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The potential regulatory role of mannose phosphotransferase system EII in alkaline resistance of *Enterococcus faecalis*

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ABSTRACT

Objective: To investigate the role of mannose phosphotransferase system (Man-PTS) EII in the alkaline resistance of *Enterococcus faecalis* (*E. faecalis*).

Methods: The Man-PTS EIID (*mptD*) deletion (Δ*mptD*) and overexpressing (+*mptD*) strains of *E. faecalis* were constructed. The contribution of the Man-PTS EII to alkaline resistance was investigated by assessing growth and biofilm formation of *E. faecalis* at pH 7 and 10, as well as changes in intracellular ATP levels, pH, potassium ion concentration, membrane potential and membrane permeability. The effects of *mptD* on the expression of other Man-PTS EII components and membrane transport-related genes were evaluated by RT-qPCR at pH 10. In addition, six clinical strains were isolated. Three strains of *E. faecalis* with high to low alkaline resistance were screened, and RNA sequencing was performed to further explore the role of Man-PTS EII in the alkaline resistance.

Results: *mptD* enhanced the alkaline resistance of *E. faecalis* by maintaining pH homeostasis in an alkaline environment. Overexpression of *mptD* induced membrane hyperpolarization, reduced membrane permeability, decreased intracellular K^+ levels, lowered cytoplasmic pH, and elevated ATP production, while the loss of *mptD* reversed these effects ($p < 0.05$). *mptD* coordinated the expression of Man-PTS EII components and upregulated genes encoding membrane transporter components ($p < 0.01$). All Man-PTS EII components were positively related to the degree of alkaline resistance of *E. faecalis* ($p < 0.05$).

Conclusion: Man-PTS EII is a key factor in the interaction between metabolism and ion membrane transport during the alkaline resistance process of *E. faecalis*. This study may provide new insights for understanding the alkaline resistance of *E. faecalis*.

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Alkaline resistance; phosphotransferase system; mannose; *Enterococcus faecalis*

Introduction

Persistent root canal infection and reinfection are significant factors contributing to the failure of root canal treatment in human teeth [1]. *Enterococcus faecalis* (*E. faecalis*) is a Gram-positive facultative anaerobe that is one of the most frequently detected bacteria in the polymicrobial biofilm community of persistent root canal infection and reinfection [2]. Its detection rate and abundance in the pathogenic biofilm community vary among different studies (ranging from 2.5% to 78.3%), depending on the method used for bacteria detection [3–5]. Despite discrepancies in the detection rate, most previous and recent published literature supported the presence and pathogenic role of *E. faecalis* in the persistent root canal infection, even when assessed with contemporary molecular detection methods (e.g. next-generation sequencing methods) [6–8]. This particular bacterium possesses the capability of withstanding nutrient-deficient conditions and alkaline stress [9,10]. *E. faecalis* can form biofilms in root canals, which in turn can lead to treatment failure due to the persistence of infections and higher drug resistance [2]. Therefore, the efficacy of alkaline disinfectants in root canal treatment, such as calcium hydroxide, may be compromised by the alkaline resistance exhibited by *E. faecalis*.

As reported, *Enterococci* could maintain proton motive force (PMF) and intracellular pH homeostasis via adenosine triphosphate synthetase (ATPase) and proton antiporter mechanisms, consequently yielding alkaline resistance [11]. The alkaline resistance of *E. faecalis* may be affected by the nutrient availability in the biofilm [12]. When in a nutrient-deficient environment, substances such as glycerin can stimulate the metabolism of *E. faecalis*, which in turn leads to increased alkaline resistance [12]. The development of alkaline resistance in *E. faecalis* is accompanied by the changes to metabolic activities, and its ability to tolerate extreme alkaline conditions is influenced by differences in physiological processes and metabolic pathways [11,13,14]. Therefore, inhibition

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of energy metabolic activity is essential for reducing alkaline resistance in *E. faecalis*. To date, the detailed molecular mechanisms behind energy metabolism of bacteria related to alkaline resistance remain unclear.

The phosphoenolpyruvate (PEP) carbohydrate phosphotransferase system (PTS) plays an important role in the survival of bacteria in adverse environments [15,16]. PTS is also reported to be involved in maintaining bacterial ion homeostasis as an adenosine triphosphate (ATP)-dependent regulator [17]. The mannose phosphotransferase system (Man-PTS) , as a part of PTS regulatory network, is crucial for the stress resistance of bacteria, and enzyme II (EII) is a sugar-specific protein complex consisting of EIIA, EIIB, EIIC and EIID, in which EIID plays a key role in the selection and use of PTS-sugars by bacteria [16,18,19]. Man-PTS could regulate various adaptive activities of bacteria through EII, including virulence gene expression and biofilm formation, which are closely related to the adaptation of bacteria to external environment [15,16,20]. There are reports that *E. faecalis* selectively metabolizes mannose to produce ATP in response to alkaline stress [21]. However, the role of Man-PTS EII in maintaining the alkalinity resistance of *E. faecalis* remains elusive.

Based on the above concerns, this study was designed to explore whether and how Man-PTS EII could regulate the alkaline resistance of *E. faecalis*.

Materials and methods

Bacterial strains and culture media

Bacterial strains used in this study are listed in Table 1. Two standard laboratory *E. faecalis* strains ATCC 29212 and ATCC 51299 were obtained from the American Type Culture Collection. The Man-PTS EIID (*mptD*) deletion (Δ*mptD*) and overexpressing (+*mptD*) strains of *E. faecalis* were constructed in our previous study and used for this study [22]. Δ*mptD* and +*mptD* strain were confirmed using PCR and quantitative real-time PCR as shown in Figure S1 and S2 of Supplementary Materials. Six clinical *E. faecalis* strains (XS 001, XS 002, XS 003, LY 001, LY 002 and LY 003) were isolated from root canals of teeth with failed root canal treatment under the approval of the Ethics Committee of School of Stomatology (No. [2022]A20), following Barbosa-Ribeiro's radiological and clinical examinations [22–24]. The identification method for clinical *E. faecalis* strains was described in Supplementary Materials. Under anaerobic conditions, *E. faecalis* was incubated in brain heart infusion (BHI) (BD, USA) at 37°C. BHI media with $\text{pH} = 10$ was prepared using 0.2 M $NaHCO_3$ and 0.2 M K_2CO_3 (Sinopharm, China) buffer solutions as previously described [25]. All BHI media were sterilized using PES membrane filter units (0.22 μm, Millipore, USA).

Table 1. Strains used in this study.

Strains	Relevant characteristics	Reference of source
ATCC 29212	Standard strain of *E. faecalis*	Laboratory preservation
ATCC 51299	Standard strain of *E. faecalis*	Laboratory preservation
XS 001	Clinical strain of *E. faecalis*	Clinical collection, this study
XS 002	Clinical strain of *E. faecalis*	Clinical collection, this study
XS 003	Clinical strain of *E. faecalis*	Clinical collection, [22]
LY 001	Clinical strain of *E. faecalis*	Clinical collection, this study
LY 002	Clinical strain of *E. faecalis*	Clinical collection, this study
LY 003	Clinical strain of *E. faecalis*	Clinical collection, this study
Δ*mptD*	*mptD* deletion of *E. faecalis*	Self-constructed, [22]
+*mptD*	*mptD* overexpression of *E. faecalis*	Self-constructed, [22]

Dynamic growth curves

The dynamic growth curve method was used to observe the growth patterns of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*). Briefly, 5 mL suspension (1×10^5 CFUs/mL) of *E. faecalis* was incubated in BHI with different pH (7 and 10). At 3, 6, 9, 12 and 24 h after incubation, 200 μL of the suspension was retrieved into a 96-well plate and measured at 600 nm using a microplate reader (Synergy H1, Biotech, USA). The test was repeated six times.

Biofilms quantification

The biofilm formation ability of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) was evaluated using crystal violet (CV) staining. Briefly, as mentioned above, *E. faecalis* was adjusted to a concentration of 1×10^5 CFUs/mL in BHI with different pH (7 and 10), and the final volume was 200 μL per well in 96-well polystyrene microtiter plates. Following 48 h of incubation at 37°C, non-adherent cells were removed by gentle PBS washing (×3), and the adherent bacteria were fixed with anhydrous methanol for 15 min. Subsequently, the biofilms were stained with 0.1% (w/v) CV dye for 15 min. After staining, the samples were gently rinsed with PBS (×3) to remove excess crystal violet. Then the dye was dissolved with anhydrous methanol for 10 min and the optical density at

560 nm was measured using a microplate reader (Synergy H1, Biotech, USA). The test was repeated six times.

Bacterial morphology

Field Emission Scanning Electron Microscope (FE-SEM) was used to observe the morphology of *E. faecalis* (ATCC 29212, Δ*mptD* and + *mptD*) in different pH (7 and 10) conditions. Briefly, mid-log phase bacterial cells ($n = 3$) were harvested, resuspended in phosphate-buffered saline (PBS), spotted on coverslip and air-dried to pellicles. Pellicles from each strain were fixed with 2.5% glutaraldehyde for 12 h, serially dehydrated in ethanol and sputter-coated with gold. Specimens were imaged using FE-SEM (Zeiss Gemini SEM 500, UK) at specified magnifications.

Intracellular pH

The assays of intracellular pH (pH_{in}) of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) were performed as previously described [22]. Briefly, *E. faecalis* suspension ($OD_{600} = 0.5$) was incubated in 5 μM of pH fluorescent probe BCECF AM for 1 h at 37°C. And the intracellular pH calibration buffer kit (Invitrogen, USA) was used to generate calibration curves. The cells were washed twice and incubated in BHI with different pH (7 and 10) for 1 h. After the cells were centrifuged and resuspended, their fluorescence was detected using a microplate reader (Synergy H1, BioTek, USA). pH was assessed by the ratio of emission intensity excited at 490 nm versus at 440 nm, using a calibration curve. The test was repeated six times.

