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# Identification of two novel type II topoisomerase mutations in *Enterococcus* spp. isolated from a hospital in China

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**Abstract:** Type II topoisomerases, including DNA gyrase (GyrA) and topoisomerase IV (ParC), contribute to fluoroquinolone resistance in *Enterococcus* spp. This study investigated the mutational status of the quinolone resistance-determining regions (QRDRs) of GyrA and ParC in the clinical isolates of enterococci from a hospital in Baotou, China. We analyzed 110 enterococcal isolates, including 57 *Enterococcus faecalis* and 53 *Enterococcus faecalis faecium*. The resistance rates of *E. faecalis* and *E. faecium* to ciprofloxacin were 63.16% and 84.91%, respectively. We found that 32 samples of *E. faecalis* and 42 of *E. faecium* had single or combined mutations in *gyrA* and/or *parC*, which were all resistant to ciprofloxacin. Only two ciprofloxacin-resistant *E. faecalis* isolates had no mutation. No mutations in *gyrA* and *parC* genes in all ciprofloxacin-susceptible isolates were found. Ciprofloxacin minimal inhibitory concentrations (MICs) in the mutation group were significantly higher than those of the non-mutation group, indicating that mutations in the QRDRs of *gyrA* and *parC* were correlated with MIC elevation. Two novel substitutions (GyrA Ser83Phe and ParC Ser80Leu) of *E. faecalis* were identified herein. Three-dimensional modeling revealed that these novel amino acid substitutions could disrupt the water/metal-ion bridge and decrease the interaction between the enzymes and ciprofloxacin. The data showed a diversity of mutation types in QRDRs of type II topoisomerases whose association with fluoroquinolone resistance in clinical isolates of enterococci warrants further investigation.

**Keywords:** *Enterococcus* spp.; type II topoisomerase; GyrA; ParC; fluoroquinolone resistance

## INTRODUCTION

Enterococci are important pathogens causing hospital- and community-acquired abdominal infections, with *Enterococcus faecalis* and *Enterococcus faecium* accounting for most enterococcal infections in humans, including urinary tract infections, surgical site infections, bacteremia, endocarditis and tissue damage [1,2]. *Enterococcus* species are of clinical importance because they lead to a fulminant and destructive disease course [3]. A recent report showed that *Enterococcus* accounted for 20% of bloodstream infections in China, with a mortality rate of 24% [4]. In recent years, some reports have demonstrated that the emergence and spread of multi-drug-resistant *Enterococcus* species is a serious problem for clinical anti-infective therapy [5,6]. With the wide application of fluoroquinolones such as norfloxacin, ciprofloxacin and ofloxacin, the rates of fluoroquinolone resistance among *Enterococcus* over the past three decades has considerably increased in many countries [7,8]. The development of ciprofloxacin resistance in clinical isolates of enterococci in China during 2008-2019 is presented in Supplementary Table S1 (China Antimicrobial Resistance Surveillance System, CARSS, http://www.carss.cn).

Fluoroquinolones are a series of synthetic antibacterial agents that are widely used in the treatment of human and animal infections. They exhibit high antimicrobial activity against a broad range of pathogenic bacteria; they possess advantageous pharmacokinetic characteristics and low toxicity [9]. The target enzymes for fluoroquinolones are the bacterial type II topoisomerases, including DNA gyrase (composed of GyrA and GryB subunits) and topoisomerase IV

(composed of ParC and ParE subunits) [10]. Crystallographic studies have been performed to investigate fluoroquinolone-enzyme interactions [11]. Recent structural studies have indicated that fluoroquinolone-topoisomerase binding was facilitated through a water-metal ion bridge, which is formed by a non-catalytic metal ion chelated by the C3/C4 keto acid of the fluoroquinolone and stabilized by four water molecules [12]. Two water molecules are coordinated by the conserved serine and acidic residues localized at the amino-terminal domains of GyrA (residues 67 to 106 for *Escherichia coli* numbering) or ParC (residues 63 to 102) [13], referred to as quinolone resistance-determining regions (QRDRs) [14]. Resistance to fluoroquinolones in *Enterococcus* species is mostly caused by changes in these amino acids. Major mutations occur at the conserved serine residues within QRDRs in both GyrA and ParC (initially described as Ser83 in *E. coli* GyrA and Ser80 in *E. coli* ParC) [15]. Alterations of conserved residues have been associated with reduced binding of the fluoroquinolones to enzyme-DNA complexes.

