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In vitro antibacterial and antibiofilm activities of isobavachalcone against *Enterococcus faecalis* clinical isolates from China

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Abstract

Background The pharmacological activities of the natural product isobavachalcone, such as antimicrobial activity, reverse transcriptase blockade, and antioxidant property have been extensively reported. Whereas, its antimicrobial and biofilm-inhibitory effects on clinical *E. faecalis* strains in China, along with its potential mechanisms, are still not fully clear. This research is intended to assess the in vitro antibacterial and anti-biofilm effects of isobavachalcone against clinical *E. faecalis* isolates sourced from China. Moreover, it further explores the potential target site of it within *E. faecalis*.

Results It was found that the minimum inhibitory concentrations (MICs) of isobavachalcone ranged from 6.25 to 12.5 μM against 220 *E. faecalis* clinical strains obtained from a tertiary hospital in China. The antibiofilm activity of it with sub-MIC concentration (1/2 × MIC) against the biofilm formation of *E. faecalis* was demonstrated and Time -killing curve assay revealed the quick bactericidal effect of isobavachalcone against *E. faecalis* planktonic cells. However, no synergetic bactericidal activity of isobavachalcone co-administered with vancomycin, or ampicillin was observed for eradicating the biofilm. Moreover, isobavachalcone-resistant *E. faecalis* was isolated by in vitro induction of isobavachalcone, and whole genome sequencing was performed to determine the genetic mutations of ten functional proteins in isobavachalcone-resistant *E. faecalis*, including PurH and FlgJ, with the other eight proteins being related to cell wall or cell membrane biogenesis, DNA synthesis, and energy metabolism. In addition, molecular docking results indicate that there is a potential binding of isobavachalcone and PurH protein in *E. faecalis*.

Conclusion This research highlights the potential of isobavachalcone as a potent antibacterial agent against *E. faecalis* clinical isolates, capable of significantly inhibiting biofilm formation at sub-MIC concentrations. PurH protein in

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E. faecalis might serve as a potential target of isobavachalcone and the specific action mechanism of isobavachalcone needs further study.

Keywords Isobavachalcone, *Enterococcus faecalis*, Antibacterial, Biofilm

Introduction

Enterococcus faecalis is a conditional pathogen responsible for nosocomial infections, including urinary tract, abdominal, and device-related infections, etc. Lately, the rising challenges posed by antimicrobial-resistant *E. faecalis* have emerged as a major hurdle for healthcare professionals. This issue is largely attributed to the spread of *E. faecalis* strains that possess either inherent or developed resistance to numerous antibiotics [1, 2]. During the 1970s, resistance in enterococci to aminoglycosides was first reported. By the 1980s, β-lactam and glycopeptide resistance emerged, with vancomycin-resistant enterococci (VRE) first identified in 1986 [3–5]. The increasing prevalence of antimicrobial medications has contributed to the global rise of multidrug-resistant (MDR) *E. faecalis*, such as VRE, linezolid-resistant varieties, and daptomycin-resistant strains, frequently resulting in failures in clinical treatment [6]. Consequently, the swift progression of MDR in enterococcal species underscores the critical necessity to discover novel therapeutic options exhibiting strong antimicrobial efficacy against MDR *E. faecalis*.

E. faecalis shows an intrinsic resistance to numerous antibiotics, attributable to its robust cell walls and limited membrane permeability. Moreover, it has a strong capacity to form biofilms, allowing it to thrive even in adverse environmental conditions [7, 8]. In addition, *E. faecalis* often employs survival tactics by entering a dormant state to withstand harsh conditions, such as exposure to antibiotics or other stressors. These so-called persistent bacteria possess a special knack for resisting drug pressure by moving through phases of dormancy, growth, and multiplication. This clever strategy allows them to evade the lethal effects of antibiotics, ensuring their survival in challenging environments [9]. Biofilms are intricate clusters of microorganisms that get ensconced within a polymer web made up of polysaccharides and eDNA. When biofilms form, it generally leads to bacteria becoming less responsive to medications and evading the host's immune system's efforts to eliminate them. All in all, this leads to subpar treatment outcomes [10, 11]. In other words, the development of biofilms aids the survival and resilience of infectious microorganisms by bolstering their defenses against the host's immune system. Currently, commonly used antibiotics in clinical settings frequently fall short of effectively targeting both persistent bacteria and the bacterial biofilm. Although many studies have explored the inhibition of bacterial biofilms or persisters, there are still no antibacterial drugs with good results in clinical anti-biofilm or anti-persister treatment [12–14]. Therefore, exploring natural bioactive compounds derived from plants that possess antimicrobial properties—capable of eradicating persisters and eliminating biofilms—offers a highly promising avenue to develop novel antimicrobial treatments [15, 16].

Isobavachalcone, a naturally occurring compound extracted from plants like *Fatouapilosa* and the fruits of *Psoralea corylifolia*, has demonstrated notable antibacterial properties, particularly against methicillin-resistant *S. aureus* (MRSA) strains. Multiple studies suggest that its effectiveness stems from its ability to disrupt bacterial cell membranes, making it a promising candidate for treating antibiotic-resistant infections [17–19]. Isobavachalcone has demonstrated a broad spectrum of pharmacological effects, like antibacterial, antitubercular, and antioxidant effects. For antibacterial effect, it has shown activity against Gram-positive, MDR, and mycobacteria [17]. Some scholars have identified the primary antibacterial mechanisms of isobavachalcone as follows: increased membrane permeability, along with the leakage of alkaline phosphatase (AKP) that results from the damage inflicted on both the cell wall and cell membrane, the suppression of the synthesis of biomolecules, including proteins, DNA, and RNA, and the disruption of energy metabolism [18, 19]. Recently, Yan Chen found that combining with gentamicin obviously enhanced its antibacterial performance on *S.aureus* biofilm [20]. Although a considerable amount of literature exists, the information regarding the bactericidal effects, biofilm-inhibiting capabilities, and the underlying antibacterial mechanisms of isobavachalcone when used against *E. faecalis* remains extremely limited. Consequently, the present study was designed to assess the antibacterial and anti-biofilm efficacy of isobavachalcone against *E. faecalis* and to identify the potential target genes of isobavachalcone within this bacterial species.

