Research Article

Antimicrobial Resistance, Virulence Profiles, and Public Health Significance of Enterococcus faecalis Isolated from Clinical Mastitis of Cattle in Bangladesh

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1. Introduction

Bovine mastitis is one of the most costly production diseases affecting dairy industries globally [1]. In Bangladesh, mastitis was estimated to cause an economic loss of 2.11 million US dollars annually due to reduced milk production and deteriorating milk quality [2]. The disease also has major public health importance because of the possibility of transmission of mastitis-causing pathogen to humans through milk.
occurrence of this pathogen was reported as 18% in mastitic cow's milk [4]. Besides, they are potential zoonotic pathogens [5]. *E. faecalis* was reported in animal-originated food including meat, milk, and their products and linked to human diseases such as urinary tract infection [5–10]. Their frequent incidence in food could indicate a zoonotic route for *E. faecalis* transmission to humans preferably through raw milk [11].

Enterococci are well known for having a high level of resistance against a wide variety of antimicrobial substances, developed by both intrinsic and acquired mechanisms. They are intrinsically resistant to virtually all cephalosporins, aminoglycosides, clindamycin, and trimethoprim-sulfamethoxazole [12]. *E. faecalis* are intrinsically susceptible to carbapenems, vancomycin, tetracycline, and fluoroquinolones; moreover, acquired resistance to the antibiotics through transposons or plasmids has been reported [12]. Due to their evolving resistance, mastitis caused by *E. faecalis* might be difficult to treat with most commercial antimicrobials.

In addition to antimicrobial resistance, virulence factors contribute to the pathogenesis of *E. faecalis*. Many researchers have reported several virulence factors in *E. faecalis* that might be involved in the severity of diseases in humans and animals [13]. Studies have reported the presence of virulence factors that facilitate adherence and colonization (*agg, ace*) and cytolysis and dissemination of *E. faecalis* into the host (*cyl, gelE*, and *sprE*) [14]. Furthermore, *E. faecalis* has biofilm-forming machinery (*pili, gelE*, and *fse* quorum-sensing systems) that allows the bacterium to adhere to biotic and abiotic surfaces and confers additional antimicrobial resistance [14]. The multifaceted virulence determinants enable this opportunistic pathogen to cause infections in the urinary tract, skin, soft tissue, abdomen, pelvis, central nervous systems, etc. in humans [15]. As enterococci can easily spread antimicrobial resistance or virulence genes to other bacterial species via horizontal transfer, the presence of enterococci in milk can enhance the emergence of MDR, transfer pathogenic *E. faecalis* to humans, and eventually affect the choice of drug [16].

To the best of our knowledge, no data yet exist from Bangladesh on the evaluation of antimicrobial resistance and virulence patterns in *E. faecalis* isolated from bovine clinical mastitis. Therefore, we aimed to conduct the present study to determine the antimicrobial resistance and virulence determinants in *E. faecalis* isolated from clinical mastitis of cattle having potential public health significance.

2. Materials and Methods

2.1. Sampling Site and Sampling. The present study was conducted in Dhaka (23.8105°N, 90.3372°E), Mymensingh (24.7539°N, 90.4073°E), and Sirajganj (24.3141°N, 89.5700°E) districts of Bangladesh between January 2019 and June 2021. A total of 105 milk samples were collected comprising 80 from cattle with clinical mastitis and 25 apparently healthy cattle. Cow with clinical mastitis was identified by the residential farm veterinarian based on gross changes in the udder (redness, swelling, and sensitive udder) and/or milk (flakes and/or clots). For the identification of apparently healthy cattle, the California Mastitis Test was used [17]. Cow's milk showing a negative CMT score, i.e., no visible precipitate on paddle movement, was considered apparently healthy. The milk samples were collected from the major dairy farms having a history of persistent mastitis. During a single visit to each farm, 10 ml of composite milk sample was aseptically collected by the residential veterinarian directly from the udder of a cow and transported to the laboratory in an icebox for microbiological analysis.