To better understand the mechanisms of *Enterococcus* resistance to fluoroquinolone, we characterized the resistance-conferring mutations in the QRDRs of GyrA and ParC and discovered two new mutations that have not previously been reported.

## MATERIALS AND METHODS

### Bacterial isolates and identification

A total of 110 clinical isolates of enterococci were collected from the Second Affiliated Hospital of Baotou Medical College in Baotou, China, between September 2016 and September 2019 and stored at -80°C until use. The phoenix100 microbiologic identification system (Bethesda, MD, USA) was used to identify the isolates as enterococci as described previously [1]. A multiplex PCR assay was designed to further confirm two species, *E. faecalis* and *E. faecium* [15]. PCR products were analyzed under UV light after separation by 1% agarose gel electrophoresis stained with ethidium bromide on a DYY-6D gel electrophoresis apparatus (Beijing Liuyi Instrument Factory, Beijing, China).

### Susceptibility testing

Antimicrobial susceptibility tests were performed using the broth microdilution method and the results were interpreted with reference to the Clinical and Laboratory Standards Institute (CLSI) criteria [16]. The following drugs were tested: ciprofloxacin (5 μg/mL), gentamicin (120 μg/mL), tetracycline (30 μg/mL), ampicillin (10 μg/mL), erythromycin (15 μg/mL), vancomycin (30 μg/mL), teicoplanin (30 μg/mL), linezolid (30 μg/mL) and rifampin (5 μg/mL). *E. faecalis* isolate ATCC 29212 was used as the quality control.

### Sequence analysis of the QRDRs of the topoisomerases encoding gene

DNA was extracted from enterococci using bacterial DNA extraction kits (Tiangen Biotech Co., Ltd., Beijing, China). Regions containing the QRDR sequences of *gyrA* and *parC* were amplified by PCR in a final reaction volume of 50 μL containing 5 μL of template DNA, 2 μL of each primer and 25 μL of a 2×Taq PCR Master Mix (Solarbio Science & Technology Co., Ltd., Beijing, China). A C1000 Touch thermocycler (Bio-Rad, USA) was used. PCR conditions were as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50-55°C for 30 s and elongation at 72°C for 1 min, and final extension at 72°C for 5 min. The following primers were used [17]: for *gyrA*: forward, 5'-CGG GAT GAA CGA ATT GGG TGT GA-3', reverse, 5'-AAT TTT ACT CAT ACG TGC TTC GG-3'; for *parC*: forward, 5'-AAT GAA TAA AGA TGG CAA TA-3', reverse, 5'-CGC CAT CCA TAC TTC CGT TG-3'. PCR products were analyzed using 1% agarose gel electrophoresis. The size of each PCR product was as follows: 241 bp for *gyrA* and 191 bp for *parC*.

### Homology modeling of *E. faecalis* GyrA and ParC

Amino acid sequences of *E. faecalis* GyrA and ParC were obtained from the databank in the National Center for Biotechnology Information (accession number CP003351). Three-dimensional models of *E. faecalis* GyrA and ParC were constructed using the program Modeller [18]. The crystal structure of the fluoroquinolone-DNA cleavage complex of type IV topoisomerase from *Streptococcus pneumoniae* (PDB

code 3RAE) was used as the reference protein. The obtained structure was evaluated with a Ramachandran plot [19].

### Molecular docking

Molecular Operating Environment (MOE) software (ver. 2015) was used for the docking of ciprofloxacin into the QRDR of GyrA and ParC. The MMFF94x was selected prior to performing docking calculations. The drug-binding pockets of GyrA and ParC were analyzed using MOE software by determining the volumes of the pockets, solvent accessible areas, and hydrophobic/hydrophilic characteristics. The ligand was docked into the active site using the triangular matching docking method. The predicted ligand-protein complexes were ranked based on the scoring function of GBVI/WSA (generalized-born volume integral/weighted surface area).

### Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software, ver. 17.0. Correlations between ciprofloxacin sensitivities and QRDR mutations of type II topoisomerases were analyzed using the chi-square test. $P<0.05$ was considered statistically significant.