Cellular ATP

The ATP levels assays of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) were performed as previously described [22]. In brief, 100 μL bacterial suspensions ($OD_{600} = 0.5$) were added into a 96-well plate with black bottom, then 100 μL of the BacTiter-Glo Reagent (Promega, USA) was added and incubated for 5 min before recording the luminescence through a microplate reader (Synergy H1, BioTek, USA) after treating bacteria with BHI at different pH (7 and 10) for 1 h. The ATP level in a single cell was calculated according to the previously described method [22]. The test was repeated six times.

Intracellular potassium ions

Potassium ion (K^+) levels of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) were measured using EPG-2 (Maokang Biology, China). In brief, *E. faecalis* ($OD_{600} = 0.5$) were incubated with 2 μM EPG- for 30 min, fluorescence images for the FITC channel were taken with high-resolution laser scanning confocal fluorescence microscopy (Leica TCS SP8 STED CW, Germany) after treating bacteria with BHI at different pH (7 and 10) for 1 h. Intracellular K^+ was measured by the fluorescence intensity of FITC channel. The test was repeated three times.

Membrane potential and permeability

The membrane potential and permeability assays of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) were performed as previously described with minor modifications [22,26]. In brief, the suspension of *E. faecalis* ($OD_{600} = 0.5$) was cultured for 24 h in BHI medium at pH 7 and 10. Subsequently, 10 μL of 3 mm $DiOC_2(3)$ (Invitrogen, USA) and 10 μL of 50 μM TO-PRO-3 (Invitrogen, USA) were added simultaneously and incubated at room temperature for 1 h. Fluorescence signals were detected by flow cytometry (Beckman Coulter Inc, USA). The membrane potential was determined by the red/green fluorescence ratio of $DiOC_2(3)$, and the membrane permeability was evaluated by the ratio of TO-PRO-3 red/$DiOC_2(3)$ green fluorescence. The test was repeated six times.

Alkaline resistance evaluation

The alkaline resistance evaluation of different *E. faecalis* strains was performed as previously described with minor modifications [22]. Dynamic growth curves and CFU count were used to evaluate the growth of different *E. faecalis* strains. The dynamic growth curve method was used to observe the growth patterns of *E. faecalis*, and the CFU count was used to observe the number of *E. faecalis* surviving in the suspensions. Briefly, 5 mL suspension (1×10^5 CFUs/mL) of *E. faecalis* (two lab strains and six clinical isolates strains) were incubated in BHI with different pH (7 and 10). Dynamic growth curves were obtained at 3, 6, 9, 12 and 24 h after incubation, as described previously. After 24 h, the suspension was diluted in equal proportions and inoculated on BHI agar plates, incubated at 37°C for 24 h, and the number of colonies was counted. The test was repeated six times. Based on the evaluation, the alkaline resistance of all strains was ranked and three strains representative of high, medium and low alkaline resistance were used for the following experiments.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Following 24 h incubation of *E. faecalis* (ATCC 29212, Δ*mptD* and +*mptD*) in BHI at pH 10 or *E. faecalis* (ATCC 29212, LY 002 and XS 003) in

BHI at different pH (7 and 10), the bacteria were resuspended with BHI and adjusted to $OD_{600} = 0.5$. The bacteria were resuspended in lysozyme (20 mg/mL) to lyse the cell wall. Bacterial total RNA was extracted with TRIzol® reagent (Invitrogen, USA), and cDNA was synthesized using the Hiscript II Q RT SuperMix cDNA synthesis kit (Vazyme Biotech, China) following the manufacturer's instructions. The QuantStudio 6 Real-Time PCR System (Life Technologies) was used to perform RT-qPCR with SYBR Green master mix (Vazyme Biotech, China), to determine relative mRNA expression levels. The $2^{-\Delta\Delta CT}$ method was used to quantify fold induction, and transcription levels of each gene were compared to the expression of the 16S rRNA gene, which was used as a reference gene. The test was repeated three times. The primers used in this study are listed in Table 2.

RNA-Seq transcriptome analysis

E. faecalis (ATCC 29212, XS 003 and LY 002) were incubated in BHI (pH = 10) for 24 h and then adjusted with BHI to $OD_{600} = 0.5$. RNA extraction, purification and modification were carried out according to standard protocols. RNA-Seq was performed by Beijing Tsingke Biotech Co., Ltd. Briefly, the integrity of the RNA was first verified, and after RNA library construction using Illumina (San Diego, CA) TruSeqTM RNA sample preparation Kit, RNA-seq paired-end sequencing was conducted using the Illumina NovaSeq 6000. The raw data format is FASTQ format. Quality control filtering of raw sequencing reads, including splice removal and the elimination of low-quality sequences, was performed using fastp software (https://github.com/OpenGene/fastp, version fastp-v0.23.0) with the following parameters: fastp -i sample.R1.fastq -I sample.R2.fastq -o out.R1.fastq -p out.R2.fastq -q 20 -l 20 -n 15. After processing, CleanData were obtained and then aligned to the *E. faecalis* ATCC 29212 genome. The software used for differential expression was DESeq2. The default screening criteria for significantly differentially expressed genes were FDR < 0.05 & $|\log2FC| \geqq 1$. Differentially expressed genes (DEGs) were identified for analysis using the Clusters of Orthologous Groups of proteins (COG) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) in the Goatools and KOBAS software. Three samples were included in each group, with the reference genome acquired from the National Center for Biotechnology Information (GenBank: NZ_CP008816).

Statistical analysis

Transcriptome analysis was performed as described in the RNA-Seq section. For the analysis of other data, GraphPad Prism 8 statistical software was used and the Shapiro–Wilk and Kolmogorov –Smirnov tests were applied to assess normal distribution. All data were normally distributed and were shown as mean ± standard deviation (SD). Group comparisons were conducted using the unpaired t-test and one-way ANOVA. Statistical significance was defined as $p < 0.05$.

Results

mptD promoted alkaline resistance in *E. faecalis*

To investigate the potential role of Man-PTS EII in alkaline resistance of *E. faecalis*, the Δ*mptD* and +*mptD* strains from our previous study were used in this study [22]. Observation of the bacterial suspensions incubated in glass tubes for 24 h revealed that at pH 7, Δ*mptD* and +*mptD* suspensions were uniformly turbid similar to ATCC 29212, but at pH 10, +*mptD* showed much more aggregated bacteria at the bottom of the glass tubes than ATCC 29212 and Δ*mptD*, and the suspensions of +*mptD* were more turbid than Δ*mptD* (Figure 1(a)). Only the turbidity of Δ*mptD* was significantly affected at pH 10 when compared to pH 7 (Figure 1(a)). Besides, at both pH 7 and 10, the crystal violet staining indicated that *mptD* promoted the biofilm-forming ability of *E. faecalis* (Figure 1(b,c)). FE-SEM showed that at pH 7, Δ*mptD* and +*mptD* showed similar morphology to ATCC 29212, while at pH 10, ATCC 29212 exhibited cell aggregation, and Δ*mptD* showed cell aggregates as well as a larger cell size, while +*mptD* showed normal cell morphology (Figure 1(d)). Meanwhile, at pH 7, expression level of *mptD* did not affect the growth of *E. faecalis*, while at pH 10, overexpression of *mptD* promoted the growth of

Table 2. Primer sequences used in this study.

gene	Primer sequences 5′-3′ (forward)	Primer sequences 5′-3′ (reverse)
16S rRNA	AGCGGTGAAATGCGTAGATATATGGAG	CTCATCGTTTACGGCGTGGACTAC
V-atpA (RS03135)	ACTAATCGCCGTAATGCTTCCTTCAC	GAAATGAGTGGTCGGTTAGAAGAAATGC
V-atpI (RS03110)	TGAAGGAACATCAGCAGAGTGTAACC	AGGCGGAACATCATAACGGTATTGG
mptA (RS11130)	TTAATCGAACAAGCAGCTCCTCCAG	TTTCTACGCCGCCTTCAACCAC
mptB (RS11125)	ACAGGCAAGCGAATGATTACGAATG	TGGCTGTCTGCTGCGACTTTTC
mptC (RS11135)	ACTCGTTGCCTTTCTAGCTGGTATG	TGCTACTAACGGTTGGTGGAATTGG
mptD (RS11140)	ATACACACCCATACATTGCCTCACC	GGACCCATCATCCCAACTTTAACCC
AA-ABC (RS12135)	GGTGGACAAAAGCAACGGGTAG	TAGCACTTCGCCAACCATTTCAG
AAP (RS15175)	GTCCAGGGCTCGTCTTATCATTTC	CATCCAAGCCACAATTTCTCCAAAG
ktrC (RS09125)	TGTCCCGTATATTGATGCATTTTGG	TGATTGCTGCAACTCTACCTAATGG
trkH (RS12510)	CAACTTCCGCCGTCTGTGTTAC	AAGCCTAAGCCGCCTAATTCAATC