2.2. Isolation and Identification of *E. faecalis*. Enrichment of milk samples was performed in Luria Bertani (LB) broth as described earlier [18], 100 μl of the enriched sample was spread onto modified Edwards medium (MEM) (Himedia, India) and incubated aerobically overnight at 37°C. Black color colonies characteristic of *Enterococcus* spp. obtained on MEM were screened for bacterial morphology by Gram’s staining. At least three colonies showing characteristics of *Enterococcus* spp. were purified by subsequent streaking onto MEM. Genomic DNA was extracted from the purified colonies by boiling method and subjected to PCR targeting *ddl* gene [19] for the identification of *E. faecalis* using the primers provided in Supplementary Table S1. PCR reaction was adjusted to 20 μl volume with 10 μl 2X GoTaq® G2 Green Master Mix (Promega, USA), 10 pmol of each primer (Supplementary Table S1), and 2 μl of DNA templates. PCR was conducted in an ASTEC 482 thermal cycler (Japan) with an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. Representative isolates which were positive for the *ddl* gene were further confirmed by sequencing of 16S rRNA using the primers 8F and 1492R [20] (Supplementary Table S1).

2.3. Antimicrobial Susceptibility Testing. Disc diffusion method [21] was performed to determine the susceptibility of *E. faecalis* isolates to antimicrobials commonly used to treat animal and human diseases including mastitis in Bangladesh. The results (resistant and susceptible) were interpreted following the guidelines of the Clinical and Laboratory Standards Institute [22]. A total of nine (9) different antimicrobials (Oxoid, UK) were employed such as β-lactams (amoxicillin 10 μg (AMX), ampicillin 10 μg (AMP), and penicillin G 10 μg (P)), aminoglycosides (gentamicin 30 μg (GEN)), glycopeptides (vancomycin 30 μg (VA)), macrolides (azithromycin 15 μg (AZM)), phenicols (chloramphenicol 30 μg (C)), polypeptides (bacitracin 10 units (B)), and tetracyclines (tetracycline 30 μg (TE)). *E. coli* strain ATCC25922 was used as the control bacterial strain, and kanamycin (30 μg) was used as the control antibiotic in each experiment. Each experiment was performed three times to confirm the reproducibility of the results. Isolates resistant to three or more antimicrobial classes were considered multidrug resistant (MDR) [23].

2.4. Minimum Inhibitory Concentration. All the isolates were subjected to broth microdilution, to determine the
Electrophoresis was performed with 1.5% LE agarose. 

**minimum inhibitory concentration (MIC)** against gentamicin (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) and tetracycline (Nacalai Tesque Inc., Kyoto, Japan) following CLSI guidelines [22]. Test plates consisted of 2-fold dilutions of gentamicin and tetracycline ranging from 2 to 512 μg/ml and from 0.5 to 128 μg/ml, respectively. Plates were interpreted according to CLSI guidelines, and MIC breakpoint of each sample against their respective antibiotic was recorded where growth was significantly reduced, ignoring tiny buttons or light or faint turbidity [22]. Each test was performed three times to examine its reproducibility. *E. coli* ATCC 25922 was included in each trial as the quality control strain. Isolates exhibiting MIC > 500 μg/ml gentamicin are considered as high-level gentamicin resistant (HLGR), otherwise considered as wild or low-level resistance to gentamicin [22].

**2.6. Detection of Virulence Genes.** *E. faecalis* isolated in this study were PCR screened for the presence of virulence genes previously described in human clinical isolates [14]. A total of nine virulence genes were screened including *ace, agg, cyl, fsrA, fsrB, fsrC, gelE, pil, and sprE*. Primers used for this study are enlisted in Supplementary Table S1. PCR reaction mixture and PCR conditions were followed as per Section 2.2 except for the annealing temperature, which was varied depending on the primers used (Supplementary Table S1).

**2.7. Sequencing and Analysis.** 16S rRNA gene of the representative *E. faecalis* isolates was sequenced using 8F and 1492R primers (Supplementary Table S1) on an Applied Biosystems 3500 series genetic analyzer (Thermo Fisher Scientific, USA) using Sanger’s dideoxy sequencing technique [18]. Confirmation of detection of the *E. faecalis* sequences was done by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank.

**2.8. Statistical Analysis.** Data obtained from this study were incorporated into Excel 2013 (Microsoft Office 2013, Microsoft, Los Angeles, CA, USA) and then transferred to statistical tools for further analysis.

