**Enterococcus faecalis** thrives in dual-species biofilm models under iron-rich conditions

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**Abstract**

*Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*) are pathogenic strains that often coexist in intestinal flora of humans and are prone to cause biofilm-associated infections, such as gastrointestinal tract and urinary tract infections. Earlier studies have demonstrated that *E. faecalis* biofilm can metabolize ferrous ions in iron-rich environments and promote biofilm growth under *in-vivo* conditions. However, the influence of iron transporters on dual-species biofilm growth and the nature of molecular-level interactions between iron transporter proteins and Fe²⁺ remains unknown. Therefore, in this work, co-culture studies were performed and the study indicates that Fe²⁺ at concentrations of 50–150 µM promotes the colonization of *E. coli*, and Fe²⁺ concentrations of 50–200 µM promote the growth of *E. faecalis* and dual-species colonies. Atomic absorption spectroscopy results reveal that Fe²⁺ ion augmentation in bacterial cells was increased to 4 folds in the single-species model and 11 folds in the dual-species model under iron-supplemented conditions. Furthermore, Fe²⁺ augmentation increased the antibiotic resistance of *E. faecalis* in both single- and dual-species bacterial cultures. In addition, *in-silico* docking were performed to determine a three-dimensional (3D) structure of ferrous iron-transporter proteins FeoB of *E. faecalis* and its affinity to extracellular Fe²⁺. Our model suggests that the FeoB facilitates the Fe²⁺ uptake in *E. faecalis* cells in the absence of iron chelator, 2,2-bipyridyl.

**Keywords** Dual-species biofilm · Fe²⁺ augmentation · Ferrous iron transporter · Homology modeling · Molecular docking

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**Introduction**

Biofilm formation is a multi-step and sequential process that begins with the attachment of a single cell (planktonic bacterium) to a conditioned surface (Garrett et al. 2008; Govindarajan and Kandaswamy 2022; Shanmugasundarasamy et al. 2022). Biofilms can consist of a single (predominantly studied in the laboratory) or multiple (polymicrobial) species. In addition, Mixed-species biofilms are hallmarks of clinical infections (Govindarajan et al. 2020). For instance, surgical, urinary tract, and wound infections are typically caused by a combination of bacterial species, such as *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*) and *Pseudomonas aeruginosa* (*P. aerugionsa*) (Kay et al. 2011; Dalton et al. 2011; Grabe et al. 2015; Tan et al. 2022). For instance, *E. faecalis* along with *E. coli* often colonizes clinical devices such as catheters and leads to recurrent Urinary Tract Infection (UTI) (Shanmugasundarasamy et al. 2022; Stamm et al. 1991; Croxall et al. 2011). Mixed species biofilm infections can better tolerate antibiotic treatment.
and often produce higher biomasses that can escape from immune responses and leads to recurrent infections (Lopes et al. 2012; Radlinski et al. 2017).

*E. faecalis* often co-exists with *E. coli* and other microbial species as polymicrobial biofilm in wound infection (Hughes and Winter 2016) and majority of those bacterial species require iron for growth and survival (Nairz et al. 2010). However, within mammalian hosts, availability of free iron is very limited due to intracellular iron sequestration either within cells or attached to secreted proteins, such as transferrin or lactoferrin (Anders 2000). Most bacteria have multiple systems for iron acquisition. In particular, siderophores are well-studied iron transporters in bacteria that binds to ferric iron with extremely high affinity (Kramer et al. 2012). In addition, ferrous iron transport system (Feo) was first identified in *E. coli* and conserved in majority of bacterial species, and the *feo* operon consists of three open reading frames *feoa*, *feob*, and *feoc* (Hantke 1987). In *Salmonella enterica*, it was shown that, FeoA directly binds to FeoB and the interaction of these two proteins is crucial for uptake of Fe$^{2+}$ (Kim et al. 2012). In *Vibrio cholerae*, it was shown that the C-terminal domain of FeoB could be embedded in inner membrane and iron acquisition is done by all three proteins (FeoA, B, and C) (Weaver et al. 2013). Taken together, it was clear that Feo iron transporter system is crucial for survival of bacterial species. However, the role of iron transporters in biofilm growth and its effect on antibiotic tolerance in mixed species was unclear and poorly understood. In addition, previous studies do not provide adequate information on the structural information of metal ion transporter proteins and their interactions with metal ions.