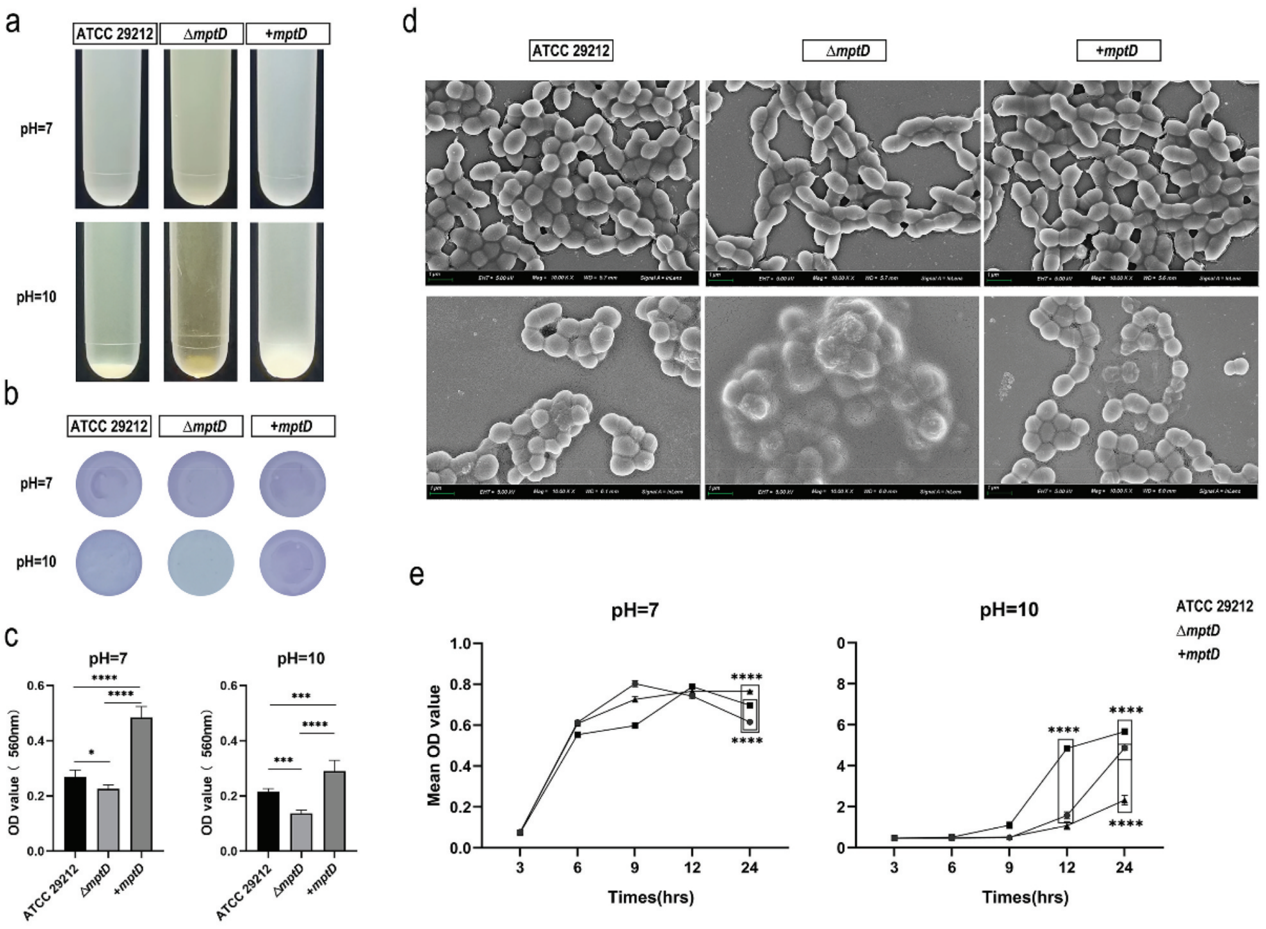

Figure 1. Biological characteristics, growth kinetic assay and morphology of *E. faecalis* at pH 7 and 10. (a) Bacterial suspensions of ATCC 29212, Δ*mptD* and +*mptD*. (b, c) crystal violet staining biofilms and biofilm mass analysis of ATCC 29212, Δ*mptD* and +*mptD*. (d) Cell morphologies of ATCC 29212, Δ*mptD* and +*mptD* at exponential growth phase (10,000×). (e) Growth kinetics of ATCC 29212, Δ*mptD* and +*mptD* for 24 h. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

E. faecalis (Figure 1(e)). Based on the survival status and biofilm quality of Δ*mptD* and +*mptD* strains after 24 h, it was confirmed that Man-PTS EII has a regulatory effect on the alkaline resistance of *E. faecalis*.

mptD *promoted energy metabolism and maintained pH homeostasis in* E. faecalis

To further elucidate the regulatory role of Man-PTS EII in alkaline resistance and pH homeostasis maintenance in *E. faecalis*, we conducted comparative analyses of membrane potential dynamics, ion flux homeostasis, and energy metabolism between Δ*mptD* and +*mptD* strains. The membrane potential of all *E. faecalis* exposed to pH 10 showed different degrees of hyperpolarization compared to pH 7 (Figure 2(a)). At pH 7, the expression level of *mptD* did not affect the membrane potential and permeability of *E. faecalis*, but at pH 10, overexpression of *mptD* enhanced membrane hyperpolarization, and membrane permeability was significantly decreased. Loss of *mptD* resulted in reduced hyperpolarization and significantly increased membrane permeability (Figure 2(a-c)). The intracellular K^+ concentration increased at pH 10 compared to pH 7. The expression level of *mptD* did not affect the intracellular K^+ concentration at pH 7, while at pH 10, overexpression of *mptD* caused a decrease in intracellular K^+ concentration (Figure 3(a, b)). Intracellular pH measurements showed no significant difference among different strains at pH 7, while at pH 10, intracellular pH of Δ*mptD* was significantly higher and intracellular pH of +*mptD* was significantly lower than ATCC 29212 (Figure 3(c)). At pH 7, the expression level of *mptD* did not affect the intracellular ATP content in *E. faecalis*, while at pH 10, overexpression of *mptD* showed a significant increase in intracellular ATP content (Figure 3(d)). These results indicated that Man-PTS EII is an ATP-dependent regulator of pH equilibrium that enhances intracellular pH homeostasis of *E. faecalis* in alkaline environments through regulating ion transport and membrane dynamics.

mptD *regulated the expression of Man-PTS EII and membrane transporter components in* E. faecalis

To investigate the effect of Man-PTS EII on genes related to membrane transport of *E. faecalis*, we evaluated the expression of other Man-PTS EII components and

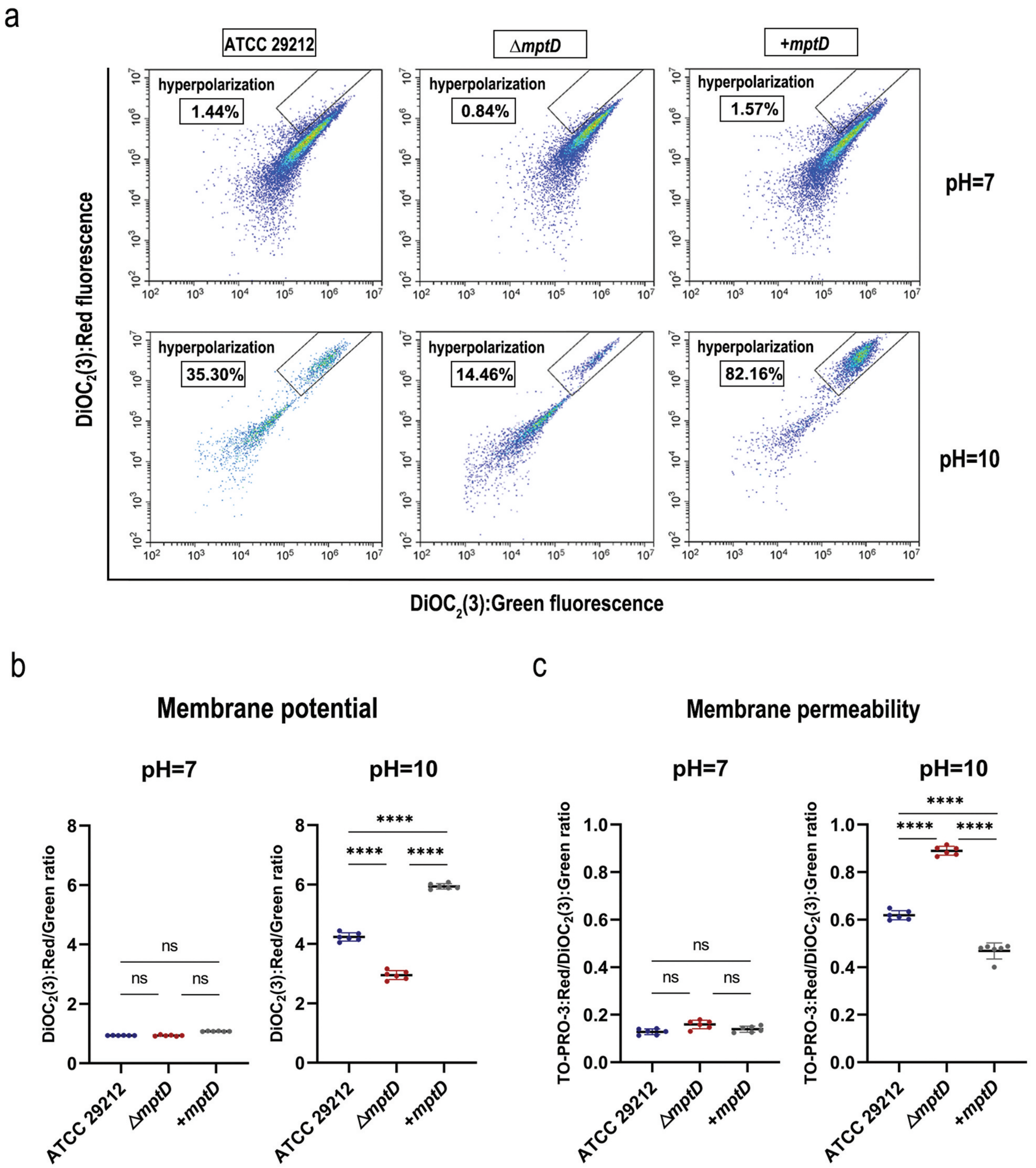

Figure 2. The membrane potential of *E. faecalis* at pH 7 and 10. (a) Flow cytometry dot plots showing membrane potential of ATCC 29212, Δ*mptD* and +*mptD*, gates indicate the proportion of the hyperpolarized cell population. (b, c) membrane potential and permeability of ATCC 29212, Δ*mptD* and +*mptD*. ****$p < 0.0001$.

membrane transport-related genes (V-ATPase, proton/potassium ion countertransporters, and amino acid transporters) in Δ*mptD* and +*mptD* strains using RT-qPCR at pH 10. The expression of *mptD* was positively correlated with that of the other three Man-PTS EII constituent factors (*mptA*, *mptB* and *mptC*) (Figure 4(a)). This correlation attests to the role of *mptD* in the positive regulation of the expression of other Man-PTS EII components. Furthermore, the results indicated that *mptD* promoted the expression of genes related to V-ATPase, proton/potassium ion countertransporter, and amino acid transporter (Figure 4(b-d)). These findings suggest that Man-PTS EII, functioning as an ATP-dependent regulatory hub, plays a pivotal role in facilitating the translocation of protons and amino acids through the action of V-ATPase.