## RESULTS

### Antimicrobial susceptibility

The antibiotic resistance rates of enterococci species isolated from clinical specimens are shown in Table 1. Out of 57 *E. faecalis* strains examined for antimicrobial susceptibility, 61.40%, 49.12%, 35.09%, 70.18%, 63.16% and 80.70% of the strains showed resistance to tetracycline, high-level gentamicin, ampicillin, rifampin, ciprofloxacin and erythromycin, respectively. In contrast, the resistance rates of *E. faecium* to tetracycline, high-level gentamicin, ampicillin, rifampin, ciprofloxacin and erythromycin were 41.51%, 49.06%, 77.36%, 77.36%, 84.91% and 90.57%, respectively. All isolates were sensitive to teicoplanin, vancomycin and linezolid. The ciprofloxacin MICs were further measured for clinical isolates based on CLSI principles. Of 57 *E. faecalis* isolates, 36 were resistant, 12 showed intermediate susceptibility, and 9 were susceptible to ciprofloxacin. Similarly, of 53 *E. faecium* isolates, 6 were susceptible, 2 were intermediate, and 45 were ciprofloxacin resistant. The ciprofloxacin MIC distribution for enterococci is shown in Fig. 1. The $MIC_{50}$ and $MIC_{90}$ of *E. faecalis* were 64 and 512 mg/L, respectively; the $MIC_{50}$ and $MIC_{90}$ of *E. faecium* were 256 and 512 mg/L, respectively.

**Table 1**. Antibiotic resistance rates of enterococci species.

| Antibiotics | *E. faecalis* (n=57) | | *E. faecium* (n=53) | |
|---|---|---|---|---|
| | Number of resistant strains (n) | Resistance rates (%) | Number of resistant strains (n) | Resistance rates (%) |
| Tetracycline | 35 | 61.40% | 22 | 41.51% |
| Gentamicin (High-level) | 28 | 49.12% | 26 | 49.06% |
| Ampicillin | 20 | 35.09% | 41 | 77.36% |
| Rifampin | 40 | 70.18% | 41 | 77.36% |
| Ciprofloxacin | 36 | 63.16% | 45 | 84.91% |
| Erythromycin | 46 | 80.70% | 48 | 90.57% |
| Teicoplanin | 0 | 0 | 0 | 0 |
| Vancomycin | 0 | 0 | 0 | 0 |
| Linezolid | 0 | 0 | 0 | 0 |

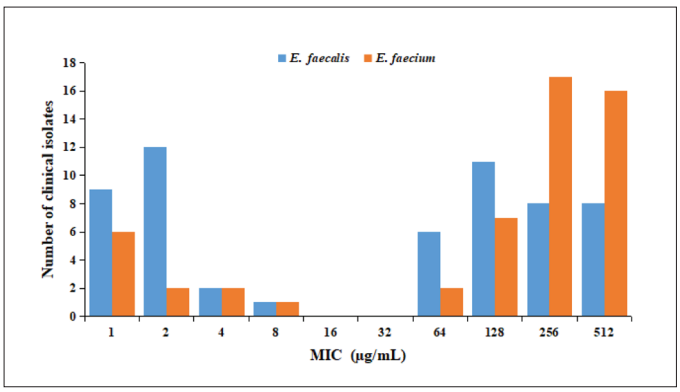


**Fig. 1.** Ciprofloxacin MIC distribution for the 110 clinical isolates of enterococci. MIC – minimal inhibitory concentration.

### PCR amplification of *gyrA* and *parC* genes in enterococci

Regions containing the QRDR sequences of *gyrA* and *parC* were amplified by PCR. For each target gene, PCR analysis confirmed the expected PCR product size by agarose gel electrophoresis (Fig. 2).

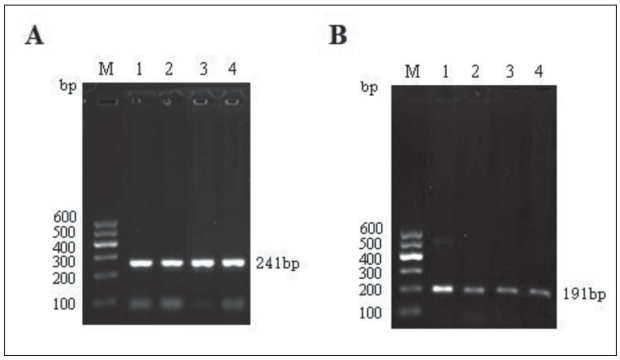


**Fig. 2.** Agarose gel electrophoresis of PCR products. **A** – *gyrA*. **B** – *parC*. Lanes 1-2 are PCR products of *E. faecalis*. Lanes 3-4 are PCR products of *E. faecium*. M – 100 bp DNA marker.