Materials and methods

Bacterial isolates and culture conditions

Between 2011 and 2015, a total of 220 non-replicative strains of *E. faecalis* were gathered from Shenzhen Nanshan People's Hospital. The identification of these bacteria was carried out employing the Vitek 2 compact system, following the guidelines provided by the manufacturer. Additionally, the *E. faecalis* strains ATCC29212 and OG1RF were acquired from the ATCC to serve as reference strains.

The collected strains were preserved at -80℃ in 40% TSB for future analysis. Unless otherwise specified, they were cultivated in TSB at a temperature of 37 °C under an agitation speed of 180 rpm. For antimicrobial susceptibility tests, cultures were grown in CAMHB at the same temperature and agitation. A biofilm assay utilized TSBG at 37 °C. All CAMHB experiments included a 50 mg/L Ca2 + supplement to evaluate daptomycin effectiveness [21]. All procedures were conducted following the moral guidelines of the present Hospital and the 1964 Helsinki Declaration. For this particular type of study, the acquisition of formal consent is not necessary.

Antimicrobial susceptibility testing

The sensitivity of *E. faecalis* to isobavachalcone and common antibiotics (tetracycline, doxycycline ampicillin, linezolid, vancomycin, ciprofloxacin, nitrofurantoin) was detected via broth microdilution following relevant Clinical and Laboratory Standards Institute recommendations (M100 - S30) [22]. Isobavachalcone and the aforementioned antibiotics were bought from Macklin Enterprise, and the MICs were measured using the broth microdilution approach [23].

Growth curve analysis

Two isolates of *E. faecalis* (16C51, sourced from blood; 16C106, obtained from urine) were chosen for one-step growth tests involving isobavachalcone. The bacterial strains were cultured in MHB at 37 °C for 24 h and then thinned to achieve an OD of 0.1 at 600 nm (OD600 = 0.1). Isobavachalcone was added at varying concentrations: 1/32, 1/16, 1/8, 1/4, 1/2 × MIC. OD values were measured every hour for 24 h with the use of an automated growth curve analyzer. To ensure reliability, the experiment was conducted in triplicate.

Antimicrobials inhibit the biofilm formation and eradicate the formed biofilms of *E. faecalis*

Two *E. faecalis* strains (16C51 and 16C106), earlier characterized by their strong biofilm-forming capacities, were selected to evaluate the inhibitory effect of isobavachalcone on biofilm formation. The bacterial cultures were inoculated into 96-well polystyrene microtiter plates filled with TSBG medium, which was supplemented with a series of different concentrations of antimicrobials (range of 1/2 to 1/32 × MIC). Control wells, devoid of antibiotics, were included for comparison. After a 24-hour static incubation phase, the biomass of the biofilm was measured via crystal violet staining. Each experimental condition was replicated three times to ensure reliability.

The potential of isobavachalcone to eradicate biofilms was evaluated using four isolates of *E. faecalis*, namely FB − 1, 16C51, 16C102, and 16C166. The *E. faecalis* strains were grown in TSB at 37 °C for 12 h and subsequently diluted 1:200 in 200 μL of TSBG, and incubated in 96-well plates for 24 h at 37 °C to form mature biofilms. Unattached cells were removed by 0.9% saline. Antimicrobials (8 × MIC) in TSBG were added, with a blank control, and culture for 48 h (medium replaced at 24 h). Biofilm biomass was determined by employing crystal violet staining [24]. Triplicate experiments were conducted.

Time-kill assay

To investigate the quick bactericidal efficacy of the antibiotic against *E. faecalis* planktonic cells, two isolates of *E. faecalis* were tested in time-kill assays using isobavachalcone alongside several common antibiotics, such as ampicillin, vancomycin, and linezolid, in accordance with earlier studies [25]. The strains were incubated in MHB at 37 °C for 12 h, followed by a 100-fold dilution in CAMHB, after which antibiotics were introduced to achieve final concentrations of 4 × MIC, and were cultured at 37∘C. Samples of 1 ml aliquots were taken at 2, 4, 6, 12, and 24 h, respectively, and subsequently washed with a 0.9% saline solution. Following this, ten-fold serial dilutions of the samples were prepared and spread onto Muller-Hinton agar plates. Finally, the number of Colony - Forming Units (CFU) was counted. Each trial was conducted in triplicate.

Persister assay

In order to evaluate the supplementary anti-persister efficacy of isobavachalcone against *E. faecalis*, two isolates of *E. faecalis*, specifically 16C51 and 16C106, were chosen and underwent persister assays with the participation of isobavachalcone and multiple first-line antibiotics. Strains of *E. faecalis* were cultivated in MHB at a temperature of 37 °C for a period of 12 h. Subsequently, the culture was diluted a hundred-fold using CAMHB until it reached the exponential phase. Then, 7 mL of this diluted culture was subjected to exposure to a variety of antibiotics at a concentration of 10× MIC at 37 °C [26]. After 12-hour incubation with 200-rpm shaking, the samples were washed twice with 0.9% NaCl. Then they were serially diluted and spread on TSB plates. The number of surviving persisters was counted by the dilution plating method.

To determine if the remaining colonies were persister cells, we took two colonies from each sample and put them back into CAMHB broth. Then we tested the antimicrobial susceptibility of isobavachalcone in vitro and found that the MIC value stayed the same.

In vitro, induction of isobavachalcone-resistant *E. faecalis* with potential mutations

To investigate the potential target genes of isobavachalcone in *E. faecalis*, we induced and screened *E. faecalis* strains that displayed resistance to isobavachalcone and possibly harbored mutations, following methods established in earlier research [27]. The *E. faecalis* isolates underwent serial subculturing in TSB supplemented with isobavachalcone. We began with a beginning concentration of 1/2 × MIC, which was subsequently escalated to higher levels. Each strain of *E. faecalis* was cultured across three to five passages at each concentration before progressing to the subsequent generation. Upon the conclusion of the final sub-culturing step at each concentration, the Enterococcus faecalis isolates were retrieved and subsequently transferred onto tryptic soy agar plates without isobavachalcone. These isolates underwent an additional three rounds of sub-culturing on these plates. Next, they were re-identified with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (IVD MALDI Biotyper, Bruker, Germany). Also, their MIC for isobavachalcone was checked again. Up until the 21st generation, the isobavachalcone-resistant *E. faecalis* were preserved at minus 80 degrees Celsius in a 35% TSB solution.