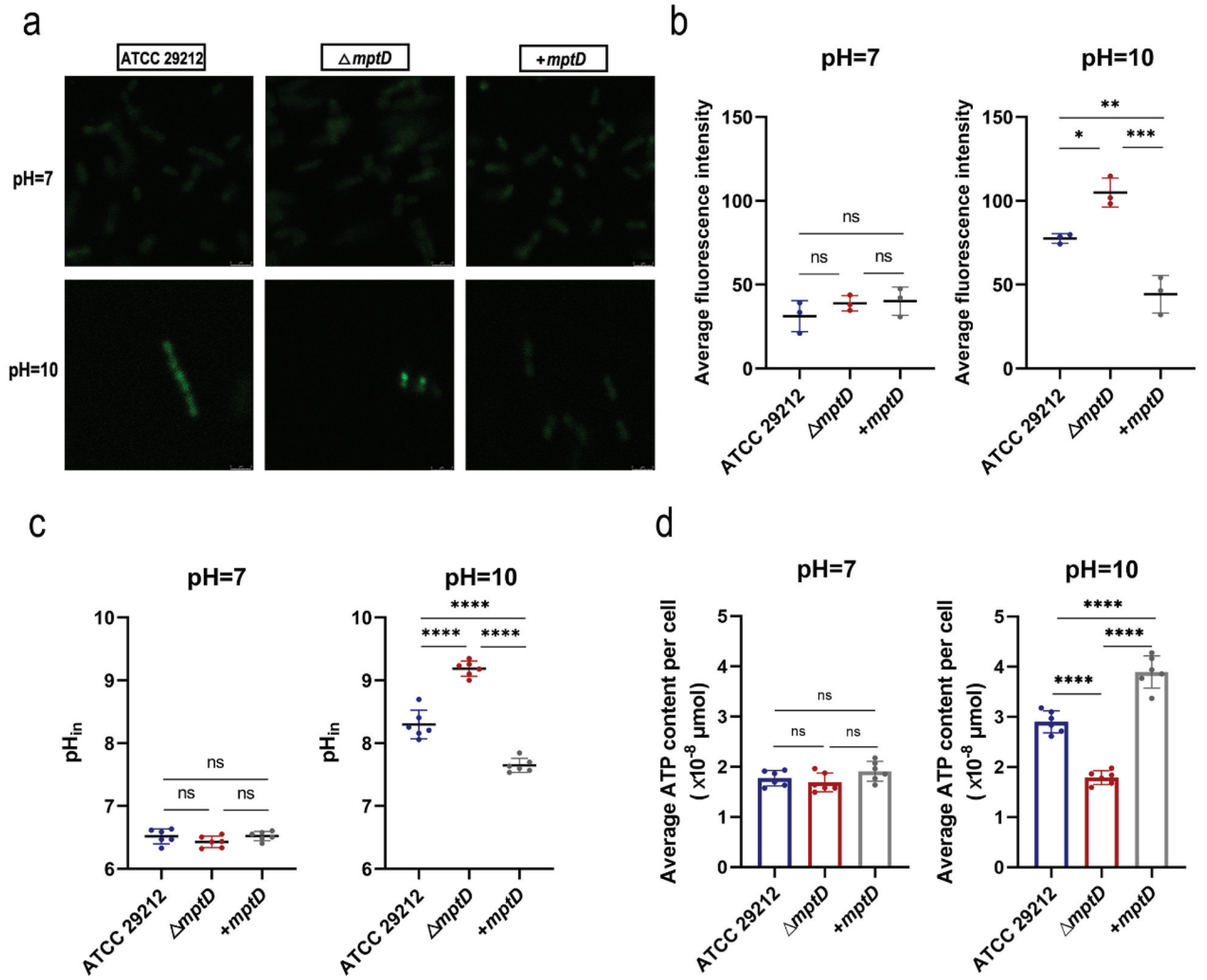

Figure 3. The intracellular potassium ions concentration and pH, as well as cellular energy metabolism of *E. faecalis* at pH 7 and 10. (a) Intracellular potassium ion (K^+) concentration of ATCC 29212, Δ*mptD* and +*mptD* are indicated by fluorescence intensity (green). (b) Quantification of intracellular K^+ concentration of ATCC 29212, Δ*mptD* and +*mptD*. (c) Intracellular pH (pH_{in}) of ATCC 29212, Δ*mptD* and +*mptD*. (d) Cellular ATP concentration of ATCC 29212, Δ*mptD* and +*mptD*. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Ranking and selection of E. faecalis *with high, medium and low alkaline resistance*

Depending on the results that Man-PTS EII is a key factor in the interaction between metabolism and ion membrane transport during the alkaline resistance process of *E. faecalis*, we propose that the alkaline resistance capacity of *E. faecalis* is intricately linked to the Man-PTS EII. To confirm this hypothesis, we isolated six clinical *E. faecalis* strains (XS 001, XS 002, XS 003, LY 001, LY 002 and LY 003) from root canals of teeth with failed root canal treatment. We compared the alkaline resistance of these six strains, along with two standard laboratory *E. faecalis* strains (ATCC 29212 and ATCC 51299), through alkaline resistance analysis. Considering that bacteria suspensions in alkaline media may contain non-viable bacterial cells, a combination of dynamic growth analysis and CFUs counting method was used for this analysis. The alkaline resistance profiles of eight strains were quantitatively assessed through dynamic growth curve analysis and CFU count methods (Figure 5(a, b)). Using ATCC 29212 as a reference, three strains of *E. faecalis* with high (XS 003), medium (ATCC 29212) and low (LY 002) alkaline resistance were selected for subsequent experiments.

DEGs of the three E. faecalis *strains with different alkaline resistance*

To further verify the regulatory effect of Man-PTS EII on the alkaline resistance of *E. faecalis*, the gene expression patterns of *E. faecalis* ATCC 29212 (moderate resistance, M group), XS 003 (high resistance, H group) and LY 002 (low resistance, L group) were compared using RNA-seq analysis. The overall distribution of DEGs among the three groups is shown in Figure 6(a). Specifically, 1,300 DEGs were identified between

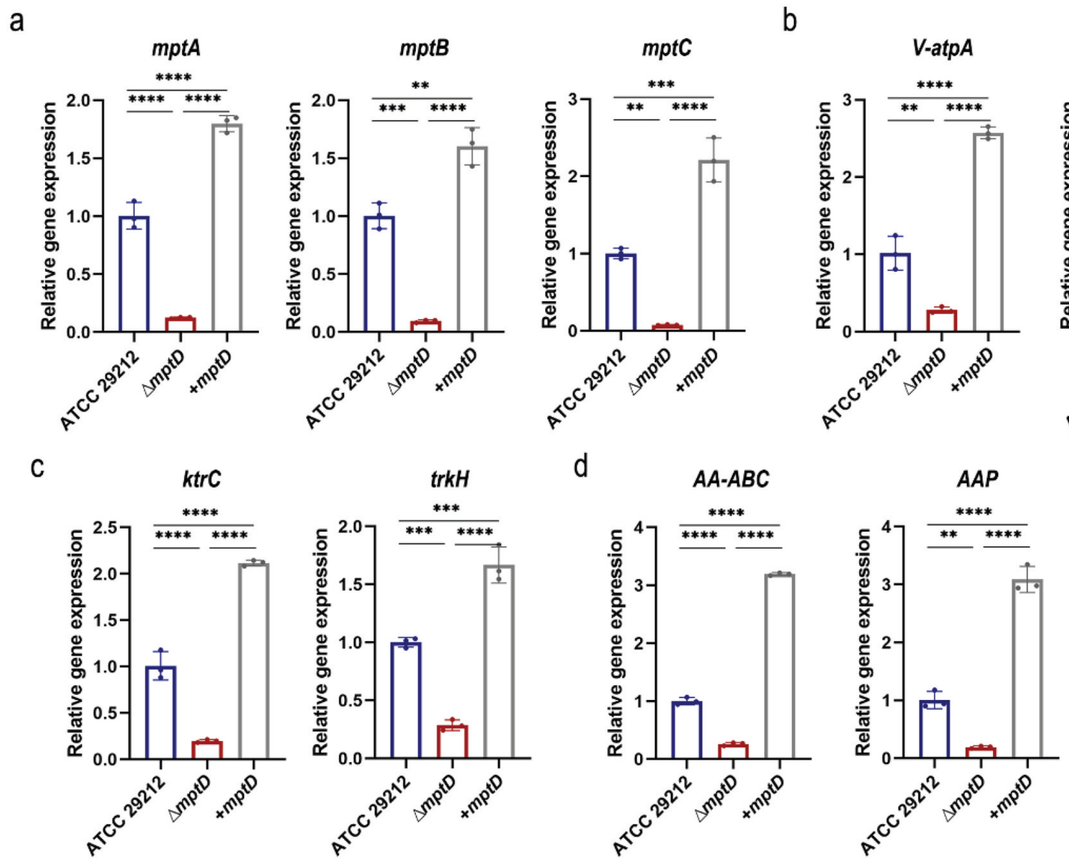

Figure 4. *mptD* regulon expression in membrane translocation and amino acid metabolic components of *E. faecalis*. (a) RT-qPCR analysis of gene expression of Man-PTS EII. (b) RT-qPCR analysis of gene expression of V-ATPase. (c) RT-qPCR analysis of gene expression of proton/potassium ion countertransporter. (d) RT-qPCR analysis of gene expression of amino acid transporter. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

