

The susceptibility of *Proteus mirabilis* and *Enterococcus faecalis* to various antimicrobial agents in polymicrobial biofilms formed using a drip flow reactor

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Submitted: 30.06.2019 Accepted: 15.08.2019

ABSTRACT

Objectives: Interspecies interactions in poly-species biofilm are substantial. Our aim is to set up dynamic biofilm models of *Enterococcus faecalis* and *Proteus mirabilis* using Drip Flow Biofilm Reactor (DFR) and to evaluate the effect of these dual population on anti-biofilms of some antimicrobials.

Materials and Methods: *E. faecalis* and *P. mirabilis* biofilms were formed in a DFR. Influences of the dual interactions on their susceptibilities to antimicrobial agents (disinfectants, antibiotics and probiotic strains) were determined.

Results: Gluteraldehyde and quaternary ammonium compounds (QAC) effectively killed the cells in both biofilms of *E. faecalis* and *P. mirabilis*. However, the efficacy of hydrogen peroxide (H_2O_2) was dependant on the microbial species present. *P. mirabilis* was less susceptible to the ampicillin and ciprofloxacin in co-culture compared to when cultured alone. Here, the influence of the presence of *E. faecalis* on *P. mirabilis* susceptibility was determined. For high concentrations of ciprofloxacin (1024 and 512 $\mu\text{g/ml}$), the log reduction in *P. mirabilis* cells was determined as approximately 4.5 and 3.5 in mono and dual-species biofilms respectively. Compared to *B. lactis*, *L. acidophilus* was found to be more effective both on single and dual species.

Conclusion: The effect of antimicrobial agents on microbial cells in a polymicrobial biofilm may depend on the composition of the biofilm.

Keywords: Polymicrobial biofilm, *E. faecalis*, *P. mirabilis*, Antimicrobial, Drip flow reactor, Probiotic strains

1. INTRODUCTION

Cells in biofilms are embedded in a self-produced extracellular polymeric matrix (EPM) that mainly consist polysaccharides, DNA, proteins, and dead cells [1]. In biofilms, the limited penetration of antimicrobial agents and accumulation of antibiotic-degrading enzymes are observed due to EPM production. EPM formation and the transfer of resistance genes in the presence of high cell density lead to an increased biofilm resistance [1,2].

Multi-species biofilms that exist in the human body are complex communities in which cells of various microorganisms are present and live together [1]. These biofilms are commonly encountered clinical concern and are found in multiple body sites (such as the skin, teeth and mucosa) in chronically infected wounds and on indwelling medical devices such as prostheses, stents, implants, catheters and endotracheal tubes [3].

P. mirabilis is associated with a large number of human infections such as catheter-associated urinary tract infection (CAUTI), cystitis, pyelonephritis, wound, eye and burn infections [4]. It is frequently found as coisolates with other pathogens, such as

Enterococcus species, *Candida* spp. in the samples of patients with biofilm related infections [5]. It was shown that expression of the virulence factors increased and resulted in greater tissue damage in parallel with the presence of polyspecies pathogens in the biofilm environment [5,6].

Enterococcus species are gram positive bacteria and the harmless members of gut flora in humans and animals. They are among the most frequent causes of nosocomial infections since they have the ability to create colony in different human body sites as well as on the surface of medical devices and may exhibit resistance to many antibiotics [7]. It is now well known that these infections are mainly associated with biofilm formation and are difficult to treat [8].

Interspecies interactions in biofilms have been extensively studied in human body and environments [9-11]. Different microbial interactions could effect the biofilm composition. A study reported that the biofilm cell counts of *Escherichia coli* decreased when co-cultured with *Pseudomonas aeruginosa*, but *Paeruginosa* obtained some advantages when grown in

How to cite this article: Kart D. The susceptibility of *Proteus mirabilis* and *Enterococcus faecalis* to various antimicrobial agents in polymicrobial biofilms formed using a drip flow reactor. Marmara Med J 2019; 32: 137-143. doi: 10.5472/marumj.637153

dual-species biofilms [12]. However, there is little information about antimicrobial susceptibility profile (such as antibiotics, disinfectants etc.) of polymicrobial biofilms. The results of a study showed that a multispecies biofilm with six different bacterial species including *Acinetobacter calcoaceticus* had the highest resistance to sodium hypochlorite (NaOCl), while a multispecies biofilm without *A.calcoaceticus* was more susceptible to NaOCl compared to the monospecies biofilms. No significant difference was reported in a previously published study in susceptibility to ethanol between mono – and multispecies biofilms of *Candida albicans* and *Staphylococcus aureus* [13]. Kart et al. concluded that the effect of the disinfectants tested in a multispecies biofilm depends on the type of the strain and the kind of the disinfectant used [14].