Genome sequence of mutations

In this experiment, genomic DNA was extracted from two isobavachalcone-resistant *E. faecalis* isolates (16C51-T1.1 and 16C51-T1.2). For the sample preparations, each isolate utilized 1 μg of DNA. The whole genomes are sequenced using an Illumina HiSeq 4000 system, following the methodology outlined in prior research [28]. The analysis involved predicting a variety of genetic elements, including coding genes, genomic islands, and CRISPR sequences. The genomes of each sample were compared against the reference genome of *E. faecalis* OG1RF (GenBank: NC_017316.1) using MUMmer and LASTZ for genomic alignments. These alignments enabled the identification of single-nucleotide polymorphisms, insertions, deletions, and structural variations, which were annotated according to the results from MUMmer and LASTZ. The data are available in the NCBI with accession NO PRJNA722586.

Molecular docking

The PDB files of the molecular configurations of PurH and FlgJ were generated using the cutting-edge deep-learning algorithms provided by AlphaFoldDB, a leading platform in protein structure prediction [29]. That file for isobavachalcone was obtained from the PubChem database, a comprehensive resource for chemical information. The molecular docking method was applied to analyze the binding structure between these two proteins and isobavachalcone, so as to determine the preferred orientation of linking. The optimal binding pockets of these two proteins were identified using cavity detection algorithms, which analyze the protein's surface to locate potential binding sites. The optimal binding site for isobavachalcone was determined using AutoDock Vina, a widely used docking program that employs a scoring function founded on the Vina score (kcal/mol) to evaluate the strength of the interaction of ligand and protein in the identified active site [30].

Statistical analysis

In this research, statistical processing was carried out using the t-test method. A notability threshold of $P < 0.05$ was set for judging statistical relevance. The dataset was processed and evaluated through the SPSS statistical software.

Results

In vitro activity of isobavachalcone against clinical *E. faecalis* isolates

We investigated the antibacterial effect of isobavachalcone on *E. faecalis* by determining its MICs using *E. faecalis* ATCC29212 and OG1RF as reference strains. Subsequently, we evaluated the MICs of isobavachalcone in 22 clinical isolates of *E. faecalis* (obtained from urine, exudate, or blood). All of these isolates exhibited MICs of 12.5 μM, as shown in Table 1. Among these, the MICs of isobavachalcone were determined to be 12.5μM for two linezolid-resistant *E. faecalis* strains as well as ten daptomycin-nonsusceptible strains, highlighting its potent antibacterial efficacy against MDR *E. faecalis*. Among these, the MICs of isobavachalcone were determined to be 12.5μM for two linezolid-resistant *E. faecalis* strains as well as ten daptomycin-nonsusceptible strains, highlighting its potent antibacterial efficacy against MDR *E. faecalis*. We conducted a broader range of antimicrobial activity validation experiments for the potential use of isobavachalcone. The experiment included 198 isolates of *E. faecalis* from different clinical sources, which were collected from patients' urine, blood, bile and other sites. After experimental analysis, it is encouraging to note that isobavachalcone demonstrated a stable and significant antibacterial activity - its MIC was generally distributed in a relatively low range of 6.25 to 12.5 μM, both the MIC50 and MIC90 remained at 12.5 μM, a finding that provides experimental support for the clinical application of this compound (Table S1).

Growth curve and the anti-biofilm activity of isobavachalcone

To explore the impact of that antibiotic on the *E. faecalis* biofilm formation, the impact of sub-MIC concentrations of it on the planktonic growth of *E. faecalis* (16C51,

Table 1 Antimicrobial susceptibilities of *E. faecalis*

Strains	MIC(mg/L)								MIC
	Amp	Van	Lin	Dap	Rif	Min	Gen	FOS[a]	Iso
OG1RF	2	2	2	4	128	1	16	64	12.5μM(4.05 mg/L)
ACTCC29212	2	2	2	4	1	4	4	8	12.5μM(4.05 mg/L)
FB-1	2	1	8	4	8	1	>512	32	12.5μM(4.05 mg/L)
16C35	4	1	2	8	8	16	16	64	12.5μM(4.05 mg/L)
16C51	2	2	4	8	8	16	8	64	12.5μM(4.05 mg/L)
16C54	2	1	2	16	16	8	>512	64	12.5μM(4.05 mg/L)
16C68	4	1	2	4	4	16	>512	64	12.5μM(4.05 mg/L)
16C102	4	1	8	4	8	8	32	32	12.5μM(4.05 mg/L)
16C106	2	1	2	8	8	16	>512	64	12.5μM(4.05 mg/L)
16C124	1	1	2	4	8	16	8	64	12.5μM(4.05 mg/L)
16C125	2	1	2	4	2	16	>512	64	12.5μM(4.05 mg/L)
16C137	2	0.5	2	8	32	16	>512	64	12.5μM(4.05 mg/L)
16C138	2	1	2	8	8	16	>512	32	12.5μM(4.05 mg/L)
16C152	2	1	2	2	4	16	>512	64	12.5μM(4.05 mg/L)
16C166	2	1	2	8	4	16	16	32	12.5μM(4.05 mg/L)
16C168	4	1	2	8	2	16	>512	64	12.5μM(4.05 mg/L)
16C186	2	0.5	2	8	8	8	16	64	12.5μM(4.05 mg/L)
16C201	2	1	2	4	8	16	>512	32	12.5μM(4.05 mg/L)
16C274	4	1	2	8	16	16	16	64	12.5μM(4.05 mg/L)
16C289	2	1	2	2	2	16	8	64	12.5μM(4.05 mg/L)
16C350	2	1	2	4	2	16	>512	32	12.5μM(4.05 mg/L)
16C353	2	1	2	16	16	16	16	32	12.5μM(4.05 mg/L)
16C385	2	0.5	2	4	16	16	>512	64	12.5μM(4.05 mg/L)
16C405	2	1	2	4	2	16	>512	64	12.5μM(4.05 mg/L)

16C106) planktonic cells was detected via automatic growth curve analyzer. During the logarithmic growth phase, isobavachalcone at a concentration of 1/2 × MIC was found to exert inhibitory effects on the planktonic growth of *E. faecalis*, as presented in Fig. 1a. Conversely, isobavachalcone at a series of concentrations, specifically 1/4 ×, 1/8 ×, 1/16 ×, and 1/32 × MICs, did not show any inhibitory effects on *E. faecalis* (Fig. 1a, b). The inhibitory effect of isobavachalcone on *E. faecalis* biofilm formation was further investigated (shown in Fig. 1d, c). Notably, it was observed that the biofilm formation of *E. faecalis* 16C51 was significantly inhibited by 1/2 × or 1/4 ×MICs of isobavachalcone. Although no significant inhibition of planktonic growth of *E. faecalis* 16C106 was observed at either 1/2 × or 1/4 × MIC concentrations, 1/2 × MIC of the antibiotic effectively inhibited the biofilm formation of the strain 16C106.