the H and M groups, with 772 upregulated and 528 downregulated genes. Similarly, 802 DEGs were observed between the L and M groups, including 375 upregulated and 427 downregulated genes. Additionally, 1,430 DEGs were detected between the H and L groups, with 804 upregulated and 626 downregulated genes. KEGG enrichment analysis suggested that DEGs between the three groups were enriched in pathways related to metabolism, especially carbohydrate metabolism and amino acid metabolism (Figure 6(b)). COG analysis showed DEGs for 19 predicted pathways. The number of up- and down-regulated genes in each functional category is shown in Table 3. Among the three groups, significant differences were found in genes encoding carbohydrate transport and metabolism, amino acid transport and metabolism, inorganic ion transport and metabolism, energy production and conversion, transcription and nucleotide transport and metabolism. The enrichment of fructose and mannose metabolism was positively related to the rank of alkaline resistance of three groups (Figure 7(a)).

Alkaline resistance of E. faecalis *positively correlated with the expression of Man-PTS EII, membrane transport and amino acid metabolism genes*

Based on the RNA-seq analysis results, we focused on the relationship between the expression level of Man-PTS EII and the level of alkaline resistance in *E. faecalis*. We observed that the higher the alkaline resistance of *E. faecalis*, the higher the expression of Man-PTS EII genes (Figure 7(b)). Meanwhile, the expression of membrane translocation and amino acid metabolic components was correspondingly elevated (Figure 7(b)). Additionally, we compared the expression of constituent factors of Man-PTS EII in ATCC 29212, XS 003 and LY 002 strains at pH 7 and 10 using RT-qPCR. The results showed that the expression of Man-PTS EII constitutive factors in ATCC 29212, XS 003 and LY 002 was all up-regulated (Figure 7(c)). Notably, compared with LY 002, the expression of Man-PTS EII-related genes showed significant upregulation in XS 003. These findings indicate that the overall gene expression level of Man-PTS EII was positively correlated with the alkaline resistance of *E. faecalis*.

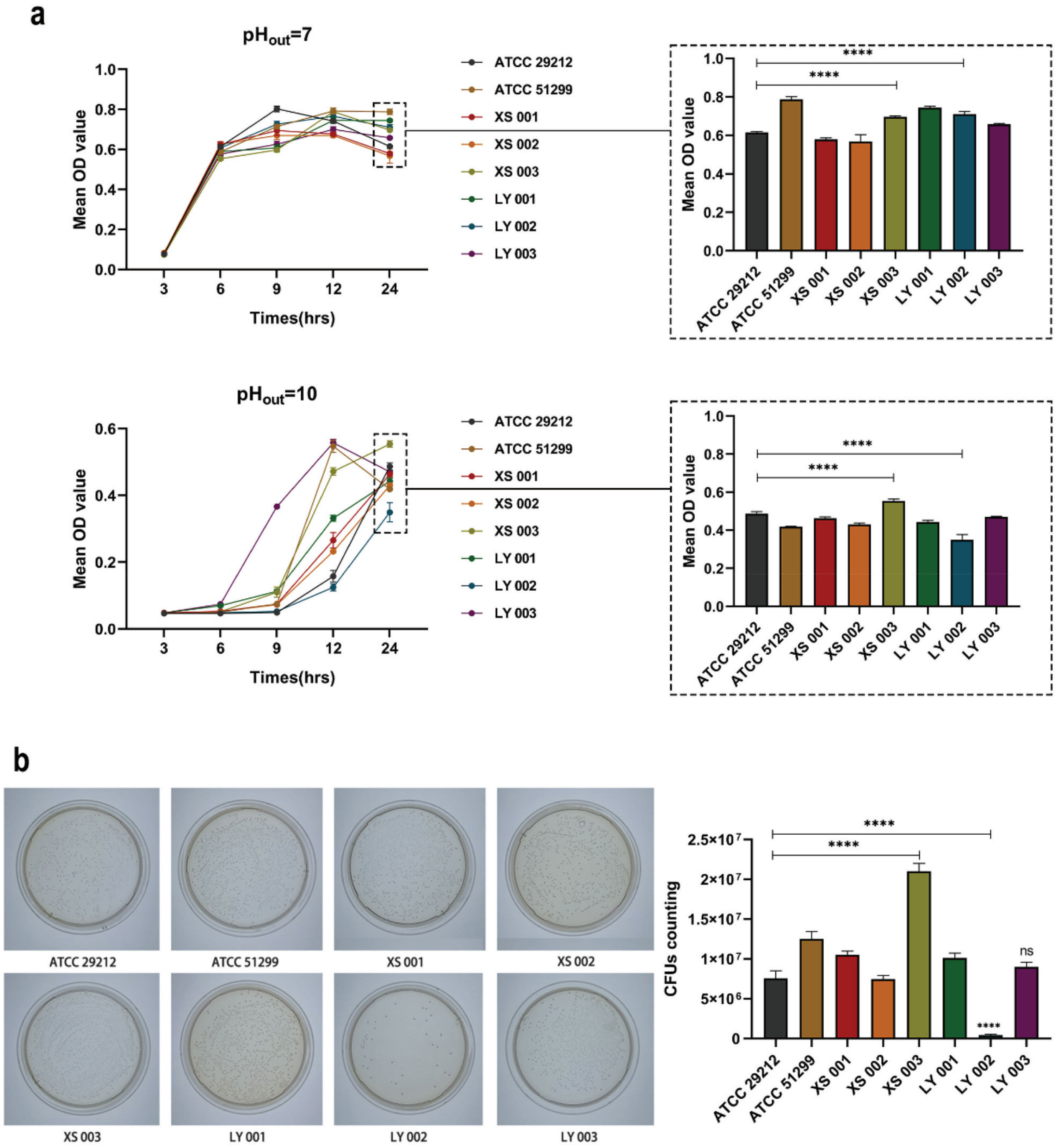

Figure 5. The alkaline resistance evaluation of *E. faecalis*. (a) Dynamic growth curves of *E. faecalis* at pH 7 and 10. (b) Representative images of CFUs and CFUs-counting comparison among groups after incubation at pH 10 for 24 h. ****$p < 0.0001$.

Discussion

The post-treatment apical periodontitis is associated with persistent or secondary endodontic infection in the root canal system and periapical tissues [27]. Although discrepancies exist, the detection of *E. faecalis* in root canals from patients with persistent periapical infections or failed root canal treatment is mostly supported by both previous and recent studies [2,12]. *E. faecalis* has been reported to be a highly alkaline-resistant bacterium [28,29]. Due to the buffering effect of dentin, the most often used intracanal medicament calcium hydroxide can only produce an alkaline environment with a pH of about 10 in the root canal [30,31]. Therefore, an alkaline environment with a pH of 10 was used in this study to explore the mechanisms behind the alkaline resistance of *E. faecalis*.

Man-PTS, as a part of PTS regulatory network, plays an important role in stress survival of bacteria, and Man-PTS EII is the key complex of the Man-PTS. Previous studies have revealed that the Man-PTS EII could act as a regulator to promote the adaptation of *E. faecalis* to stressful conditions, thereby enhancing the capability of bacterial survival