Dynamic mono and dual species biofilm models of *E. faecalis* and *P.mirabilis* which were reported as frequently isolated species from catheter-associated polymicrobial infections were developed in this study in a repeatable style using Drip Flow Biofilm Reactor (DFR) [15].

The aim of this study is to evaluate the effect of dual species interactions between *E.faecalis* and *P.mirabilis* in the developed biofilm models on individual susceptibility against various antimicrobial agents such as antibiotics (ampicillin and ciprofloxacin), disinfectants (Quarternary Ammonium Compound (QAC), hydrogen peroxide, glutaraldehyde) and probiotic strains.

2. MATERIALS and METHODS

Bacterial strains

P. mirabilis ATCC 29906, *E. faecalis* ATCC 47077/OG1RF, *B. lactis* ATCC 27536 and *L. acidophilus* ATCC 4356 were used in the study. *P.mirabilis* ATCC 29906 and *E.faecalis* ATCC 47077/OG1RF were grown overnight at 37°C in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK).

B.lactis ATCC 27536 and *L.acidophilus* ATCC 4356 were cultured in Man-Rogosa-Sharpe broth (MRSB; Merck, Darmstadt, Germany) and incubated at 37 °C in an anaerobic jar for 18 h and maintained on MRS agar plates (MRSA; Merck, Darmstadt, Germany).

Preparation of cell-free culture supernatant of probiotic strains

After incubating *L. acidophilus* and *B.lactis* strains in MRS broth at 37 °C for 18 h, the cultures were centrifugated at 8000xg for 20 min at 4 °C to obtain the cell-free supernatants. Then, the supernatants were filtered through 0.2-µm syringe filter (Millipore, Bedford, MA, USA) [9].

Antimicrobial agents

Disinfectants, antibiotics and probiotic strains tested in the study were shown in Table I. All disinfectant solutions were prepared using water of standard hardness (WSH), filter-sterilized and stored at 4°C for up to 1 month. Sterile WSH

distilled water and MRS broth were used as positive controls for antimicrobial activities of the disinfectants, antibiotics and probiotic supernatants, respectively.

Table I. Antimicrobials used in the study

Disinfectants/ Other antimicrobials	Concentration (%/mg/ml)	Contact Times (minutes/ hours)
Quarternary Ammonium Compound (QAC)	3%	15 min.
Quarternary Ammonium Compound (QAC)	1.5%	15 min.
Hydrogen peroxide (H ₂ O ₂)	1.5%	5 min.
Hydrogen peroxide (H ₂ O ₂)	0.75%	5 min.
Gluteraldehyde	2%	15 min.
Gluteraldehyde	1%	15 min.
Ampicillin	32-1024 µg/ml	24 h.
Ciprofloxacin	32-1024 µg/ml	24 h.
Probiotic supernatants (<i>B. lactis</i> ATCC 27536 and <i>L. acidophilus</i> ATCC4356)	-	24 h.

Development of mono – and dual-species biofilms in Drip Flow Reactor Model

Biofilms consisting of *P.mirabilis* and *E.faecalis* were grown in DFR (Bio Surface Technologies Corp, Montana, USA) with low shear and continous flow approved by the American Society for Testing and Materials International (ASTM) standard setting organization [15]. Final inoculum suspensions (10⁶ CFU/ml) of both bacteria were prepared in 10 ml BHI broth and placed into the DFR, which comprises six individual, parallel test channels including sterile slide (Figure 1). After operating the reactor in batch mode for 6 h, the flow was started and maintained for another 48 h with a continuous flow rate of 0.82 ml/min per channel [16]. During continuous flow, the media was dripped onto the surface of slides set at a 10° angle and mature biofilms were formed on these slides. The microorganisms were cultured alone (for mono species biofilms) and together (for dual-species biofilms).



Figure 1. Drip flow reactor used in the study

Anti-biofilm evaluation

The prescribed concentrations and contact times of each agents were applied to the slides which mono and dual-species biofilms of the strains were formed on (Table I). After the contact time, the disinfectant was neutralized with Dey-Engley neutralizing broth, as described previously [13,14]. For the ampicillin and ciprofloxacin, all concentrations (ranging from 1024 to 32 mg/ml) were tested in each of the six channel, individually, at same time. The supernatants of *B. lactis* ATCC 27536 and *L. acidophilus* ATCC 4356 were obtained after centrifugating the overnight cultures of both strains in MRS broth (Merck, Darmstadt, Germany) and were transferred onto the pre-formed mature biofilms to evaluate their anti-biofilm effects.