Time-killing curve and biofilm elimination of *E. faecalis* by isobavachalcone alone or in combination

A comparison was drawn to explore the bactericidal and anti-biofilm potency of isobavachalcone, with its performance examined both when used alone and in combination with other antibiotics, focusing on two isolates of *E. faecalis*, namely 16C51 and 16C106. Firstly, time-killing curve analysis were performed for isobavachalcone and linezolid, vancomycin, and ampicillin. Exposure to antibiotics at 4 × MIC caused a decrease in log10CFU/mL over the 0-24-hour period (Fig. 2a, b), which reflects that similar bactericidal effect of isobavachalcone alone and combined other common antibiotics in the two isolates. It is noteworthy that the time-killing curve of isobavachalcone alone against *E. faecalis* was significantly lower than that of linezolid or vancomycin, and that the combination of isobavachalcone with these two drugs did not further enhance its bactericidal activity. Figure 2c presents the anti-persister assay of isobavachalcone in the *E. faecalis* 16C51 isolate, clearly demonstrating its activity in eradicating persister cells. Notably, the anti-persister activity of isobavachalcone against the 16C51 isolate of *E. faecalis* was comparable to that of vancomycin and linezolid. Even more interestingly, when isobavachalcone was combined with these three commonly used antibiotics, a significantly enhanced anti-persister effect was achieved. Similar results were found for the *E. faecalis* 16C106 isolate, which suggests that such a combination strategy can completely eliminate the persister cells after 48 h (Fig. 2d). Moreover, the efficacy of isobavachalcone combination with other three antibiotics (all for t 8 × MIC) to treat formed *E. faecalis* biofilms (FB-1, 16C51, 16C102, 16C166) were explored. The results indicated that it combined with daptomycin had an obvious elimination effect on the 16C51, but was not observed in other isolates (Fig. 3).

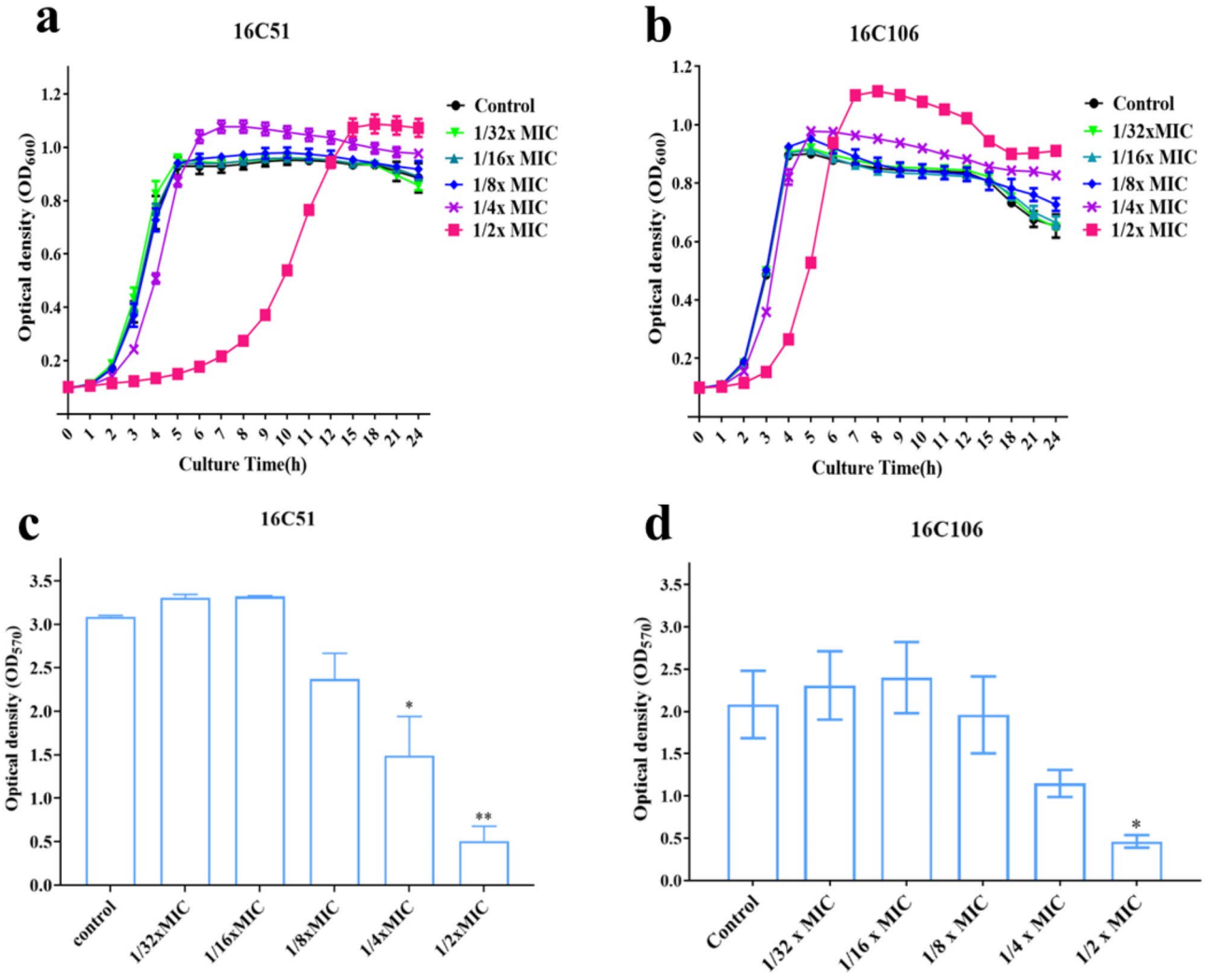

Fig. 1 *E. faecalis* strains 16C51 (**a**) and 16C106 (**b**) were treated with sub-MIC concentrations of isobavachalcone for 24 h or left untreated. The growth of planktonic cells was detected by OD600. Panels (**c**) and (**d**) show the effect of sub-MIC isobavachalcone on biofilm formation of the two isolates. Data in (**a**, **b**) are means ± SD of three replicates; spots mark the mean. Biofilm formation was determined by crystal violet staining and absorbance measurement at 570 nm for 24 h (Table S2). Data in (**c**, **d**) are means ± SEM of three independent experiments. Each treatment was compared with the control; significant differences (*$P < 0.05$, **$P < 0.01$; Student's t-test) were marked, $n = 3$