**2.8.1. Descriptive Analysis.** By the Statistical Package for the Social Sciences (SPSS) software (IBM SPSS version 25.0, USA), a Fisher’s exact test was conducted to find the possible variations between the occurrences of *E. faecalis*. A p value less than 0.05 was considered statistically significant in all tests.

**2.8.2. Bivariate Analysis.** Pearson correlation coefficient was enumerated by a bivariate analysis to assess the correlations between antimicrobials resistant to isolated *E. faecalis*, to evaluate the associations between resistance genes of *E. faecalis* isolates, and finally to determine the interrelation between different virulence genes of *E. faecalis* isolates. A statistically significant p value was less than 0.05. The bivariate analysis was performed in SPSS software (version 25).

**3. Results.**

**3.1. Occurrence of E. faecalis.** In the present study, 9.52% (10/105) of milk samples were positive for *E. faecalis* following culture and PCR targeting the *ddl* gene (Figure 1, Table 1). Representatives of the isolates were further confirmed by sequencing, BLASTn homology, and phylogenetic
Table 2: Antimicrobial resistance and virulence properties of the E. faecalis isolated in this study.

| Isolate ID | Sources | Phenotypes | Antibiotic resistance Genotypes | MIC (μg/ml) | Virulent genes |
|------------|---------|------------|---------------------------------|-------------|----------------|
|            |         |            | GEN                | TE                  |                |
| 2006       | MCCM    |            |                    | ≥8.0          | <1.0           | ace, fsrB, fscC, pil |
| 2008       | MCCM    |            |                    | <2.0          | <1.0           | ace, fsrB, fscC, pil |
| 2020       | MCCM    | AZM, TE    | tetK                | ≥16          | ≥64            | ace, fsrB, fscC, pil |
| 2024       | MCCM    |            |                    | ≥16          | <1.0           | ace, fsrB, fscC, pil |
| 2029       | MCCM    |            |                    | <2.0          | <1.0           | ace, fsrB, fscC, pil |
| 2032       | MCCM    | AZM, TE    | tetL, aph(3") – IIIa | ≥16          | 128            | ace, fsrB, fscC, pil |
| 2073       | MCCM    | TE         | aacC4, tetM        | ≥8.0          | ≥64            | agg, pil |
| 2081       | MCCM    | TE         | aacC2, tetM        | ≥8.0          | ≥64            | pil |
| 2091       | MCCM    | AZM        | aacC4, vanB        | ≥8.0          | <1.0           | fscB, gelE, sprE, pil |
| 20105      | MAHC    | AZM        | aacC4               | ≥16          | <1.0           | fscB, gelE, sprE, pil |

MCCM: milk of cattle with clinical mastitis; MAHC: milk of apparently healthy cattle; AZM: azithromycin; GEN: gentamicin; TE: tetracycline.

Table 3: Correlation between the phenotypic resistances of different antimicrobials against E. faecalis.

| TE | AZM |
|----|-----|
| TE | Pearson correlation: 1  |
|    | Sig. (2-tailed): 1    |
| AZM| Pearson correlation: 0.167  |
|    | Sig. (2-tailed): 0.645 |

TE: tetracycline; AZM: azithromycin.

analysis of the 16S rRNA (Supplementary Figure S1). The sequences were submitted to GenBank (GenBank accession numbers OK187180, OK187181, OK187182, OK187183, OK187184, and OK187185). The occurrence of E. faecalis in clinical mastitis samples (11.25%, 9/80) was higher than milk samples originating from apparently healthy cattle (4%, 1/25); however, the difference was not statistically significant (p = 0.281) (Table 1).

3.2. Antimicrobial Susceptibility Patterns of the Isolated E. faecalis

3.2.1. Antimicrobial Susceptible Phenotypes. In the antibiogram, E. faecalis isolates were found sensitive to the entire nine antibiotics tested except tetracycline and azithromycin. Forty percent (40%) of the isolates were found sensitive to tetracycline and azithromycin, respectively (Table 2). None of the isolates was multidrug resistant. In MIC test, the MIC of the isolates to gentamicin was ≥8-16 μg/ml confirming their sensitivity to this antibiotic. On the other hand, tetracycline-resistant isolates revealed a MIC of ≥64-128 μg/ml (Table 2). Bivariate analysis was performed to elucidate any correlation in phenotypic resistance; however, no positive significant correlation was observed between tetracycline and azithromycin resistance (Table 3).