In the present study, we demonstrated the effects of Fe$^{2+}$ augmentation both in the single (*E. coli* or *E. faecalis*) and dual-species (*E. coli* + *E. faecalis*) biofilms. In addition, we have also found that *E. faecalis* is more resistant to antibotic (Kanamycin) than *E. coli* when Fe$^{2+}$ is supplemented in mixed species biofilms. Furthermore, using in-silico docking strategy we found that, FeoB of *E. faecalis* could potentially bind and import Fe$^{2+}$ in to the cytosol and this could promote biofilm formation and antibiotic resistance. In essence, the results from this study will not only provide a basis for understanding antimicrobial resistance in dual-species biofilm models but also serve as a potential starting point for designing novel biofilm inhibitors.

### Materials and methods

#### Bacterial strains and culture methods

Gram negative (*E. coli*: MTCC 443) and Gram positive (*E. faecalis*: OG1RF), strains were used in this study. The strains were streaked on the agar plates for the single colony isolation. The isolated single colony was inoculated in 2 ml of freshly prepared Luria–Bertani broth (LB broth, HiMedia) and incubated overnight at 37 °C in a shaker flask at 120 rpm. For dual-species biofilm studies, the cultures were inoculated in a 1:1 ratio (*E. coli*: *E. faecalis*) in LB broth and incubated overnight at 37 °C in a shaker flask at 120 rpm. In addition, all the experiments were performed using minimal media (M9 media) to check the consistency of growth media. The M9 media (100 ml) consists of 20 mL M9 salts 5X, 2 mL of 20% Glucose, 200 μL of 1 M MgSO$_4$, and 10 μL of 1 M CaCl$_2$, 1.5 g of peptone, and 78 mL of H$_2$O.

### Colony-forming units assays

Colonies Forming Units (CFU) assay was performed for both the single- and dual-species bacterial cultures (Saravanan et al. 2020). One ml of bacterial culture at 0.5 OD$_{588}$ was chosen and serially diluted from $10^{-1}$ to $10^{-8}$ in LB broth. Five microliters of the serially diluted culture ($10^{-6}$ to $10^{-8}$) were then added to sterile LB agar as a control group. In the test sample, the nutrient agar was supplemented with 50, 100, 150, 200, 250, and 300 μM of FeSO$_4$ and incubated at 37 °C for 12 h and the colonies were then counted to determine CFU/ml using the formula (Eq. 1). A similar method was performed to determine the effect of iron chelators on CFU. Chelators such as 2,2-bipyridyl (2bpy) were added to test samples in ratios such as (2bpy:FeSO$_4$; 3:1) to chelate the extracellular Fe$^{2+}$. Wherein, 3 molecules of 2bpy binds to Fe$^{2+}$ and completely chelates the extracellular Fe$^{2+}$ which in turn eradicates the extracellular Fe$^{2+}$ effect to the bacterial cells. All the experiments were performed in triplicates. To check the consistency of CFU in growth media, the entire experiment was also repeated using minimal media (M9 media) following the same conditions.

$$\text{CFU/ml} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of culture plated (mL)}} \quad (1)$$

### Estimation of doubling time for single- and dual-species biofilms

To determine the doubling time for both single (*E. coli* or *E. faecalis*) and dual-species (*E. coli* + *E. faecalis*) cultures, 5 μL of an overnight culture was inoculated in 50 mL sterile LB broth and allowed to grow at 37 °C and 120 rpm. Three mL of the sample was collected at a 30 min time interval and OD$_{588}$ was measured. The values were plotted in a graph to analyze the difference in doubling the time.
between single- and dual-species bacterial cultures using the formula (Eq. 2). All the experiments were performed in triplicates:

\[
\text{Doubling time, } t = \frac{\text{time in minutes}}{\text{number of times the cell population doubles during the time interval}}
\]

### Cell counting for dual-species cultures

The cell counting was performed in the hemocytometer to evaluate the cell proportion taken for dual-species model, 20 μL of *E. coli* and *E. faecalis* cells were taken separately at 0.5 OD₅₈₈ for hemocytometer and covered with cover slip. The cells located in the hemocytometer grids was observed under the light microscope at 100 X magnification (Carl ZEISS Primo Star Light Microscope). The microscopy images were recorded using the Zen blue software by Zeiss and the cells were counted manually.