Genetic mutations in the isobavachalcone-resistant *E. faecalis* and potential targeting were identified through molecular docking

To research the potential target site, the isobavachalcone-resistant *E. faecalis* isolates 16C51-T1 were selected under the pressure of isobavachalcone. Then, the whole genome sequencing in 16C51-T1 was conducted to detect possible genetic mutations associated with isobavachalcone resistance. This analysis revealed 10 nucleotide mutations in such isolate (Table 2). *And the relevant* mutations were found in PurH and FlgJ proteins (Fig. 4a). Moreover, the other genetic mutations were implicated in cell wall or membrane biosynthesis, DNA replication, as well as energy transduction and assimilation. Given the pivotal roles that PurH and FlgJ proteins play in the planktonic growth phase of *E. faecalis*, the molecular docking approach was employed to evaluate the binding affinity of isobavachalcone to PurH and FlgJ. Remarkably, the results revealed that the isobavachalcone might interact with PurH (Fig. 4b, c) and FlgJ (Fig. 4d, e) in favorable conformations, with low binding energies of − 8.6 kcal/mol and − 7.1 kcal/mol respectively. It should be noted that a more negative score value (i.e., a greater absolute value of the negative value) signifies a stronger binding force, and an affinity < 7 kcal/mol suggests a good binding force. Figure 4b presents the optimal conformation of the complex formed by isobavachalcone and PurH. In the docking pocket region, it was found that the interaction between isobavachalcone and PurH comprises nine hydrogen bonds, along with five hydrophobic interactions and two ionic interactions (Fig. 4c). These findings

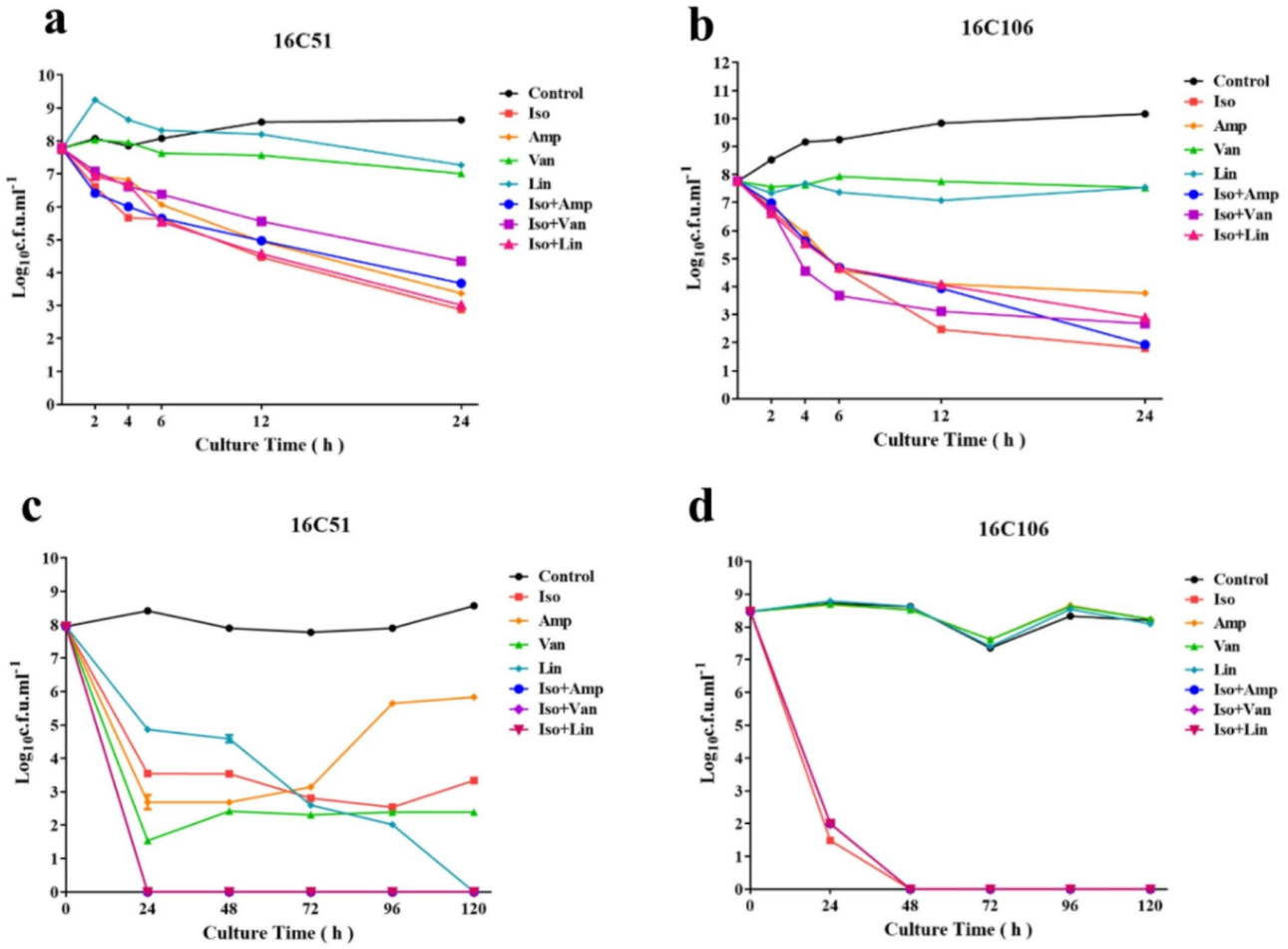

Fig. 2 For *E. faecalis* strains 16C51 (**a**) and 16C106 (**b**), the time-kill curves of planktonic cells were examined using 4 × MIC isobavachalcone combined with ampicillin, vancomycin, and linezolid. The anti-persister activity of isobavachalcone against these two strains (**c**, **d**) was also tested with the same three antibiotics and their combinations at 10 × MICs. Data in panels show the SD of means from three replicates; spots denote the mean of independent experiments. Antimicrobials were added at t = 0 and monitored for 24 h. Abbreviations: Amp for Ampicillin; Van for Vancomycin; Lin for Linezolid

indicate that isobavachalcone exhibits the potential to bind efficaciously to PurH of *E. faecalis*.