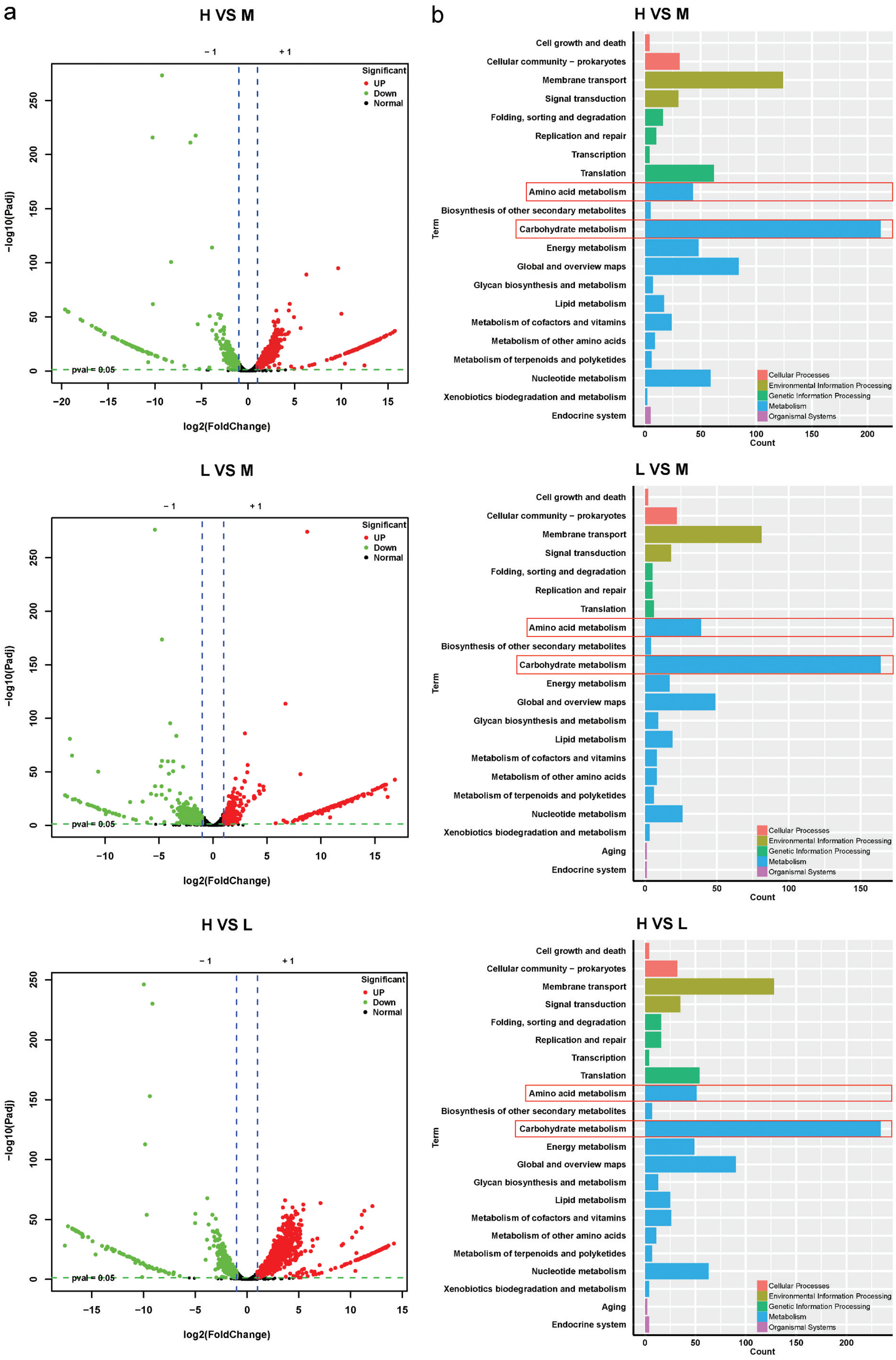

Figure 6. Transcriptomic analysis of *E. faecalis* with high, medium and low alkalinity resistance at pH 10. (a) Volcano plot depicting the distribution of DEGs. (b) KEGG classification diagram of DEGs.

Table 3. The number of differentially expressed genes of *E. faecalis* with different alkaline resistance under alkaline condition.

	H VS M			L VS M			H VS L		
COG category	Genes	DUGs	DDGs	Genes	DUGs	DDGs	Genes	DUGs	DDGs
Energy production and conversion	64	37	27	41	11	30	68	44	24
Cell cycle control, cell division, chromosome partitioning	10	2	8	3	3	0	9	0	9
Amino acid transport and metabolism	85	51	34	61	17	44	91	63	28
Nucleotide transport and metabolism	50	33	17	26	8	18	54	32	22
Carbohydrate transport and metabolism	131	95	36	123	28	95	149	122	27
Coenzyme transport and metabolism	22	17	5	9	7	2	26	16	10
Lipid transport and metabolism	21	7	14	22	18	4	28	4	24
Translation, ribosomal structure and biogenesis	84	8	76	12	7	5	81	8	73
Transcription	67	45	22	36	20	16	83	47	36
Replication, recombination and repair	33	19	14	11	7	4	36	19	17
Cell wall/membrane/envelope biogenesis	43	19	24	35	26	9	55	16	39
Cell motility	5	3	2	2	1	1	6	3	3
Posttranslational modification, protein turnover, chaperones	26	5	21	11	8	3	28	5	23
Inorganic ion transport and metabolism	68	44	24	30	16	14	73	45	28
Secondary metabolites biosynthesis, transport and catabolism	11	8	3	8	5	3	11	7	4
Function unknown	167	92	76	105	65	41	204	94	111
Signal transduction mechanisms	25	13	12	20	9	11	27	15	12
Intracellular trafficking, secretion, and vesicular transport	13	8	5	4	1	3	17	8	9
Defense mechanisms	23	16	7	19	14	5	19	9	10

[18]. As the Man-PTS EIID is reported to be crucial in the selection and use of PTS-sugars by bacteria [18], its gene *mptD* was selected to study the role of Man-PTS EII in the alkaline resistance of *E. faecalis*. Results also confirmed that the expression of *mptD* could positively regulate the expression of other Man-PTS EII components (*mptA*, *mptB* and *mptC*). Thus, *mptD* may act as a positive regulator of Man-PTS EII operon. The study utilized *mptD* deletion and overexpression strains to elucidate that the Man-PTS EII functions as an ATP-dependent regulator of pH homeostasis. By redirecting ATP flux, this mechanism enhances the survival of *E. faecalis* in an alkaline environment.

Numerous studies have shown that transmembrane ion transport plays an important role in the pH homeostasis of *Enterococcus* [32,33]. The membrane potential of bacteria is one of the important factors for maintaining membrane transport function and cellular metabolism. Alterations in membrane potential would affect the intracellular environment and functions of bacteria, such as bioenergy synthesis and ion channel activity [34,35]. The two members of the monovalent cation/proton antiporter, *trkH* and *ktrC*, contribute to potassium homeostasis, maintaining intracellular protons and expelling potassium ions from the cell [36,37]. Meanwhile, V-ATPase provides a membrane voltage (Vm) for the monovalent cation/proton antiporter by hydrolyzing ATP, thereby maintaining pH homeostasis under alkaline conditions [11,38]. The results of this study showed that in an alkaline environment, Man-PTS EII participates in carbohydrate metabolism to generate energy, thereby promoting V-ATPase to provide Vm for the monovalent cation/proton antiporter, which leads to increased potassium ion efflux, proton influx and hyperpolarization of the membrane potential.

Previous studies have demonstrated that amino acid transport and metabolic pathways contribute to the survival of *E. faecalis* under alkaline conditions [21,39]. The metabolism of amino acids facilitates the response of bacteria to osmotic changes and supports survival and growth. Amino acid metabolizing enzymes participate in the catabolic pathways of arginine and glutamate, and convert carbon into acids, which helps maintain pH levels [32,40]. Proline has a strong hydration ability, which helps maintain the osmotic balance, prevent cell deformation and remove reactive oxygen species [41]. The Vm generated by V-ATPase contributes to the PMF, which drives amino acid transmembrane transport [42]. Amino acids can generate pyruvate through deamination, which can enter the tricarboxylic acid cycle and supply energy through PTS [43]. However, amino acid metabolism remains a neglected pathway in the regulation of alkaline resistance. In this study, we found that Man-PTS EII regulates the expression of amino acid ABC transporter ATP binding protein (*AA-ABC*) and amino acid osmotic enzyme (*AAP*), thus maintaining amino acid transport and metabolism in *E. faecalis* under alkaline stress.

As shown in Figure 8, our findings reveal that under alkaline stress, *E. faecalis* utilizes Man-PTS EII to drive carbohydrate catabolism, thereby increasing intracellular ATP levels. Acting as an ATP-dependent regulatory hub, Man-PTS EII energizes V-ATPase-mediated proton translocation. The resulting Vm and PMF generated by V-ATPase activity upregulate the expression of monovalent cation/proton antiporter (*trkH* and *ktrC*), amino acid transporters (*AA-ABC*) and osmoregulatory enzymes (*AAP*). This process triggers membrane hyperpolarization. The degree of hyperpolarization positively correlates with both the energy available for membrane transport and the driving force for potassium ion efflux, proton influx and amino acid uptake. Through PMF-