3.2.2. Antimicrobial Resistance Genotypes. By PCR, 60% of the E. faecalis isolates harbored at least one out of 18 antimicrobial resistance genes examined. Tetracycline-resistant isolates carried tetK, tetL, and tetM as 25% (1/4), 25% (1/4), and 50% (2/4), respectively (Table 2). Although all the isolates were sensitive to gentamicin and vancomycin, aminoglycoside-resistant genes aph (3") – IIIa, aacC2, and aacC4 were detected in 12.5% (1/8), 12.5% (1/8), and 37.5% (3/8) of the isolates, respectively; and one (1) of the isolates carried vanB gene. Besides, none of the antibiotic-resistant gene was detected in four (4) isolates examined. In bivariate analysis, high positive significant correlations were observed in between aac2 and tetM (p = 0.035), and aph (3") – IIIa and tetL gene (p = 0.001) (Table 4).

3.3. Distribution of the Virulence Genes in the Isolated E. faecalis. All the E. faecalis isolates were found positive for at least one virulence gene, where 80% (8/10) of the isolates harbored four or more virulence genes. Occurrence of pil gene (100%) was higher than virulence genes fscB (80%), fscC (60%), ace (60%), sprE (30%), and gelE (20%), and agg (10%) (Table 2). However, no isolates carried virulence genes fsrA and cyl. Based on bivariate analysis, high and moderate positive significant correlations were revealed in between ace and fscC (p = 0.001), and gelE and sprE (p = 0.01) virulence genes of E. faecalis. On the other hand, a negative moderate significant correlation was observed between agg and fscB (p = 0.035) virulence genes of E. faecalis (Table 5).

4. Discussion

Antimicrobial resistance is a global public health concern. Indiscriminate or irrational use of medically important antimicrobials in animal production is claimed as a major driver of antimicrobial resistance transfer to humans [30]. In animal production, dairy farms are the largest user of medically important antimicrobials where mastitis comprises the single most common cause. Bovine mastitis is one of the challenging veterinary infections to control, and enterococci are important causative pathogens for mastitis. Over time, enterococci represent one of the most significant pathogens to cause infections, especially in humans, by acquiring antimicrobial resistance and virulence determinants [31]. E.
faecalis are the most common enterococci species in clinical mastitis; however, there is no study yet in Bangladesh in detecting antimicrobial resistance and virulence factors of this organism from clinical bovine mastitis. From this perspective, we undertook the present study to identify E. faecalis with their antimicrobial resistance, corresponding resistance genes, and virulence factors from cattle showing mastitis.

In this study, the occurrence rate of E. faecalis in milk samples of cattle having mastitis was 11.25% which is higher than previously reported as 0.2% in Germany and the United States [32, 33]. However, a higher prevalence of E. faecalis in clinical mastitis was reported in Belgium (20%) and South Korea (86.5%) [34, 35]. The observed differences in occurrence might be attributed to the geographical location and sample sizes. However, the presence of E. faecalis in milk samples represents a threat to human health as they can be transmitted to humans via the consumption of contaminated milk or milk products [11].

Tetracycline is one of the most commonly used antimicrobials in animal production for disease control and growth promotion. Widespread uses of this antimicrobial lead to the emergence of tetracycline-resistant bacteria [36]. In addition to tetracycline, use of azithromycin is increasingly reported in animal production in Bangladesh (personal communication). Besides, tetracycline and azithromycin are among the