MIC, minimum inhibitory concentration; Amp, Ampicillin; Van, Vancomycin; Lin, Linezolid; Dap, Daptomycin; Rif, Rifampin; Min, Minocycline; Gen, Gentamicin, Fos, Fosfomycin; Iso, Isobavachalcone. a, agar dilution method.

E. faecalis strain16C51 strain was serially subcultured in TSB isobavachalcone containing Mutations in the isolate T1 from 21 generations of 16C51 strain were detected by whole genome sequencing. NA, nucleotide; AA, amino acid.ref_gene_strand, the orientation of the gene in which the SNP is located on the reference sequence.

Discussion

Isobavachalcone, a naturally occurring flavonoid, is extracted from various medicinal plants such as those in the Fabaceae and Moraceae families, among others [31]. So far, some scholars at home and abroad have studied this problem and formed a corresponding theoretical system. These findings also provide a reference for the writing of this paper. Research has shown that it exhibits strong antibacterial characteristics, particularly against Gram-positive bacteria like Methicillin-Susceptible *S.aureus* (MSSA) and MRSA, having MIC levels of 1.56 μg/mL and 3.12 μg/mL, individually [32]. However, there are limited publications on the effect of this natural flavonoid on *E. faecalis*. Linezolid is one important choice for VRE infections [33], and recently, the prevalence of linezolid-resistant *E. faecalis* cases has risen, with their biofilm-forming ability severely restricting treatment choices [34]. Biofilm and persister cell formation are two key survival strategies employed by bacteria [35]. Current first-line antibiotics often lack the dual ability to eradicate persister cells and disrupt biofilms. Thus, it's critical for the development of more effective antibacterial drugs for selecting antibacterial compounds with both

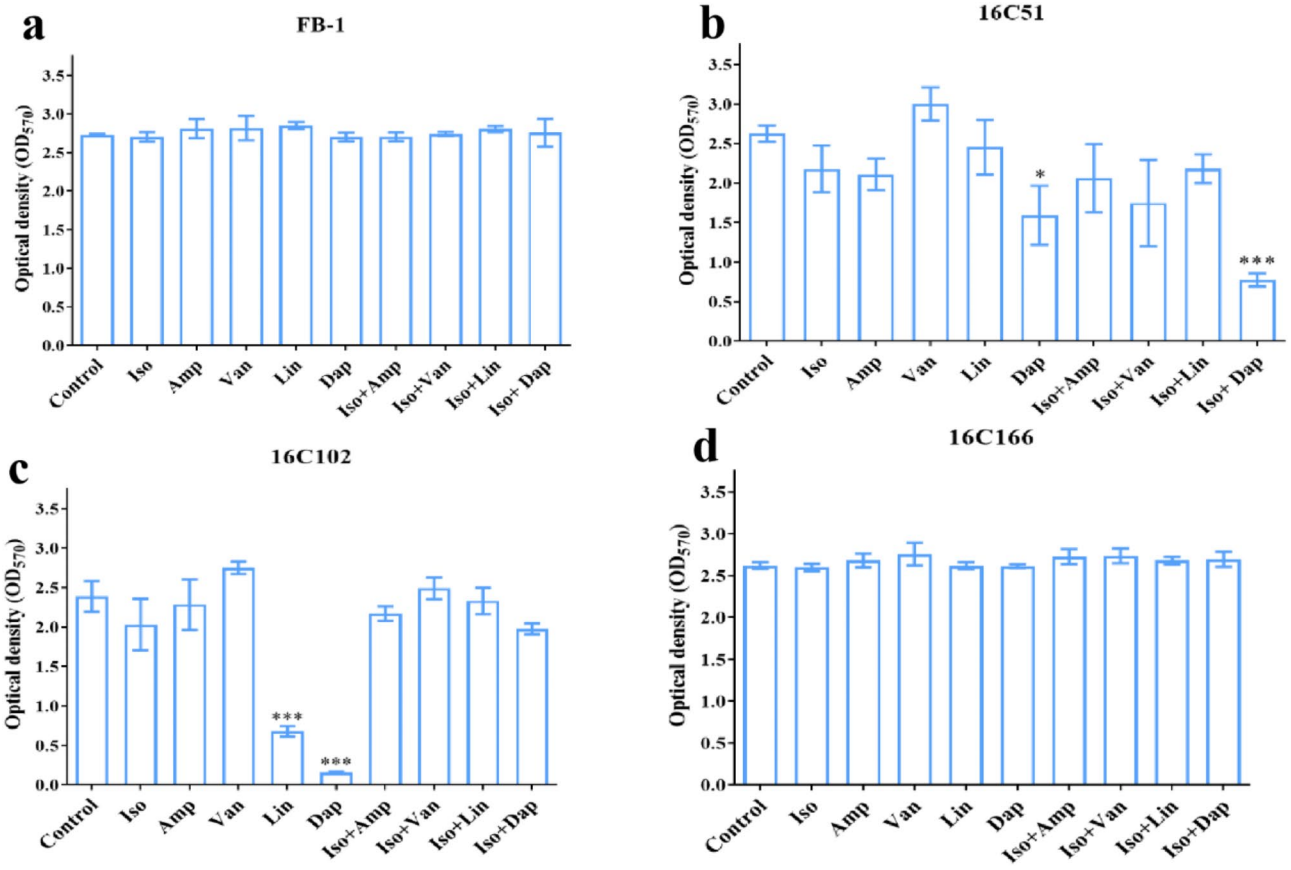

Fig. 3 The effect of isobavachalcone on formed biofilms of four *E. faecalis* strains (FB-1(**a**), 16C51(**b**), 16C102(**c**), 16C166(**d**)) was investigated. Biofilm formation at 8×MIC was measured by absorbance at 570 nm after 24 h of culture (Table S3). Data shown are means ± SD of three independent experiments. Each treatment was compared to the control, and significant differences (*$P < 0.05$; ***$P < 0.001$; Student's t-test) were marked, $n = 3$. MIC, minimum inhibitory concentration. Amp, Ampicillin; Van, Vancomycin; Lin, Linezolid; Dap, Daptomycin; Iso, Isobavachalcone