### Gram staining of dual‑species biofilms

Gram staining was performed using the method described in previous studies (Thairu et al. 2014). A loop-full of the bacterial colony from the culture plate of dual-species (*E. coli* + *E. faecalis*) grown in iron supplemented conditions (0, 50, 100, 150, 200, 250 and 300 μM) were smeared and heat-fixed in the coverslip. The sample was then stained with crystal violet (positively charged) dye for about 30 s to stain the Gram-positive bacterial cells in purple color. Then, the sample was treated with iodine solution for 30 s and washed with sterile distilled water to remove the excess crystal violet dye and iodine solution. The negatively charged iodine solution acts as a mordant which bind with positively charged crystal violet dye, together they form a complex which strengthens the crystal violet stain to Gram-positive bacteria. In a decolorizing solution, ethanol was then added to remove the unbounded dye. Then, the sample was counterstained with safranin dye to stain the Gram-negative bacterial cells in pink color and washed with sterile distilled water to remove the excess dye (Coico 2006). The slides were then allowed to dry for 10 min and then covered with a coverslip. The slides were then imaged under the light microscope at 100 X magnification (Carl ZEISS Primo Star Light Microscope). The images were processed using Zen blue software by Zeiss.

### Quantification of bacterial Fe²⁺ intake from external iron source

The bacterial cells, *E. coli*, *E. faecalis*, and dual-species cultures were allowed to grow overnight under both conditions as control (LB broth) and iron supplemented conditions (LB broth supplemented with 150 μM of FeSO₄ for *E. coli* culture; 50 mL LB broth supplemented with 200 μM of FeSO₄ for *E. faecalis*, and dual-species culture) which was incubated at orbital shaker at 37 °C, 120 rpm. After incubation, the samples were then centrifuged at 8000 rpm for 5 min to separate the pellet and supernatant. The supernatant (media) contains a reduced amount of Fe²⁺ ions that remain after bacterial growth (Liu et al. 2017). The bacterial cells were then resuspended and washed twice with distilled water to remove the culture media. Once again, the cells were resuspended with 50 mL distilled water and sonicated at 40 kHz for 2 min to break the cell wall and release the intracellular compounds. Then, the sonicated samples were centrifuged at 8000 rpm for 5 min to remove the cell debris (pellet) and to recover the intracellular compounds (supernatant). The left-over culture media and intracellular compound samples were analyzed in AAS, (Thermo Fisher scientific-iCE 3000 series AA Spectrophotometer) to trace the amount of Fe²⁺ concentration taken by the bacterial cells under iron supplemented and control conditions (Stokes et al. 2004). Initially, the parameters were selected in the SOLAAR AA software to run the AAS in terms of entering the number of samples and Fe²⁺ element selection from the periodic table to initiate the sample analysis. The inlet sample was automatically pumped from the sample tank to the analyzer followed by sample discharge in the waste tank. The values were displayed in the SOLAAR AA software after each sample.

### Pellicle biofilm formation of single- and dual-species under FeSO₄ supplemented condition

Pellicle biofilm assay was performed to study the effect of FeSO₄ on the single- and dual-species biofilms containing *E. coli* and *E. faecalis*. The overnight cultures were inoculated (inoculum:culture media; 1:20) into the culture tubes containing LB broth as a control group. In the test sample, LB broth is supplemented with 50, 100, 150, 200, 250, 300 and 350 μM of FeSO₄ and incubated at 37 °C for 48 h without shaking. After incubation, the tubes were washed thrice with sterile distilled water to remove the planktonic bacteria which does not adhere to the walls of the test tube. The tubes were then dried and stained with 0.1% (w/v) crystal violet and incubated at room temperature for 10 min. After incubation, the stain was removed and the tubes were washed three times with sterile distilled water to remove the excess dye from the sample. To each tube, 4 ml of 30% acetic acid was added to solubilize the stained adherent cells attached to the test tube walls. Then, the sample was measured under
OD$_{588}$ to estimate the biofilm formation (Mu et al. 2016). All the experiments were performed in triplicates. To check the consistency of biofilm formation in growth media, the entire experiment was repeated using minimal media (M9 media) following the same conditions.