Colony counting

After treatments, the slide samples were removed from the reactor channels and rinsed with sterile buffered saline solution to remove planktonic cells. Biofilms were scraped from the slide surfaces with the cell scrapper and clumps were disaggregated by vortexing and sonication steps (at a frequency of 42 kHz (model 2510 sonicating water bath; Branson, New Hampshire, USA) according to the published protocols [17]. After disaggregating, the biofilm suspensions were serially diluted in sterile buffered saline solution and plated triplicate on selective growth media; ie tryptic soy agar (containing 1.5% agar) supplemented with vancomycin (16 µg/ml) for *P.mirabilis* and bile esculin azid agar for *E.faecalis*, respectively. Following 24 h of incubation time, the colonies were counted as colony forming units per milliliter (cfu/ml).

Scanning electron microscopy (SEM)

After the mature biofilm formations, the slides were removed from DFR, rinsed with 1 ml of buffered saline solution and cutted with glass diamond. Subsequently, all parts were washed with sterile distilled water and fixed in a buffer containing 2% glutaraldehyde and 0.1 M cacodylate for 30 min followed by rinsing three times for 10 minutes in 0.2 M cacodylate buffer. After passing them through serial ethanol solutions, samples were dried, then coated with gold-palladium and examined by a scanning electron microscope [18].

Statistical analysis

The colony counts were recorded for each treatment as log10. All statistical calculations were performed on the log density values. Statistical analysis was performed using two-tailed t-test assuming unequal variances with $\alpha = 0.5$ and a p value < 0.05 was considered to be significant.

3. RESULTS

Single and dual-species inoculum suspensions containing 10⁶ cfu/ml of *E. faecalis* and *P. mirabilis* in BHI was added to each channel of the DFR to form mature biofilms. In single and dual-species biofilms *E. faecalis* and *P. mirabilis* were grown up to ~10⁷ cfu/ml and ~10⁸ cfu/ml respectively. Compared to single species biofilm, the results demonstrated that the survival rate of each species was

not affected with the other one. The cell numbers in the biofilm following treatment with the disinfectants were determined by plating onto the selective media concerning the results (Table II).

All cells were death after treatment with two other disinfectants. Except H₂O₂, all other disinfectants led to cell death in all biofilm formations with either *P. mirabilis* or *E. faecalis* in single species and in dual-species biofilm (Table II).

Table II. Efficacy of disinfectants on biofilm

Disinfectant	Conc. (%)	Time (min.)	<i>P.mirabilis</i>		<i>E.faecalis</i>	
			Mono	Dual	Mono	Dual
H ₂ O ₂	2	5	95.71±0.4	99.93±0.01	99.47±0.1+	88.42±1.2
H ₂ O ₂	1	5	86.91±1.5=	98.28±0.3	93.12±0.7+	73.26±1.7
QAC*	3	15	99.99±0.0	99.99±0.0	99.99±0.0	99.99±0.0
QAC*	1.5	15	99.99±0.0	99.96±0.01	99.99±0.0	99.99±0.0
GA*	2	15	100±0.0	100±0.0	100±0.0	100±0.0
GA*	1	15	100±0.0	100±0.0	100±0.0	100±0.0

The results are expressed as hundred percent effective and are shown as the average ± standard error (n = 3). *Statistically significant reduction for all biofilm conditions compared to untreated control (p < 0.05). +Significantly more cells of this organism are killed in a single-species biofilm than in a dual-species biofilm (p < 0.05). =Significantly more cells of this organism are killed in the dual-species biofilm than in single-species biofilm (p < 0.05). H2O2 : hydrogen peroxide, QAC: quarternery ammonium compounds, GA: Glutraldehyde

The ampicillin and ciprofloxacin susceptibilities of *P. mirabilis* and *E. faecalis* cells both in single and dual species biofilms were determined individually. In comparison to untreated controls, none of the tested concentrations of ampicillin against *E. faecalis* showed anti-biofilm activity both in single and dual-species biofilms (Figure 2). In contrast, a significant decrease in cell survival of *P. mirabilis* was determined only with 1024 µg/ml among all the tested concentrations of ampicillin both in single and dual species biofilms (Figure 2). Statistically significant difference was obtained between susceptibilities of single and dual species biofilms formed by *P. mirabilis*. Accordingly, in the presence of *E. faecalis*, the biofilm cells of *P.mirabilis* were found to be less susceptible to ampicillin (1024 µg/ml).