Table 2 Mutations in *E. faecalis* 16C51-T1 isolate. Genome analysis of mutations

ref_gene_ID	NA mutations	AA mutations	ref_gene_product	ref_gene_strand
16C51T1_GM000347	T247C	Y83H	Pyrroline-5-carboxylate reductase Amino acid transport and metabolism	+
16C51T1_GM000484	G559A	A187T	dTDP-4-dehydrorhamnose reductase Cell wall/membrane/envelope biogenesis	+
16C51T1_GM000718	C187T	P63S	Adenine deaminase Nucleotide transport and metabolism	+
16C51T1_GM001010	A508G	T170A	Flagellum-specific peptidoglycan hydrolase FlgJ Cell wall/membrane/envelope biogenesis; Cell motility;	-
16C51T1_GM001244	A688G	K230E	Fatty acid/phospholipid biosynthesis enzyme Lipid transport and metabolism	+
16C51T1_GM001349	T938C	I313T	DNA-binding transcriptional regulator, FrmR family, Transcription	+
16C51T1_GM001679	G49A	E17K	Glycerol kinase Energy production and conversion	-
16C51T1_GM001760	C632A	T211K	AICAR transformylase/IMP cyclohydrolase PurH Nucleotide transport and metabolism	+
16C51T1_GM002262	A109G	K37E	Signal transduction histidine kinase Signal transduction mechanisms	-
16C51T1_GM000346	A442G	I148V	Phosphomannomutase Carbohydrate transport and metabolism	+

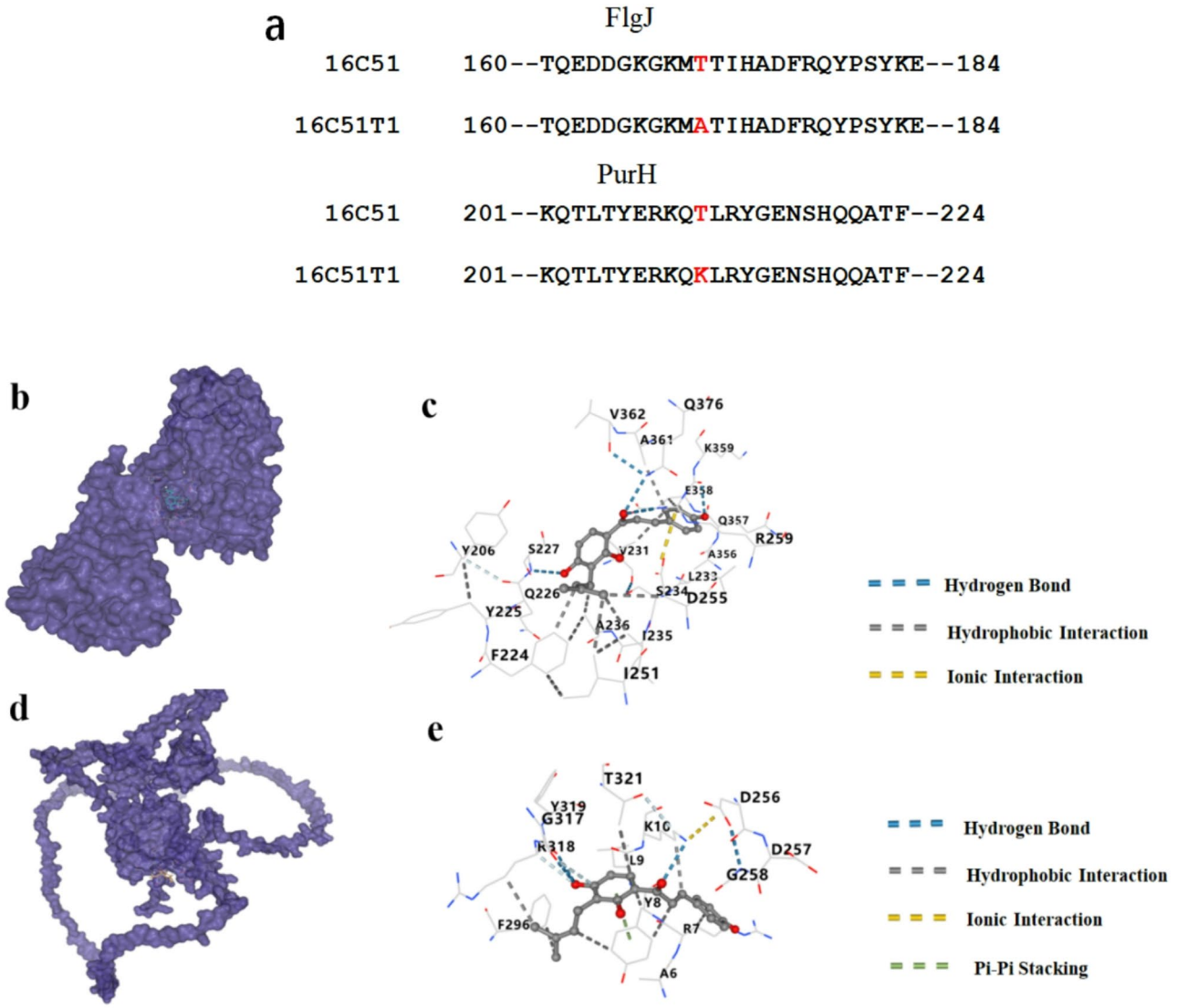

Fig. 4 (**a**) The expected mutation in the target genes occurs at a specific position in the amino acid sequence. The anticipated mutation within the target genes takes place at a precise locus in the amino acid sequence. Molecular docking was employed to explore the interaction between isobavachalcone and PurH (**b**, **c**) as well as FlgJ (**c**, **d**). Utilizing a ball-and-stick model, the isobavachalcone was visually represented with its constituent elements depicted in diverse color schemes, where blue was used to signify a nitrogen atom, red for an oxygen atom, and green for a fluorine atom

persister-killing and biofilm-eliminating [15]. It has been reported that certain small molecules and natural products possess the ability to suppress growth, impede biofilm formation, and inhibit exopolysaccharide synthesis of *E.faecalis* [36, 37]. Here, our study demonstrated that isobavachalcone exhibited superior antibacterial potency against *E. faecalis* clinical isolates from China and effectively inhibited *E. faecalis* biofilm formation at sub-MIC concentrations. Notably, isobavachalcone seemed to have similar MIC values against linezolid - or daptomycin - nonsusceptible *E. faecalis* to those of the ones susceptible to antibiotics. The obtained results suggest that isobavachalcone shows promise as a potent antibacterial substance for combating drug-resistant *E. faecalis*. It is commonly acknowledged that bacterial persister cells can escape the killing effect triggered by antimicrobials by transitioning into a state of physiological dormancy. This phenomenon is regarded as a crucial element contributing to the inefficacy of antimicrobial interventions and the recurrence of infections [38]. Therefore, a diminution in the production of bacterial persister cells is prone to induce a reduction in the incidences of chronic or relapsing infections and, furthermore, it is anticipated that such a reduction will concomitantly augment the effectiveness of anti-infection treatments [9]. *E. faecalis* often exhibits resistance to vancomycin and β-lactam antibiotics, making combination therapy a common treatment approach. Research employing biofilm time-kill assays and in vitro