**Iron chelating activity on the bacterial cells and biofilms under niche concentrations of FeSO$_4$**

To investigate the effect of the iron chelator (2bpy), the cells were grown under FeSO$_4$ supplemented conditions in the presence of 2bpy. The niche concentrations obtained from the CFU and pellicle biofilm assays were used as a guideline, in addition 2bpy was added in the ratio of 1:3 (iron:2bpy). In single-species studies, *E. coli* cells were grown in the iron-chelator complex at concentration, such as 0, 50, 150 and 250 μM. Similarly, *E. faecalis* and dual-species cells grown in the iron-chelator complex at concentration, such as 0, 100, 200, and 300 μM. The samples were then incubated at 37 °C for 12 h and the colonies were then counted to determine CFU/ml. All the experiments were done in triplicates.

Similarly, the chelator effect on the biofilm formation was investigated by incubating the monocultures, *E. coli*, and *E. faecalis* when supplemented with niche concentration of FeSO$_4$, 250 μM of FeSO$_4$:2bpy (1:3), and dual species when supplemented with 300 μM of FeSO$_4$:2bpy (1:3) and incubated at 37 °C for 48 h without shaking. After incubation, the tubes were washed thrice with sterile distilled water (5–10 mL) to remove the planktonic bacteria which does not adhere to the walls of the test tube. The tubes were then dried and stained with 0.1% (w/v) crystal violet (5 mL) and incubated at room temperature for 10 min. After incubation, the stain was removed and the tubes were washed three times with sterile distilled water to remove the excess dye from the sample. To each tube, 4 ml of 30% acetic acid was added to solubilize the stained adherent cells attached to the test tube walls. Then, the sample was measured under OD$_{588}$ to estimate the biofilm formation (Mu et al. 2016). All the experiments were performed in triplicates.

**Minimum inhibitory concentration of bacteria under iron supplemented conditions**

Minimum Inhibitory Concentration (MIC) assay was performed on single- and dual-species cultures of *E. coli* and *E. faecalis*. One mL of overnight cultures was mixed with 4 mL of LB broth containing 0.8% sterile agar and poured over the nutrient agar plate and incubated at room temperature to enable the formation of bacterial lawns. For dual-species biofilms (*E. coli* and *E. faecalis*) were mixed in the ratio of 1:1, 1:3, and 3:1 and for the test sample, the nutrient agar was supplemented with 150 μM of FeSO$_4$ for *E. coli* cultures, 200 μM of FeSO$_4$ for *E. faecalis* and dual-species cultures. Once the plates were solidified, 5 μL of Kanamycin with various concentrations (1000, 500, 100 and 50 μg/ml) was pipetted over the agar surface and allowed to incubate overnight at 37 °C. The culture plates were then observed for the zone of inhibition (Saravanan et al. 2020). All the experiments were done in triplicates.

**In-silico analysis on Fe$^{2+}$ ion interaction to FeoB protein of *E. faecalis***

Previous studies suggested that ferrous ion transporter protein facilitates extracellular ion transportation in bacterial cells (Kim et al. 2012; Morrison et al. 2013). The homology protein structures of the FeoB were modeled in the ModWeb server using the amino acid sequence (Accession ID: AEA93047.1; 716 amino acid residues) of *E. faecalis* (OG1RF) as a query sequence. The query sequence of FeoB was modeled in comparison with the three-dimensional crystal structures of FeoB proteins of *Klebsiella pneumoniae* (PDB ID: 2WIC, 2WIA), *Thermoto maritima* (PDB ID: 3A1V), and *Streptococcus thermophilus* (PDB ID: 3B1V, 3LX5), were used as template structure to construct five protein model. The best protein model was selected based on the scoring function available in MODELLER program—DOPE (Discrete Optimized Protein Energy) score and GA431 (Eswar et al. 2008). Model with lowest DOPE scores was selected for further studies. The best scoring modeled FeoB structure was validated in PROCHECK, Ramachandran plot for confirming the Van Der Waals force with no steric hindrance on parallel/antiparallel β-sheets and left/ right-handed α-helix conformations. Modeled FeoB protein was superimposed using the ‘align’ tool in PyMOL with the crystal structure of 3A1V, 2WIC, 2WIA, 3B1V, and 3LX5 posing ion interaction. The atomic coordinates of the ion interactions were located and sequentially matched to the query FeoB protein model to produce the final FeoB homology model bound to the Fe$^{2+}$ ion. The FeoB–Fe$^{2+}$ ion docking was carried out using the AutoDock Vina software (Sivaramakrishnan et al. 2020). Based on the predicted Fe$^{2+}$ ion binding residues, the grid was fixed in the FeoB protein for docking. Docked FeoB–Fe$^{2+}$ ion complex was analyzed in the LigPlus software to identify the Fe$^{2+}$ ion interaction (Kothandan et al. 2021).