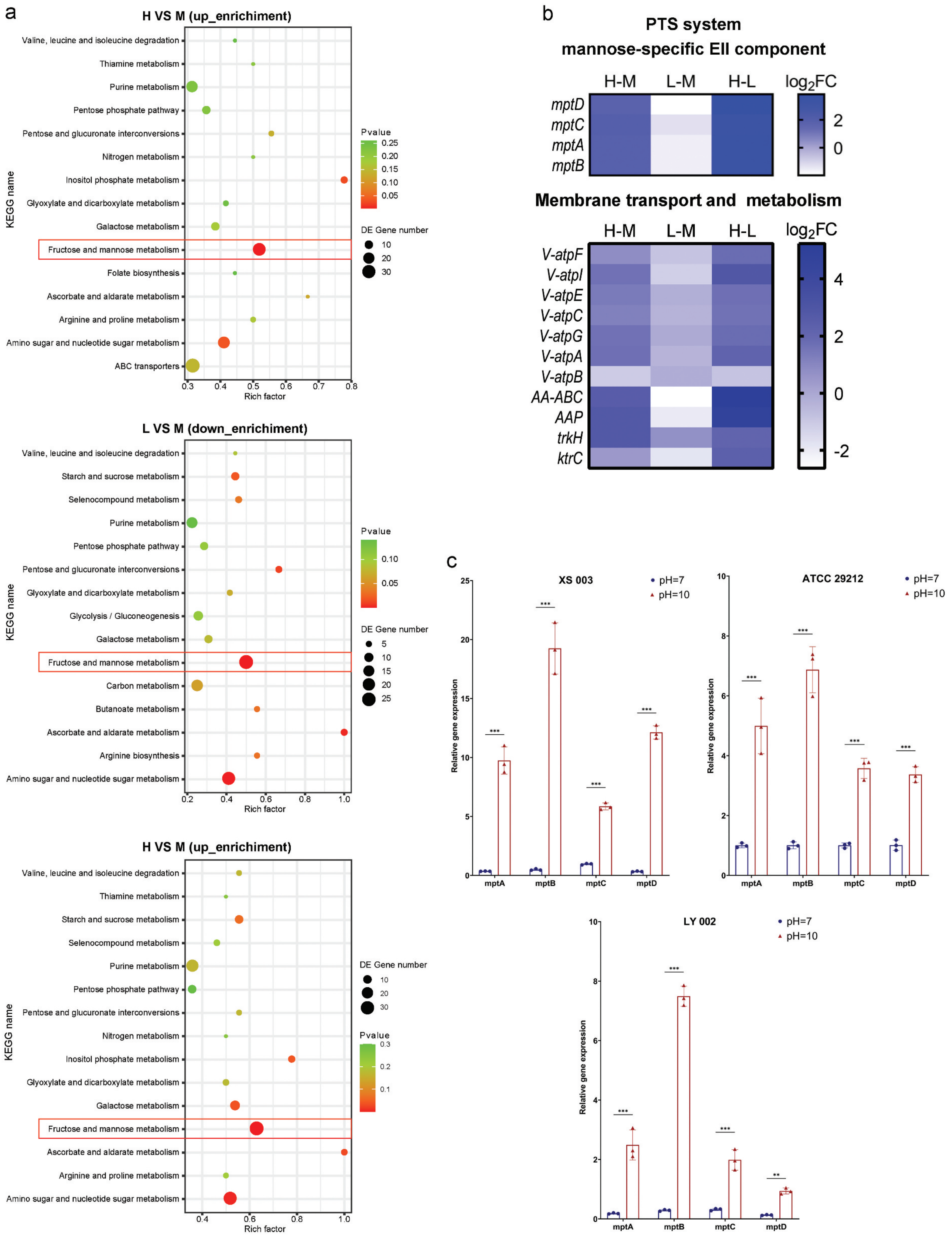

Figure 7. Alkaline resistance of *E. faecalis* positively correlated with the expression of Man-PTS EII, membrane transport and amino acid metabolism genes. (a) KEGG enrichment analysis of upregulated or downregulated DEGs. (b) Selected differential expression genes involved in Man-PTS EII and membrane transport and amino acid metabolism. (c) Comparison of mRNA expression levels of Man-PTS EII at pH 10 by RT-qPCR in *E. faecalis* compared to pH 7. ***$p < 0.001$; **$p < 0.01$.

dependent influx of pH-neutralizing solutes and amino acids, the system coordinately regulates metabolic adaptation, ion transport and osmotic homeostasis to maintain intracellular pH homeostasis. This process enhances alkaline resistance and ultimately ensures bacterial survival in alkaline environments.

Previous studies have demonstrated that clinical *E. faecalis* strains exhibit higher alkaline resistance

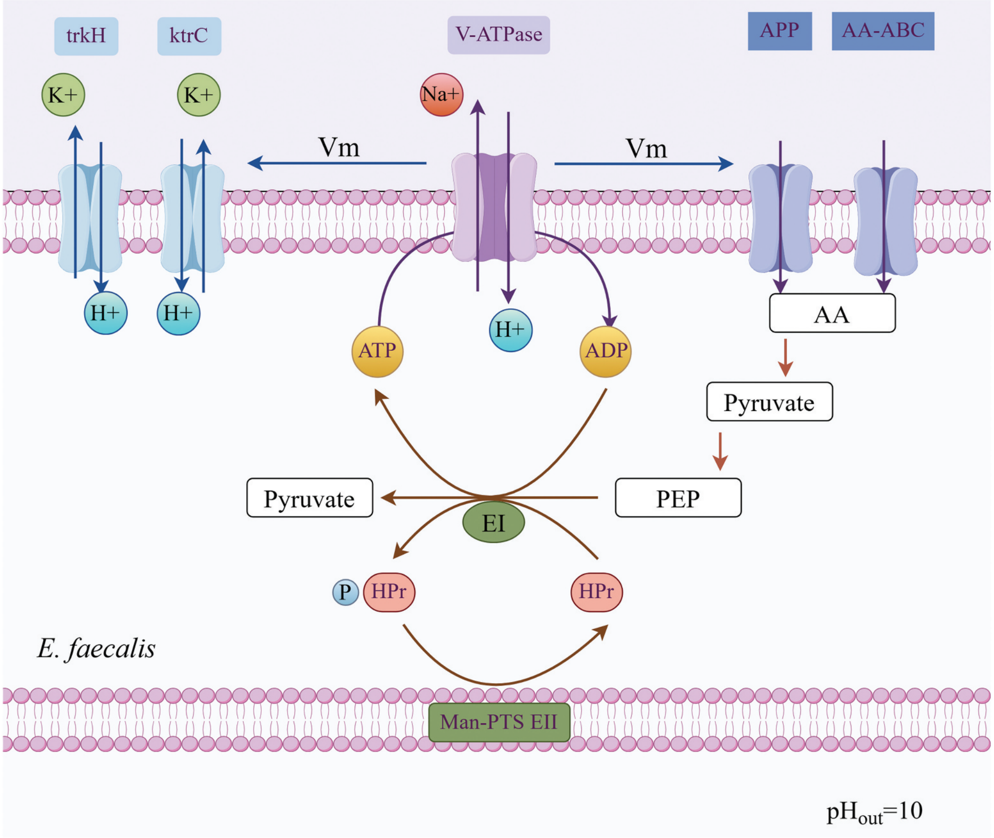

Figure 8. Illustration of potential role of Man-PTS EII in the alkaline resistance of *E. faecalis*.

compared to standard laboratory strains [12,13]. In this study, alkaline resistance varied significantly among clinical *E. faecalis* strains, with some being very high while others being very low. This variability suggests divergent survival capabilities of clinical *E. faecalis* strains in alkaline environments, likely attributable to strain-specific molecular mechanisms. To investigate the correlation between Man-PTS and the alkaline resistance, three strains of *E. faecalis* with different alkaline resistance were selected for RNA-seq analysis. Results revealed a positive correlation between *E. faecalis* alkaline resistance and Man-PTS EII expression levels, further supporting the involvement of Man-PTS EII in regulating alkaline resistance.

However, some studies have shown that genes and proteins related to the Man-PTS and glucose metabolism were downregulated in *E. faecalis* under alkaline stress [21,39]. This discrepancy likely stems from divergent experimental designs across studies, including variations in alkaline medium preparation methods and nutrient composition (e.g. carbohydrates, amino acids) [44]. Previous studies used tryptic soy broth (TSB) to prepare alkaline media to study changes in genes and proteins of *E. faecalis* under starvation and alkaline stress, while this study used BHI with full nutrients to explore changes in *E. faecalis* under alkaline stress. Although Man-PTS EII could transport both mannose and glucose, glucose is easily isomerized and converted to mannose under alkaline conditions [45,46]. Compared to TSB, the carbohydrates in BHI provide higher alkaline resistance for *E. faecalis* through the Man-PTS metabolic pathway. The glucose-containing BHI-based alkaline media of this study may help reveal the effect of carbohydrates on the alkaline resistance of *E. faecalis* through the Man-PTS EII.

Based on the findings of this study, Man-PTS EII is a key factor of the interaction between metabolic and ion membrane transport processes in the alkaline resistance of *E. faecalis*. This study may provide new insights for understanding the alkaline resistance of *E. faecalis* and develop strategies for the treatment of persistent root canal and periapical infections of human teeth.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

CRediT: **Runze Liu:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft; **Yi Luo:** Data curation, Formal analysis, Methodology, Software, Validation; **Pei Liu:** Data curation, Formal analysis, Software, Validation; **Shuo Xiao:** Data curation, Formal analysis, Validation; **Wei Fan:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing; **Bing Fan:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Data availability statement

The data that support the findings of this study are included in this published manuscript and supplementary materials. The full RNA-seq dataset has been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA1224309.

Ethics approval statement

Clinical strains of *E. faecalis* were collected under the approval of the Ethics Committee of School & Hospital of Stomatology, Wuhan University (No. [2022] A20).

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References

[1] Ricucci D, J Siqueira AB Jr., Bate AL, et al. Histologic investigation of root canal–treated teeth with apical periodontitis: a retrospective study from twenty-four patients. J Endod. 2009;35(4):493–502. doi: 10.1016/j.joen.2008.12.014

[2] Suriyanarayanan T, Lee LS, Han SHY, et al. Targeted metabolomics analysis approach to unravel the biofilm formation pathways of Enterococcus faecalis clinical isolates. Int Endod J. 2024;57(10):1505–1520. doi: 10.1111/iej.14110

[3] Keskin C, Demiryürek EÖ, Onuk EE. Pyrosequencing analysis of cryogenically ground samples from primary and secondary/persistent endodontic infections. J Endod. 2017;43(8):1309–1316. doi: 10.1016/j.joen.2017.03.019

[4] Ordinola-Zapata R, Costalonga M, Nixdorf D, et al. Taxonomic abundance in primary and secondary root canal infections. Int Endod J. 2022;56(2):278–288. doi: 10.1111/iej.13864

[5] Özok AR, Persoon IF, Huse SM, et al. Ecology of the microbiome of the infected root canal system: a comparison between apical and coronal root segments. Int Endod J. 2012;45(6):530–541. doi: 10.1111/j.1365-2591.2011.02006.x

[6] Gaeta C, Marruganti C, Ali IAA, et al. The presence of Enterococcus faecalis in saliva as a risk factor for endodontic infection. Front Cell Infect Microbiol. 2023;13 :1061645. doi: 10.3389/fcimb.2023.1061645