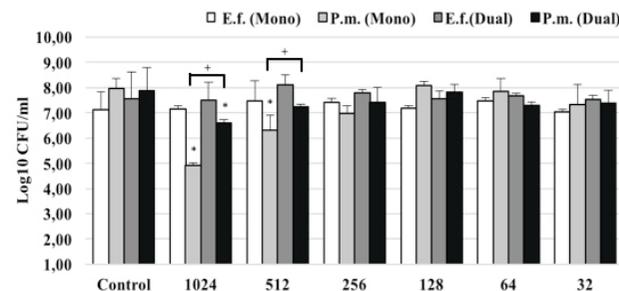


Figure 2. The effect of ampicillin on mono and dual species biofilms of *E.faecalis* and *P.mirabilis*

*Statistically more significant decrease in the cell counts than control (p < 0.05) +Significantly more cells of this organism were killed in a monospecies biofilm than in a dual-species biofilm (p < 0.05)

The significant decrease in the growth of *E. faecalis* cells both at 512 and 1024 mg/ml concentrations of ciprofloxacin were obtained in single and also in dual-species biofilm when compared to the untreated control (Figure 3). It was concluded that the ciprofloxacin showed more inhibitory activity against *P. mirabilis* than *E. faecalis* as the significant decrease in cell growth in *P. mirabilis* single species biofilm was obtained even at the lowest tested concentration (32 mg/ml) (Figure 3). Furthermore, the cells in single species biofilm formed by *P. mirabilis* were more susceptible to both tested antibiotics when compared to dual species biofilm (Figure 3).

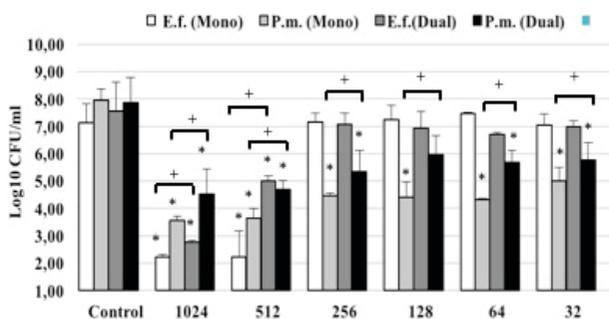


Figure 3. The effect of ciprofloxacin on mono and dual species biofilms of *E. faecalis* and *P. mirabilis*

*Statistically more significant decrease in the cell counts than control ($p < 0.05$)
+Significantly more cells of this organism were killed in a monospecies biofilm than in a dual-species biofilm ($p < 0.05$)

When the results of the effect of two probiotic supernatants on the cell survival were reviewed, *L. acidophilus* was found to be more effective both on single and dual species compared to *B. lactis*. None of the probiotic supernatants showed anti-biofilm effect on *E. faecalis* cells in dual species biofilm. However the significant decrease in the cell growth of *E. faecalis* was obtained by *L. acidophilus* supernatant treatment in the single species biofilm (Figure 4). The dense cell populations of *P. mirabilis* and *E. faecalis* were observed in mono and also in dual-species biofilms by SEM images (Figure 5).

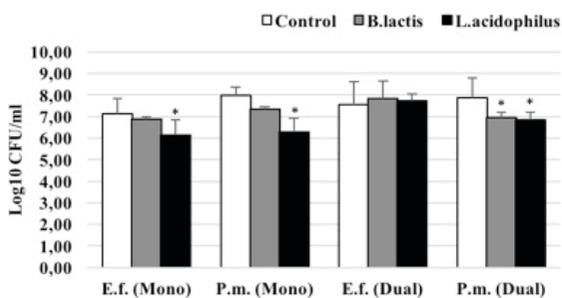


Figure 4. The antimicrobial effects of probiotic supernatants on the biofilms of *E. faecalis* and *P. mirabilis*.