**Results**

**Fe$^{2+}$ ion promotes the single- and dual-species colonies**

In vitro CFU assay was performed to determine the effect of FeSO$_4$ on bacterial growth. Where, *E. coli*, *E. faecalis*, and dual microbial community (*E. coli*: E. faecalis; 1:1; v/v)
inoculated at different dilutions such as $10^{-4}$, $10^{-5}$, and $10^{-6}$ on the LB agar plates supplemented with FeSO$_4$ concentration ranging between 0 and 300 µM (Fig. 1). The extracellular Fe$^{2+}$ concentration from 50 to 150 µM increases the colony count of E. coli cultures and the following concentrations of FeSO$_4$ (200–300 µM) reduces the colony count. Similarly, the extracellular Fe$^{2+}$ concentration from 50 to 200 µM increases the colony count of E. faecalis and dual-species cultures and the following concentrations of FeSO$_4$ (200–300 µM) reduces the colony count. This signifies that the niche concentrations of extracellular Fe$^{2+}$ such as 150 and 200 µM promotes the bacterial colonies of single and dual species, respectively. In GraphPad Prism 8.4.3, statistical significance was determined by paired t test for column wise comparison. $n=3$ (three biological replicates). $P \leq 0.0001$ (****), $P \leq 0.001$ (**), $P \leq 0.01$ (**) and $P \leq 0.05$ (*) and the error bar represents the standard deviation from the mean.

In addition, to ensure the true effect of extracellular iron supplementation, the minimal media (M9 media) (Abdul-Tehrani et al. 1999) was used and the results showed an increase in E. coli colonies from $14.67 \pm 2.49 \times 10^9$ to $28.67 \pm 3.39 \times 10^9$ CFU/mL under 150 µM of extracellular iron supplementation. Similarly, E. faecalis colonies were increased from $2 \pm 1.41 \times 10^9$ to $27.33 \pm 4.11 \times 10^9$ CFU/mL under 250 µM of extracellular iron supplementation, and the dual-species colonies were increased from $21 \pm 2.16 \times 10^9$ to $30 \pm 2.83 \times 10^9$ CFU/mL under 250 µM of extracellular iron. The higher extracellular iron concentrations ≥ 300 µM reduces the E. coli, E. faecalis, and dual-species colony formation (Supplementary Fig. 1). The extracellular iron increases CFU in both LB and M9 media. From the aforementioned CFU, data (obtained from both LB growth media and M9 minimal media) clearly show that extracellular iron promotes the colony formation in both single- and dual-species cultures only at specific iron concentration.

On the other hand, the 2bpy chelation on Fe$^{2+}$ does not promote/inhibit the colony formation of both single- and dual-species biofilms (Supplementary Fig. 2). Results in this study is in good agreement with the previous findings that 2bpy as a widely used neutral ligand to chelate the metal ions and possess high chelation affinity to Fe$^{2+}$ ion (Tan et al. 2022).
Doubling time for dual-species culture is same as single species

The doubling for the bacterial cultures was found to be 21 ± 0.3 min for *E. coli* cultures, 18 ± 0.2 min for *E. faecalis*, and 22 ± 0.2 min dual-species cultures. In the aforementioned doubling time, the growth rate of mixed species is nearly similar to single species which represents no growth defects found in mixed bacterial cultures (Fig. 2).

*E. coli* cells and *E. faecalis* cells found homogenous in hemocytometer

The cell count in the hemocytometer grid under microscope at 100 X magnifications shown that 25 ± 3.21 *E. coli* cells and 33 ± 4.04 *E. faecalis* cells were at 0.5 OD588 (Supplementary Fig. 3). This result represents that nearly same number of *E. coli* and *E. faecalis* cells were present at 0.5 OD588. These are the cell proportions that was present in the inoculum used for the dual-species model.