[7] Hou Y, Wang L, Zhang L, et al. Potential relationship between clinical symptoms and the root canal microbiomes of root filled teeth based on the next-generation sequencing. Int Endod J. 2021;55(1):18–29. doi: 10.1111/iej.13640

[8] Gomes BP, Berber VB, Kokaras AS, et al. Microbiomes of endodontic-periodontal lesions before and after chemomechanical preparation. J Endod. 2015;41(12):1975–1984. doi: 10.1016/j.joen.2015.08.022

[9] Ran S, He Z, Liang J. Survival of Enterococcus faecalis during alkaline stress: changes in morphology, ultrastructure, physiochemical properties of the cell wall and specific gene transcripts. Arch Oral Biol. 2013;58(11):1667–1676. doi: 10.1016/j.archoralbio.2013.08.013

[10] Ran S, Wang J, Jiang W, et al. Assessment of dentinal tubule invasion capacity of Enterococcus faecalis under stress condition sex vivo. Int Endod J. 2015;48(4):362–372. doi: 10.1111/iej.12322

[11] Gaca A, Lemos J. Adaptation to adversity: the intermingling of stress tolerance and pathogenesis in enterococci. Microbiol Mol Biol Rev. 2019;83(3):e00008–19. doi: 10.1128/MMBR.00008-19

[12] Salem AS, Tompkins GR, Cathro PR. Alkaline tolerance and biofilm formation of root canal isolates of Enterococcus faecalis: an in vitro study. J Endod. 2022;48(4):542–547. doi: 10.1016/j.joen.2022.01.006

[13] Weckwerth PH, Zapata RO, Vivan RR, et al. In vitro alkaline pH resistance of Enterococcus faecalis. Braz Dent J. 2013;24(5):474–476. doi: 10.1590/0103-6440201301731

[14] Chen D-D, Ahmad M, Liu Y-H, et al. Transcriptomic responses of haloalkalitolerant bacterium Egicoccus halophilus EGI 80432T to highly alkaline stress. Extremophiles. 2021;25(5–6):459–470. doi: 10.1007/s00792-021-01239-8

[15] Lim S, Seo HS, Jeong J, et al. Understanding the multifaceted roles of the phosphoenolpyruvate: phosphotransferase system in regulation of Salmonella virulence using a mutant defective in ptsI and crr expression. Microbiol Res. 2019;223–225:63–71. doi: 10.1016/j.micres.2019.04.002

[16] Jeckelmann J-M, Erni B. The mannose phosphotransferase system (Man-PTS) - mannose transporter and receptor for bacteriocins and bacteriophages. Biochim Biophys Acta (BBA) - Biomembr. 2020;1862(11):183412. doi: 10.1016/j.bbamem.2020.183412

[17] Prell J, Mulley G, Haufe F, et al. The PTS ntr system globally regulates atp-dependent transporters in Rhizobium leguminosarum. Mol Microbiol. 2012;84(1):117–129. doi: 10.1111/j.1365-2958.2012.08014.x

[18] Peng Z, Ehrmann MA, Waldhuber A, et al. Phosphotransferase systems in Enterococcus faecalis OG1RF enhance anti-stress capacity in vitro and in vivo. Res Microbiol. 2017;168(6):558–566. doi: 10.1016/j.resmic.2017.03.003

[19] Galinier A, Deutscher J. Sophisticated regulation of transcriptional factors by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. J Mol Biol. 2017;429(6):773–789. doi: 10.1016/j.jmb.2017.02.006

[20] Le Bouguenec C, Schouler C. Sugar metabolism, an additional virulence factor in enterobacteria. Int J Med Microbiol. 2011;301(1):1–6. doi: 10.1016/j.ijmm.2010.04.021

[21] Ran S, Liu B, Jiang W, et al. Transcriptome analysis of Enterococcus faecalis in response to alkaline stress. Front Microbiol. 2015;6:795. doi: 10.3389/fmicb.2015.00795

[22] Liu R, Liu P, Luo Y, et al. Metformin reduced the alkaline resistance of Enterococcus faecalis against calcium hydroxide via man-pts EII: in vitro and in vivo studies. Clin Oral Investig. 2024;28(10):520. doi: 10.1007/s00784-024-05909-7

[23] Zhu X, Wang Q, Zhang C, et al. Prevalence, phenotype, and genotype of Enterococcus faecalis isolated from saliva and root canals in patients with persistent apical periodontitis. J Endod. 2010;36(12):1950–1955. doi: 10.1016/j.joen.2010.08.053

[24] Sakamoto M, Siqueira JF Jr, Rôças IN, et al. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. Oral Microbiol Immunol. 2008;23(4):275–281. doi: 10.1111/j.1399-302X.2007.00423.x

[25] 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. Microb Pathog. 2018;115:117–122. doi: 10.1016/j.micpath.2017.12.028

[26] Fan W, Sun Q, Li Y, et al. Synergistic mechanism of Ag±Zn2+ in anti-bacterial activity against Enterococcus faecalis and its application against dentin infection. J Nanobiotechnology. 2018;16(1):10. doi: 10.1186/s12951-018-0336-3

[27] Matoso FB, Montagner F, Grecca FS, et al. Microbial composition and diversity in intraradicular biofilm formed in situ: new concepts based on next-generation sequencing. Mol Oral Microbiol. 2024;39(5):393–406. doi: 10.1111/omi.12463

[28] Kayaoglu G, Erten H, Ørstavik D. Growth at high pH increases Enterococcus faecalis adhesion to collagen. Int Endod J. 2005;38(6):389–396. doi: 10.1111/j.1365-2591.2005.00957.x

[29] Chen W, Liang J, He Z, et al. Differences in the chemical composition of Enterococcus faecalis biofilm under conditions of starvation and alkalinity. Bioengineered. 2017;8(1):1–7. doi: 10.1080/21655979.2016.1226655

[30] Haapasalo H, Siren E, Waltimo T, et al. Inactivation of local root canal medicaments by dentine: an in vitro study. Int Endod J. 2000;33(2):126–131. doi: 10.1046/j.1365-2591.2000.00291.x

[31] Evans M, Davies J, Sundqvist G, et al. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J. 2002;35(3):211–228. doi: 10.1046/j.1365-2591.2002.00504.x

[32] Mamo G. Challenges and adaptations of life in alkaline habitats. Adv Biochem Eng Biotechnol. 2020;172:85–133. doi: 10.1007/10_2019_97

[33] Padan E, Bibi E, Ito M, et al. Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta. 2005;1717(2):67–88. doi: 10.1016/j.bbamem.2005.09.010

[34] Strahl H, Hamoen LW. Membrane potential is important for bacterial cell division. Proc Natl Acad Sci USA. 2010;107(27):12281–12286. doi: 10.1073/pnas.1005485107

[35] Prindle A, Liu J, Asally M, et al. Ion channels enable electrical communication in bacterial communities. Nature. 2015;527(7576):59–63. doi: 10.1038/nature15709

[36] Acciarri G, Gizzi FO, Torres Manno MA, et al. Redundant potassium transporter systems guarantee the survival of Enterococcus faecalis under stress conditions. Front Microbiol. 2023;14:1117684. doi: 10.3389/fmicb.2023.1117684

[37] Stautz J, Hellmich Y, Fuss MF, et al. Molecular mechanisms for bacterial potassium homeostasis. J Mol Biol. 2021;433(16):166968. doi: 10.1016/j.jmb.2021.166968

[38] Roh H, Kim DH. Genotypic and phenotypic characterization of highly alkaline-resistant Carnobacterium maltaromaticum V-Type ATPase from the dairy product based on comparative genomics. Microorganisms. 2021;9(6):1233. doi: 10.3390/microorganisms9061233

[39] Cathro P, McCarthy P, Hoffmann P, et al. Enterococcus faecalis V583 cell membrane protein expression to alkaline stress. FEMS Microbiol Lett. 2022;369(1):fnac082. doi: 10.1093/femsle/fnac082

[40] Stancik LM, Stancik DM, Schmidt B, et al. pH-dependent expression of periplasmic proteins and amino acid catabolism in Escherichia coli. J Bacteriol. 2002;184(15):4246–4258. doi: 10.1128/JB.184.15.4246-4258.2002

[41] Mahipant G, Paemanee A, Roytrakul S, et al. The significance of proline and glutamate on butanol chaotropic stress in Bacillus subtilis 168. Biotechnol Biofuels. 2017;10:122. doi: 10.1186/s13068-017-0811-3

[42] Harvey WR, Boudko DY, Rheault MR, et al. NHEVNAT: an H+ V-ATPase electrically coupled to a Na+: nutrient amino acid transporter (NAT) forms an Na+/H+ exchanger (NHE). J Exp Biol. 2009;212(3):347–357. doi: 10.1242/jeb.026047

[43] Chen R, Hatzimanikatis V, Yap WM, et al. Metabolic consequences of phosphotransferase (PTS) mutation in a phenylalanine-producing recombinant Escherichia coli. Biotechnol Prog. 1997;13(6):768–775. doi: 10.1021/bp970060h

[44] Ake FM, Joyet P, Deutscher J, et al. Mutational analysis of glucose transport regulation and glucose-mediated virulence gene repression in listeria monocytogenes. Mol Microbiol. 2011;81(1):274–293. doi: 10.1111/j.1365-2958.2011.07692.x

[45] Keffeler EC, Iyer VS, Parthasarathy S, et al. Influence of the alternative sigma factor RpoN on global gene expression and carbon catabolism in Enterococcus faecalis V583. MBio. 2021;12(3):e00380–21. doi: 10.1128/mBio.00380-21

[46] Topper YJ, Stetten D. The alkali-catalyzed conversion of glucose into fructose and mannose. J Biol Chem. 1951;189(1):191–202. doi: 10.1016/S0021-9258(18)56109-3