PK/PD models has shown that β-lactams can boost the effectiveness of daptomycin when targeting vancomycin-resistant strains of *E. faecalis* [39, 40]. Interestingly, our results demonstrated that isobavachalcone, when administered as a monotherapy, exhibited a rapid bactericidal effect against *E. faecalis*, resulting in the eradication of a greater number of planktonic cells in comparison to vancomycin, linezolid, and ampicillin. Furthermore, it was observed that isobavachalcone eliminated *E. faecalis* persister at high concentrations, and that combining it with vancomycin, linezolid or ampicillin enhanced its effectiveness. Nevertheless, despite demonstrating potential when combined with daptomycin, the combination of isobavachalcone with several other drugs failed to effectively eradicate formed *E. faecalis* biofilms. This result agrees with the findings of prior research, which suggest that once bacterial biofilms have been formed, antimicrobials often struggle to successfully remove them [41]. Consequently, additional investigations are warranted to corroborate the effectiveness of isobavachalcone in eradicating biofilms in combination with daptomycin, which, in turn, can provide theoretical reference for clinical practice.

In order to explore the potential binding sites of isobavachalcone within *E. faecalis*, an in vitro selection process of isobavachalcone-insensitive *E. faecalis* clones was implemented. This was achieved by inducing wild-type strains exposed to isobavachalcone, followed by identifying mutations within the potential target genes via genome-wide sequencing. Our current study postulates that modifications occurring in the PurH and FlgJ proteins potentially serve as the primary targets of isobavachalcone. This hypothesis is substantiated by molecular docking assays. Moreover, it was noted that additional modifications are involved in the processes of cell membrane biosynthesis and DNA replication. Previous investigation have demonstrated that ampicillin and vancomycin exert their antibacterial activities by inhibiting the cell wall synthesis of Gram-positive bacteria, whereas linezolid achieves its antibacterial efficacy by impeding the protein synthesis of such bacteria [42]. In our study, it was noted that isobavachalcone exhibited a quick bactericidal efficacy on *E. faecalis* planktonic cells. Significantly, it proved to be more effective than vancomycin, linezolid, and ampicillin. Moreover, prior research has suggested that the antibacterial efficacy of isobavachalcone is associated with membrane perturbation [32]. Consequently, this research implies that isobavachalcone might not inhibit protein synthesis, but rather appears to exercise its antibacterial efficacy by impeding the formation of the cell membrane.

In-depth analysis reveals that the present research exhibits dual limitations. Primarily, it was incapable of pinpointing the objective sites of isobavachalcone in *E. faecalis* via experimental development or serial passage methodologies. Secondly, even though such experimental procedures are instrumental in assessing the propensity of a given antimicrobial substance to engender resistance mutations, they might not unerringly disclose the precise molecular target. This is due to the inherent complexity of microbial resistance mechanisms and the potential for multiple compensatory pathways to emerge, confounding the direct identification of a single, definitive target. Consequently, additional research is essential to definitively determine the accurate target of isobavachalcone in *E. faecalis* by leveraging alternative techniques like liquid chromatography-mass spectrometry (LC-MS) and co-immunoprecipitation (CO-IP).

Conclusion

This study highlights that isobavachalcone might be a potent antibacterial agent against *E. faecalis*, capable of significantly inhibiting biofilm formation at sub-MIC concentrations. Isobavachalcone exhibited robust bactericidal effects, even possibly outperforming vancomycin, linezolid, and ampicillin against planktonic cells and effectively eradicating persister cells. Our findings suggest that the mechanism of action of isobavachalcone may involve disruption of cell membrane biogenesis, with potential molecular targets identified as the PurH proteins. These insights provide a foundation for developing isobavachalcone as a novel antimicrobial agent, specifically addressing MDR *E. faecalis* and biofilm-associated infections, which are notoriously difficult to treat. Future research should focus on in vivo studies to confirm the safety and efficacy of isobavachalcone and its combinations, as well as advanced molecular approaches to conclusively identify its precise mechanisms of action.

Abbreviations

E. faecalis	Enterococcus faecalis
VRE	Vancomycin-resistant enterococci
MDR	Multidrug-resistant
MRSA	Methicillin-resistant Staphylococcus aureus
MSSA	Methicillin-sensitive Staphylococcus aureus

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
Ouyang L was responsible for the antimicrobial susceptibility and growth curve tests and contributed to paper writing. Xu Z focused on antimicrobial susceptibility testing. Tang Y was in charge of the time-kill and persister assays. Li D collected E. faecalis samples and entered data for statistics. Wen Z handled genome sequence analysis and assisted with protein tests. Yu Z, Zhang C, and Zhang H designed the study framework, carried out data analysis, and provided key support for the paper.

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Data availability
The whole-genome sequencing data were posted in the Sequence Read Archive (SRA) database under BioProject accession numbers PRJNA722586 and PRJNA685616 in NCBI.

Declarations

Ethics approval and consent to participate
All methods were carried out by relevant guidelines and regulations and were approved by the Ethics Committee of Shenzhen Nanshan People's Hospital and the 1964 Helsinki Declaration and its later amendments, or comparable ethical standards. All experimental procedures involving human subjects were approved by the institutional ethical committee of Shenzhen Nanshan People's Hospital. Bacterial strains were collected as part of the routine clinical management of patients, according to the national guidelines in China [43]. Therefore, informed consent was not sought, and an informed consent waiver was approved by the institutional ethical committee of Shenzhen Nanshan People's Hospital.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Clinical trial number
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