*E. faecalis* thrives in dual-species cultures under iron supplemented conditions

The microscopy studies revealed that the *E. faecalis* cells were more abundant under iron niche conditions in dual-species bacterial cultures (Fig. 3). The *E. faecalis* cells (blue diploccoci) were found in a greater population than *E. coli* cells (pink rod-shaped) in dual-species cultures under 200 µM of FeSO4 supplementation. From Fig. 3, it is clear that *E. faecalis* cell proportion in the dual-species

![Fig. 2](image2.png)  
**Fig. 2** Doubling time of the bacterial cultures was found to be 21 ± 0.3 min for *E. coli* cultures, 18 ± 0.2 min for *E. faecalis* and 22 ± 0.2 min dual species

![Fig. 3](image3.png)  
**Fig. 3** Microscopy images showing the population of *E. coli* and *E. faecalis* cells in dual-species bacterial community: the dual-species cultures under the FeSO4 supplemented conditions a blank, b 50 µM, c 100 µM, d 150 µM, e 200 µM, f 250 µM and g 300 µM. The dual-species bacterial cells were counter stained with crystal violet and safranin, shows that *E. faecalis* cells in violet colour and *E. coli* cells in pink colour. The *E. faecalis* cell population was found to be increased under FeSO4 supplemented conditions from 50 to 200 µM (i.e., *E. faecalis* cell population in Blank < 50 µM < 100 µM < 150 µM < 200 µM). It is clear that *E. faecalis* cell was population found higher population than *E. coli* cells in dual-species cultures under niche FeSO4 supplemented conditions
cultures was increased when supplemented with FeSO₄ (control—200 µM). The Gram staining on the bacterial cells supplemented with 200 µM > FeSO₄ showed that safranin dye stains the E. faecalis cell (Fig. 2f, g). However, additional imaging studies with species specific dyes are needed to quantify individual species and cells.

**Bacterial cells import the external Fe²⁺ ion source**

Previous studies demonstrate the metal ion augmentation in bacterial cells through direct electron transfer and mediated electron transfer in cell membrane promotes the biofilm formation (Keogh et al. 2018). This is in good agreement with this study that external FeSO₄ concentration of about 150 µM promotes the CFU of E. coli and 200 µM of FeSO₄ promotes the CFU of E. faecalis and dual species in LB media. AAS results confirm the Fe²⁺ augmentation in bacterial cells. The calibration curve for estimation of iron concentration in AAS (Supplementary Fig. 4) and corresponding AAS absorbance values were tabulated (Supplementary Table 1). In the FeSO₄ supplemented samples, using AAS we quantified the intracellular Fe²⁺ concentration about ∼1.3, ∼1.5, and ∼4.9 µM for E. coli, E. faecalis, and dual species, respectively. Whereas in control samples, the intracellular Fe²⁺ concentration was found to be ∼0.4, ∼0.4, and ∼0.4 µM for E. coli, E. faecalis, and dual species, respectively (Fig. 4). On the other hand, the initial (before bacterial inoculation) and final (after overnight incubation of bacterial cultures) concentration of Fe²⁺ ion was reduced over time (Supplementary Fig. 5). In FeSO₄ supplemented samples, the extracellular Fe²⁺ ion concentration was decreased from ∼150 to ∼147 µM when incubated overnight with E. coli cultures. The extracellular Fe²⁺ ion concentration was decreased from ∼200 to ∼195 and ∼193 µM when incubated overnight with E. faecalis and dual-species culture, respectively (Supplementary Fig. 5). In control samples, the extracellular Fe²⁺ ion concentration was decreased from ∼6.1 µM to ∼5.1, ∼5.4, and ∼4.5 µM when incubated overnight with E. coli, E. faecalis, and dual species, respectively. These results suggest the augmentation of Fe²⁺ in E. coli, E. faecalis, and dual-species cultures. Interestingly, dual-species cultures exhibit a similar increasing trend of Fe²⁺ more than monocultures, in concordance with the microscopy images reveals that E. faecalis cells were found more predominant than E. coli under FeSO₄ supplemented conditions.