| Table 4: Correlation between antimicrobial resistance genes detected in the E. faecalis recovered in this study. |
|---------------------------------------------------------------|
| **aacC2** | **aacC4** | **tetK** | **tetL** | **tetM** | **aph(3′)-IIIa** | **vanB** |
| Pearson correlation  | 1  |  |  |  |  |  |
| Sig. (2-tailed)  | -  |  |  |  |  |  |
| **aacC4** | Pearson correlation  | -0.218  | 1  |  |  |  |
| Sig. (2-tailed)  | 0.545  | -  |  |  |  |  |
| **tetK** | Pearson correlation  | -0.111  | -0.218  | 1  |  |  |
| Sig. (2-tailed)  | 0.76  | 0.545  | -  |  |  |  |
| **tetL** | Pearson correlation  | -0.111  | -0.218  | -0.111  | 1  |  |
| Sig. (2-tailed)  | 0.76  | 0.545  | 0.76  | -  |  |  |
| **tetM** | Pearson correlation  |  |  |  |  |  |
| Sig. (2-tailed)  |  |  |  |  |  |  |
| **aph(3′)-IIIa** | Pearson correlation  | -0.111  | -0.218  | -0.111  | **1.000****  | -0.167  | 1  |
| Sig. (2-tailed)  | 0.76  | 0.545  | 0.76  | 0.645  | **0.001**  | 0.645  | -  |
| **vanB** | Pearson correlation  | -0.111  | 0.509  | -0.111  | -0.111  | -0.111  | 1  |
| Sig. (2-tailed)  | 0.76  | 0.133  | 0.76  | 0.76  | 0.645  | 0.76  | -  |

A p value less than 0.05 was deemed statistically significant *. Correlation is significant at the 0.05 level (2-tailed) **. Correlation is significant at the 0.01 level (2-tailed).

| Table 5: Correlation between the occurrences of virulence genes in the E. faecalis isolated in this study. |
|---------------------------------------------------------------|
| **agg** | **fsrB** | **fsrC** | **gelE** | **sprE** | **ace** | **pil** |
| Pearson correlation  | 1  |  |  |  |  |  |
| Sig. (2-tailed)  | -  |  |  |  |  |  |
| **fsrB** | Pearson correlation  | -0.667**  | 1  |  |  |  |
| Sig. (2-tailed)  | **0.035**  | -  |  |  |  |  |
| **fsrC** | Pearson correlation  | -0.408  | 0.612  | 1  |  |  |
| Sig. (2-tailed)  | 0.242  | 0.06  | -  |  |  |  |
| **gelE** | Pearson correlation  | -0.167  | 0.25  | -0.612  | 1  |  |
| Sig. (2-tailed)  | 0.645  | 0.486  | 0.06  | -  |  |  |
| **sprE** | Pearson correlation  |  |  |  |  |  |
| Sig. (2-tailed)  |  |  |  |  |  |  |
| **ace** | Pearson correlation  | -0.218  | 0.327  | -0.356  | **0.764**  | 1  |
| Sig. (2-tailed)  | 0.545  | 0.356  | 0.312  | **0.01**  | -  |  |
| **pil** | Pearson correlation  | -0.408  | 0.612  | **1.000****  | -0.612  | -0.356  | 1  |
| Sig. (2-tailed)  | 0.242  | 0.06  | **0.001**  | 0.06  | 0.312  | -  |  |

A p value less than 0.05 was deemed statistically significant *. Correlation is significant at the 0.05 level (2-tailed) **. Correlation is significant at the 0.01 level (2-tailed).
most frequently prescribed antibiotics for human diseases in Bangladesh [37]. Thus, the occurrence of tetracycline and azithromycin resistance in *E. faecalis* is quite alarming since it could lead to treatment failure and potential life-threatening diseases in humans if proper antimicrobials are not selected. Interestingly, the isolates described in this study were sensitive to seven (7) other antibiotics tested including penicillin and gentamicin. Gentamicin is the most widely used aminoglycoside against enterococci; however, due to the emergence of HLGR, gentamicin monotherapy becomes ineffective in such cases. In case of low-level resistance, gentamicin or other aminoglycosides are suggested in combination therapy with cell wall inhibitors like penicillin and glycopeptides against *Enterococcus* [22, 38]. Based on the findings of this study, combination therapy could be suggested in controlling mastitis caused by *E. faecalis* in the study areas. However, antibiotic sensitivity testing is warranted before prescribing any antibiotics to control mastitis as a diverse group of microorganisms are involved in this disease pathogenesis.

Tetracycline resistance is conferred by diverse tetracycline-resistant genes located on horizontally transferable elements. At least thirty different tetracycline-resistant genes have been described so far [36, 39]. In this study, we have screened the *E. faecalis* isolates for nine (9) different tetracycline-resistant genes where the isolates carried either of the three tetracycline-resistant genes tetK or tetL or tetM, indicating the diversity of the tetracycline-resistant genes in the study area. Detection of tetK, tetL, and tetM genes in *E. faecalis* isolated from subclinical or clinical bovine mastitis has been reported earlier [40, 41]. However, ascertaining the real diversity of tetracycline-resistant genes in *E. faecalis* isolated from mastitis in Bangladesh needs further investigation with more samples and isolates.

