC	TTGGGCAATCCCAGAGAACAA
E2YAA7	HMPREF9493_00603	TTTGTGTTGCGGCTTTGGGTT	CAACAATCGCCATTTCGAGCC
R3I6P6	WOU_01561	TATGCACGCTGAATTTGGTGA	TCGCTATTACTTTTCCAAGCGG