**Ferrous ion promotes the single- and dual-species biofilms**

In vitro pellicle biofilm formation of E. coli, E. faecalis, and dual species were performed under varying concentrations of FeSO₄ (0–350 µM). The biofilm-forming ability of single and mixed-bacterial strains under iron-rich conditions were measured in a spectrophotometer at OD₅₈₈. In figure 5, E. coli biofilms formation show an increasing trend of 0.481 ± 0.026, 0.506 ± 0.006, 0.574 ± 0.04, and 0.669 ± 0.058 AU under the increasing FeSO₄ supplementation, such as 0, 50, 150, and 250 µM, respectively. In contrast, higher concentrations such as 300 and 350 µM of FeSO₄ decreases the E. coli biofilm formation to 0.602 ± 0.054 and 0.528 ± 0.012 AU, respectively. At similar FeSO₄ supplementation, the E. faecalis biofilms were shown a similar increasing trend was recorded at about 0.307 ± 0.011, 0.335 ± 0.058, 0.492 ± 0.03, and 0.694 ± 0.058 AU, respectively. However, higher concentrations of FeSO₄ such as 300 and 350 µM reduces E. faecalis biofilm formation to 0.62 ± 0.043 and 0.571 ± 0.035 AU, respectively.

In the dual-species biofilm model, biofilm formation showed an increasing trend of about 0.288 ± 0.3, 0.554 ± 0.037, 0.662 ± 0.055, and 0.876 ± 0.086 AU under the increasing FeSO₄ supplementation, such as 0, 100, 200 and 300 µM, respectively. In contrast, higher concentrations such as 350 µM of FeSO₄ decrease the dual-species biofilm formation to 0.681 ± 0.057 AU. Therefore, it is clear that 250 µM of FeSO₄ promotes the single-species biofilm formation and 300 µM of FeSO₄ promotes the dual-species biofilm formation in LB media.

In similar fashion, the extracellular iron promotes the E. coli biofilm formation in minimal media (M9 media) from 0.5 ± 0.01 to 0.85 ± 0.03 AU under extracellular iron
supplementation from 0 to 150 µM, E. faecalis biofilm formation was increased from 0.36 ± 0.01 to 1.06 ± 0.1 AU under extracellular iron supplementation from 0 to 200 µM, and the dual-species biofilm formation were increased from 0.65 ± 0.06 to 1.63 ± 0.04 AU under extracellular iron supplementation from 0 to 100 µM. The higher extracellular iron concentrations 200 to 350, 250 to 350, and 150–350 µM reduces the E. coli, E. faecalis, and dual-species biofilm formation. The extracellular iron promotes the biofilm formation in both LB and M9 media (minimal media) was found to follow similar trend. From the aforementioned pellicle biofilm formation, data obtained from LB growth media and M9 minimal media clearly shows that, on the one hand extracellular iron promotes the biofilm formation of both single- and dual species, respectively.

In GraphPad Prism 8.4.3, statistical significance was determined by paired t test for column wise comparison. n = 3 (biological replicates). \( P \leq 0.0001 \) (****), \( P \leq 0.001 \) (**), \( P \leq 0.01 \) (*), \( P \leq 0.05 \) (*) and the error bar represents the standard deviation from the mean

![Graph showing OD at 588 nm vs Concentration of FeSO₄ (µM)](image)

**Fig. 5** Extracellular Fe²⁺ increases the biofilm formation of E. coli, E. faecalis, and dual species: the biofilm formation of E. coli, E. faecalis and dual species under FeSO₄ supplemented conditions (0–350 µM) shown that niche concentrations of FeSO₄, 250 and 300 µM promotes the biofilm formation of single and dual species, respectively.

**Ferrous ion promotes antibiotic resistance in E. faecalis in both single- and dual-species cultures**

A Minimal Inhibitory Concentration (MIC) assay was performed to determine the antibiotic concentration required to inhibit the single- and dual-species biofilms under FeSO₄ supplemented conditions. In the single-species model, 100 µg/mL, and 50 µg/mL of kanamycin inhibited the E. coli and E. faecalis cultures, respectively. In contrast, the E. faecalis cultures under FeSO₄ niche concentration (200 µM) offers resistance to a higher concentration of kanamycin (500 µg/mL) but the E. coli cultures under FeSO₄ niche concentration (150 µM) do not resist kanamycin. The 100 µg/mL, and 50 µg/mL of kanamycin inhibit the E. coli cultures at both control and FeSO₄ supplemented conditions. Therefore, the extracellular Fe²⁺ ion uptake in the E. faecalis cultures promotes antibiotic resistance in the single-species model (Fig. 6).