## Sequence variations in the QRDR of GyrA and ParC

Of the 57 *E. faecalis* isolates, the following known mutations were detected: GyrA Ser83Tyr, GyrA Ser-83Ile, ParC Ser80Ile and ParC Ser80Arg. In the present study, ParC Ser80Ile (n=28) and GyrA Ser83Ile (n=22) were the most common variations, followed by GyrA Ser83Tyr (n=8) and ParC Ser80Arg (n=1). No change was detected at positions GyrA Glu87 and ParC Glu84. Two novel changes at positions GyrA Phe83 and ParC Leu80 were detected in one ciprofloxacin-resistant isolate (Table 2). Three isolates harbored single mutations in either GyrA or ParC, and 29 had both GyrA and ParC mutations. The remaining 12 ciprofloxacin-intermediate, 2 ciprofloxacin-resistant and 9 ciprofloxacin-susceptible *E. faecalis* had wild-type QRDRs (Table 2). In contrast, of the 53 *E. faecium* isolates, ParC Ser80Ile (n=41) and GyrA Ser83Ile (n=28) were the most common amino acid substitutions, followed by GyrA Ser83Tyr (n=13) and ParC Ser80Arg (n=1). One isolate had only ParC QRDR mutations, and dual amino acid substitutions in both GyrA and ParC were identified in 41 of the 53 isolates. The remaining 2 ciprofloxacin-intermediate, 3 ciprofloxacin-resistant and 6 ciprofloxacin-susceptible isolates lacked both GyrA and ParC QRDR mutations (Table 3).

## Impact of the QRDR mutations of GyrA and ParC on ciprofloxacin susceptibilities

The isolates of enterococci were divided into two groups: the mutation group and the non-mutation group. The mutation group pointed to amino acid substitutions in GyrA and/or ParC QRDRs, and the non-

**Table 2.** Ciprofloxacin susceptibility data for the 57 strains of *E. faecalis* with details of GyrA and ParC amino acid substitutions.

| Amino acid (nucleotide) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GyrA | | ParC | | | No. of isolates with ciprofloxacin MICs (μg/mL) | | | | | | | |
| Ser 83 | Glu 87 | Ser 80 | Glu84 | N (n) | 1-4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| — | — | — | — | 25 | 23 | 1 | | | 1 | | | |
| — | — | Leu(CTC) | — | 1 | | | | | | 1 | | |
| Tyr(TAT) | — | — | — | 1 | | | | | | | | 1 |
| Ile(ATC/ATT) | — | — | — | 1 | | | | | | | 1 | |
| Phe(TTT) | — | Ile(ATC/ATT) | — | 1 | | | | | | 1 | | |
| Ile(ATC/ATT) | — | Arg(CGC) | — | 1 | | | | | | 1 | | |
| Tyr(TAT) | — | Ile(ATC/ATT) | — | 7 | | | | | 1 | 2 | | 4 |
| Ile(ATC/ATT) | — | Ile(ATC/ATT) | — | 20 | | | | | 4 | 6 | 7 | 3 |

**Table 3.** Ciprofloxacin susceptibility data for the 53 strains of *E. faecium* with details of GyrA and ParC amino acid substitutions.

| Amino acid (nucleotide) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GyrA | | ParC | | | No. of isolates with ciprofloxacin MICs (μg/mL) | | | | | | | |
| Ser 83 | Glu 87 | Ser 80 | Glu84 | N (n) | 1-4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| — | — | — | — | 11 | 10 | 1 | | | | | | |
| — | — | Ile(ATC/ATT) | — | 1 | | | | | | 1 | | |
| Ile/(ATC ATT) | — | Arg(CGC) | — | 1 | | | | | | 1 | | |
| Tyr(TAT) | — | Ile(ATC/ATT) | — | 13 | | | | | 1 | | 2 | 10 |
| Ile(ATC/ATT) | — | Ile(ATC/ATT) | — | 27 | | | | | 1 | 5 | 15 | 6 |