Antibiotic resistance is conferred by a diverse mechanism including the presence of respective genes or bacterial metabolism [42, 43]. However, the presence of an antibiotic-resistant gene does not always mean that it would confer resistance to the respective antibiotic due to alteration through mutation or other genetic mechanisms [44]. In this study, we have detected plasmid-encoded aminoglycoside N(3)-acyltransferases II and IV encoding genes (aacC2 and aacC4) in some of the *E. faecalis*. These genes are known to confer resistance to gentamicin [45, 46]. However, the isolates did not show gentamicin resistance nor their presence was previously described in other studies. Thus, further studies are required to ascertain the presence and role of these genes in *E. faecalis* pathogenicity. In addition, one of the *E. faecalis* isolates carried vanB gene despite phenotypic vancomycin sensitivity indicating that the gene is dormant or nonfunctional in this strain. The presence of aacC2, aacC4, and vanB genes might not be associated with antibiotic resistance in *E. faecalis* isolated in this study but poses a threat of transferring these resistance determinants to other enterobacteria having clinical significance in human infections.

Pathogenesis of *E. faecalis* is dependent on its establishment, adherence, invasiveness, and ability to overcome the host defense system and biofilm formation—an important attribute of the bacteria that facilitates its persistence in adverse environmental conditions [47]. In this study, all the *E. faecalis* isolates were positive for virulence gene pili and six for ace genes. The pili and ace are two important virulence factors whose products are associated with adhesion and colonization in the host [14]. In addition, our present study found that *E. faecalis* isolates also carried virulence genes fsrB, fsrC, sprE, and gelE, whose products are linked to biofilm formation and its strength in *E. faecalis* [14]. Furthermore, a moderate to strong association between the ace and fsr genes, as well as the sprE and gelE genes, indicates that the isolated *E. faecalis* are strong biofilm former, as evidenced by previous studies [14]. It is clear from our findings that the *E. faecalis* isolates have the characteristics of a potential human pathogen. In addition, these virulence properties might be linked to *E. faecalis* persistence in the udder environment. However, to ascertain these possibilities, phenotypic expression of the virulence properties is suggested, which could not be performed due to a lack of our laboratory facilities and isolates.

5. Conclusion

For the first time in Bangladesh, in this study, we have detected antimicrobial resistance and virulence determinants of *E. faecalis* from bovine clinical mastitis. The occurrence of these resistant isolates in milk samples from mastitis as well as apparently healthy cattle is of public health concern. *E. faecalis* isolated in this study might be strong biofilm formers. Their biofilm-associated virulence determinants and antimicrobial resistance could be the reason for their persistence in the udder environment and resistance to antimicrobial therapy. Their virulence properties and resistance to medically important antimicrobials indicate their potential to induce human or animal diseases that might be difficult to treat if proper antimicrobials are not selected. Thus, an antibiotic sensitivity test is suggested before prescribing any antimicrobials for mastitis.

Data Availability

The data will be available from the corresponding author on request.

Ethical Approval

All the milk samples in this study were collected from cows by a residential veterinarian. The researcher in this study did not use any animal subject for any pathological investigation or show any act of cruelty or invasive sampling. Thus, no ethical approval was required. However, verbal permission was taken from the farm owners or authority during the collection of milk samples.

Conflicts of Interest

The authors have no competing interests to declare.
Authors’ Contributions
Md. Abdus Sattar Bag and Mohammad Arif contributed equally.

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Supplementary Materials
Supplementary Table S1: list of primers used for the detection of antimicrobial resistance and virulent genes in the E. faecalis isolated from clinical mastitis. Supplementary Figure S1: phylogenetic relationship of E. faecalis isolated in this study. The relationship was inferred with the partial 16S rRNA gene sequences of E. faecalis (marked with red rectangles) obtained in this study using the Neighbor-Joining method (Saitiu and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 15 E. faecalis and 1 E. coli sequence as an out-group. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1452 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

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