In the dual-species cultures, the MIC of kanamycin was observed at 100 µg/mL for dual species (E. coli:E. faecalis, 1:3) and 50 µg/mL for dual species (E. coli:E. faecalis, 1:1 and 3:1) under controlled conditions. Under 200 µM of FeSO₄ supplemented conditions, the antibiotic resistance was increased, 500 µg/mL MIC of kanamycin tends to inhibit the dual species (E. coli:E. faecalis, 1:1) and 100 µg/mL MIC of kanamycin tends to inhibit the dual species (E. coli:E. faecalis, 3:1). Interestingly, under 200 µM of FeSO₄ supplemented conditions, the dual species (E. coli:E. faecalis, 1:3) were resistant to even higher concentrations of kanamycin, 500 µg/mL. Therefore, from the aforementioned results, it is clear that Fe²⁺ augmentation in the E. faecalis bacterial cultures promotes antibiotic resistance in single- and dual-species bacterial culture models.

In reference to the previous AAS results on iron augmentation in bacterial cells (Kim et al. 2012), suggesting that the presence of iron transporter protein, FeoB in E. faecalis
Archives of Microbiology (2022) 204:710

could facilitate the Fe$^{2+}$ uptake, which could enable the bacteria to grow against the antibiotics. This is probably, due to changes in local environmental pH as *E. faecalis* can express lactate dehydrogenase (*ldh1*) resulting in production of l-lactate under iron restricted condition (Tan et al. 2022) and promote l-lactate-mediated iron chelation. However, in the absence of chelators, the Fe$^{2+}$ ions could bind to iron transporters, such as siderophores with higher affinity (Chu et al. 2010). Nonetheless, a thorough investigation is needed to confirm the antimicrobial resistance of *E. faecalis* under iron augmentation. The growth of *E. faecalis* in an iron-rich environment was high owing to ferrous iron transporters proteins. A similar effect was also observed in *Vibrio Cholera* strains (Morrison et al. 2013), and *E. coli* strains (Kim et al. 2012).

**Model of *E. faecalis* FeoB 3D structure**

The FeoB protein model in *E. faecalis* was modelled using comparative homology modelling method in MODELLER software. This FeoB model validated its Phi/Psi angles of FeoB amino acid residues using Ramachandran plot, i.e., 99% of amino acid residues lies within the outlier region (Supplementary Fig. 8). FeoB protein model to mimic the ion uptake mechanisms facilitated by the ion-binding residues. Bound FeoB–Fe$^{2+}$ complex from Autodock Vina was further analyzed using Pymol to obtain better interaction poses (Fig. 7A). Docked complexes were further analyzed with Ligplot software, to identify potential interactions; plots reveal that residues—Asp54, Thr16, and Thr36 of FeoB interact with the extracellular Fe$^{2+}$ ion (Fig. 7B). Stronger interactions suggest that FeoB protein could interact with the extracellular Fe$^{2+}$ ion for their enhanced colony formation and biofilm formation (Fig. 7).

**Conclusions**

The extracellular Fe$^{2+}$ ion concentration from 50 to 150 μM and 50–200 μM promotes the growth of *E. coli* and *E. faecalis* (and dual-species colonies), respectively. Interestingly, imaging results suggest that *E. faecalis* cells were found in higher proportion when compared to *E. coli* in the dual-species model. In addition, the extracellular Fe$^{2+}$ uptake was higher in the dual-species model (~4.9 μM) compared to the single species, which was ~1.3 and ~1.5 μM for *E. coli* and *E. faecalis*, respectively. Taken together, it is clear that single- and dual-species bacterial cells consume extracellular Fe$^{2+}$ for efficient bacterial colonization and biofilm formation under niche concentrations (Fig. 8). These results are in good agreement with earlier studies (Keogh et al. 2018; Keogh et al. 2016). Similarly, niche concentration of Fe$^{2+}$ uptake increases the antibiotic resistance, for instance, it is clear that *E. faecalis* dominates both single- and dual-species biofilms, and a tenfold increase in antimicrobial resistance.
(kanamycin) was observed. Finally, in-silico docking studies showed that amino acid residues of ferrous ion transporter proteins, FeoB (of *E. faecalis*) could interact and bind strongly to the Fe$^{2+}$ ion, which is key to promote biofilm growth. However, in the current study we are unable to predict the reasons behind this increased resistance of *E. faecalis*. Further investigation with gene knockouts are required to identify the enhanced antibiotic resistance of *E. faecalis* in mixed species biofilms. Overall, this study not only provides a clear understanding of antimicrobial resistance in dual-species biofilm models but also serves as a potential starting point for designing novel biofilm inhibitors.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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