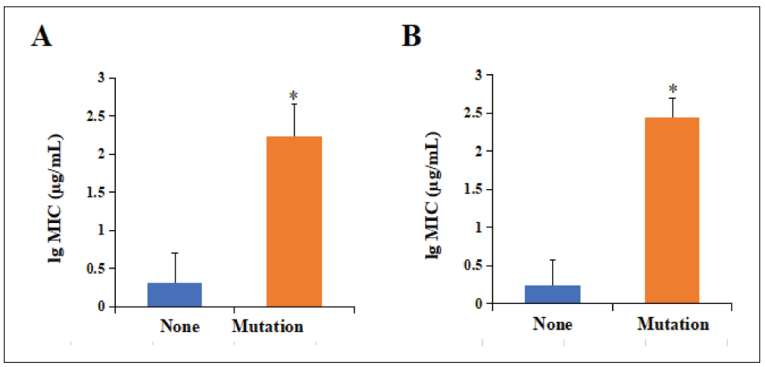


**Fig. 3.** Correlations between amino acid mutations of GyrA and ParC and ciprofloxacin susceptibility of clinical isolates. **A** – *E. faecalis*. **B** – *E. faecium*. *P<0.05 *vs* non-mutation group.

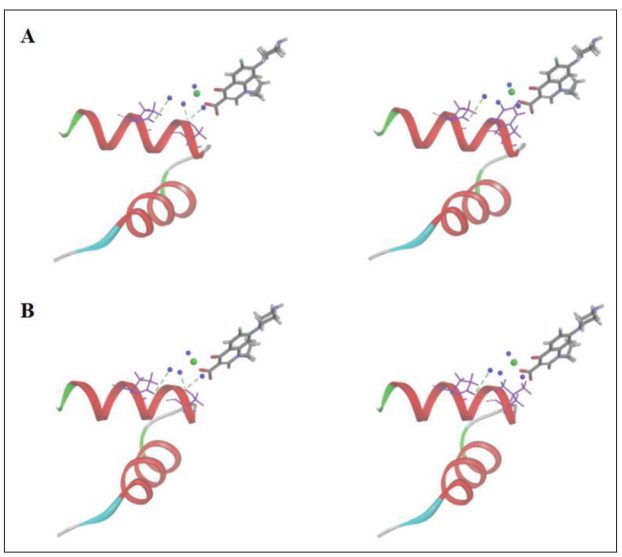


**Fig. 4.** The structures of the QRDRs of *E. faecalis* type II topoisomerases. **A** – Docking orientation of ciprofloxacin into the wild-type (left) and mutant (right) *E. faecalis* GyrA QRDR models. **B** – Docking orientation of ciprofloxacin into the wild-type (left) and mutant (right) *E. faecalis* ParC QRDR models. *E. faecalis* GyrA and ParC models are shown as a ribbon, ciprofloxacin is shown as a stick, the dashed lines represent hydrogen bonds; green spheres and blue spheres represent $Mg^{2+}$ and $H_2O$ molecules, respectively.

mutation group showed wild-type GyrA and ParC QRDRs. Our results showed that the log-transformed ciprofloxacin MICs of *E. faecalis* isolates in the mutation group (n=33) were significantly higher compared to the log-transformed MICs of isolates in the non-mutation group (n=24) (2.235±0.426 *vs* 0.301±0.397 μg/mL, t=17.409, P<0.05) (Fig. 3A). Compared to the log-transformed ciprofloxacin MICs of *E. faecium* isolates in the non-mutation group (n=11), the log-transformed MICs of *E. faecium* in the mutation group (n=42) were significantly higher (2.444±0.259 vs 0.246±0.325 μg/mL, t=23.741, P<0.05) (Fig. 3B).

### Detection of binding between ciprofloxacin and *E. faecalis* GyrA and ParC

The homology models of *E. faecalis* GyrA and ParC were constructed based on the structure of template. The Ramachandran plot showed that >90% of the amino acid residues resided in the favored region (96.0% for GyrA, 96.6% for ParC) (Supplementary Fig. S1 and S2, respectively). These results indicated that the predicted models were reliable. The docking results showed that the conserved Ser (GyrA Ser83 and ParC Ser80) and Glu (GyrA Glu87 and ParC Glu84) in the QRDRs associate with the $Mg^{2+}$ ion and two water molecules via hydrogen bonds. Also, the water-metal ion bridge coordinated ciprofloxacin binding to the enzyme (Fig. 4). However, mutation of these amino acids disrupts the bridge formation, reducing the binding affinity of the drug for the enzyme (Fig. 4).

## DISCUSSION

It is clear that the increased prevalence of drug-resistant bacteria has become a major public health problem [20,21]. In the current study, we studied 110 clinical isolates of *Enterococcus* (57 *E. faecalis* and 53 *E. faecium*). Our results showed that a high percentage of enterococci exhibited resistance to many types of antimicrobials, including erythromycin, ciprofloxacin, ampicillin, high-level gentamicin and tetracycline. However, the isolates were highly sensitive to the following antibiotics: teicoplanin, linezolid and vancomycin. These data are similar to previous reports [1,22]. In this study, the rate of ciprofloxacin resistance in *Enterococcus* species was high and most isolates exhibited high levels of resistance ($MIC_{50}$=32 mg/L, $MIC_{90}$=256 mg/L), as reported previously [23]; the rate of ciprofloxacin resistance in *E. faecium* species showed a significant difference when compared to *E. faecalis* (84.91% of *E. faecium* and 63.16% of *E. faecalis* strains), similar to a previous report [24].

One of the major mechanisms in the development of fluoroquinolone resistance involves QRDR mutations in the *gyrA* and *parC* genes. Previous studies found that the most common alterations occur at positions GyrA Ser83, GyrA Glu87, ParC Ser80 and ParC Glu84 [25,26]. However, in our region, only limited data on QRDR mutations in clinical samples are available. Herein we demonstrated that 32 *E. faecalis* resistant isolates and 42 *E. faecium* resistant isolates harbored amino acid mutation(s) at positions GyrA Ser83 and/or ParC Ser80. Alterations at positions GyrA Glu87 and ParC Glu84 were not observed. Interestingly, our results showed novel mutations in GyrA, where serine 83 was changed to phenylalanine, and in ParC serine 80 was changed to leucine. This is the first report, to the best of our knowledge, describing the GyrA Ser83Phe and ParC Ser80Leu mutations in *E. faecalis*-resistant isolates. The isolates with novel mutations had MICs of 128 mg/L. As previously described, GyrA Ser83 or ParC Ser80 anchors the water-metal ion bridge [27]. In accordance with the literature, our computational structural analysis showed that the novel mutations result in the disruption of the water-metal ion bridge and are associated with a higher level of fluoroquinolone resistance. We further found that the QRDR mutations in both GyrA and ParC were responsible for the elevated ciprofloxacin MICs, which is in agreement with previous studies [28]. Moreover, the results showed that 2 ciprofloxacin-resistant *E. faecalis* had wild-type QRDRs, suggesting that other resistance mechanisms other than mutations in *gyrA* and *parC*, may be involved in ciprofloxacin resistance.

QRDR amino acid substitutions in GyrA and ParC influence the enzyme-ciprofloxacin interaction as a result of the loss of essential protein-drug contacts, which ultimately cause antibiotic resistance [29]. To obtain additional evidence to support the hypothesis that two novel changes at positions GyrA Phe83 and ParC Leu80 were involved in ciprofloxacin-target enzyme binding, protein modelling and molecular docking were carried out to understand in atomic detail how ciprofloxacin interacts with GyrA and ParC. Our results showed that ciprofloxacin binding involved a water-magnesium ion bridge between the C3-C4 keto-acid moiety of quinolone and the amino acids equivalent to *E. faecalis* GyrA-83 and GyrA-87 (or ParC-80 and ParC-84). While this finding is similar to that observed in a previous study [30], the mutations of GyrA Ser83 to Phe and ParC Ser80 to Leu disrupt the water-magnesium ion bridge and decrease the interactions between ciprofloxacin and target enzymes.

## CONCLUSIONS

Enterococci collected from clinical samples exhibited a high level of resistance to ciprofloxacin. MIC elevations significantly correlated with QRDR mutations of GyrA and/or ParC. Moreover, the mutations of GyrA Ser83 to Phe and of ParC Ser80 to Leu in *E. faecalis* isolates reduced the affinity between the drug and target enzymes. This is the first report indicating that these two novel mutations are responsible for ciprofloxacin resistance in *E. faecalis*. Our study contributes to a better understanding of fluoroquinolone-resistant enterococci.

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**Author contributions:** RS and YP contributed equally to this work. RS and YP conducted the study, collected the data, performed the analysis of the data and prepared the manuscript; ZW conducted the study, collected the data, and performed the analysis of data; HY and QW designed and supported the study and edited the manuscript. All authors read and approved the final version of the manuscript.

**Conflicts of interest disclosure:** The authors declare that they have no competing interests.

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**Supplementary Material**

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Su%20et%20al_6731_Supplementary%20Material.pdf