*Statistically more significant decrease in the cell counts than control ($p < 0.05$)

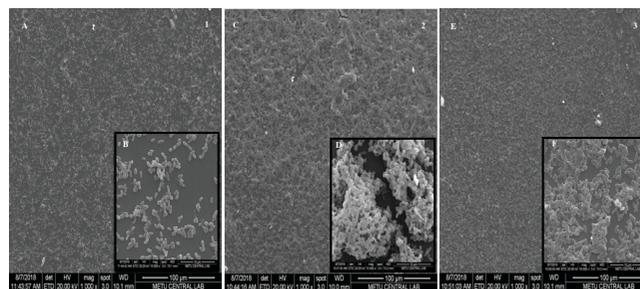


Figure 5. Scanning electron microscopy (SEM) images of biofilms

1. Dual-species biofilm of *E. faecalis* and *P. mirabilis* 2. *E. faecalis* 3. *P. mirabilis* (A,C,E): SEM images magnified by 1000X. (B,D,F): SEM images magnified by 10000X

4. DISCUSSION

The anti-biofilm susceptibilities of *E. faecalis* and *P. mirabilis* cells against three different disinfectant were determined both in single and dual species biofilms, individually. Except for H_2O_2 , our results demonstrated that all tested concentrations of glutaraldehyde and QAC effectively kill more than 99.999 % of cells both in mono and dual-species biofilms. In previously published reports it was reported that monospecies biofilms are often more susceptible to antimicrobial agents than polyspecies biofilms [3,19]. On contrary, our results clearly suggested that the efficacy of H_2O_2 was dependent on the type of microbial species present in the biofilm environment. Compared to its mono species biofilm *P. mirabilis* was found to be more susceptible to H_2O_2 when it was co-cultured with *E. faecalis*. On the other hand, *E. faecalis* was found to be less susceptible to H_2O_2 in the dual species biofilm with *P. mirabilis* compared to its mono species biofilm. These results support our previously published data indicating that the antimicrobial susceptibility of biofilm cells was dependent on both the nature of microbial species and properties of the selected disinfectant [14]. In our previous study, H_2O_2 was found to kill 96.94 % of *P. aeruginosa* cells in a triple-species biofilm consisting of *P. aeruginosa*, *S. aureus* and *C. albicans*, whereas *P. aeruginosa* cells in a single-species biofilm were not affected [14]. Alfa et al. evaluated the ability of different high-level disinfectants, frequently used for endoscope reprocessing to remove the single-species biofilms of *E. faecalis* and *P. aeruginosa*, separately. They have concluded that high-level disinfectants such as glutaraldehyde and H_2O_2 were insufficient to completely kill the biofilm cells when the high numbers of cells were found in the environment [20].

The results of glutaraldehyde activity obtained from the presented study were not consistent with the literature. This difference may be caused by using different protocols (flow based system versus static conditions) in the studies. In a study reported by Bock et al, the efficacy of H_2O_2 based disinfectants was investigated against biofilms formed by multi-drug resistant *Acinetobacter* spp., *Klebsiella pneumoniae* and *P. aeruginosa*. In comparison to planktonic forms the efficacies of recent working concentrations of disinfectants were not found to be susceptible

[21]. Majority of the previously reported studies on biocide susceptibility of planktonic or single-species biofilm cells have not taken into consideration the effect of the co-existence of bacteria in a mixed biofilm on individual biocide susceptibility. In the literature the reduced susceptibility to antibiotics in biofilm related infections formed by *E. faecalis* was attributed to biofilm-specific antimicrobial tolerance mechanisms [22]. Penetration limitation is one of these mechanisms and basically defined as the limited diffusion of the antibiotics into the biofilm matrix thus only the surface of the biofilm can get into contact with the antibiotic [23,24].

The MIC values of the antibiotics commonly used in treatment of the planktonic form of pathogen microorganisms are insufficient to eradicate the biofilm-embedded counterparts [25]. Due to the lack of standards and specific breakpoints for anti-biofilm assays biofilm susceptibility tests are not currently used in clinical diagnostic routines [26]. In this study ampicillin was found to be insufficient to eradicate the *E. faecalis* biofilm cells both in mono and dual-biofilms. For high concentrations of ciprofloxacin (1024 and 512 µg/ml), the log reduction in viable population was determined as approximately 4.5 and 3.5 in mono and dual-species biofilms respectively. These results were found to be in accordance with the results of two recently published studies [26].

In the present study the influence of coexistence of *P. mirabilis* with *E. faecalis* in dual-species biofilm on the susceptibility of *P. mirabilis* against ciprofloxacin and ampicillin was evaluated. Compared to its single-species biofilm, *P. mirabilis* showed less susceptibility to both antibiotics in dual-species biofilm (Figure 2 and 3). The interaction of two bacteria was resulted with the decrease of *P. mirabilis* susceptibility to the tested antibiotics with an unknown mechanism. Ampicillin significantly decreased the number of *P. mirabilis* viable cells in mono and also in dual-species biofilms at 1024 and 512 mg/ml concentrations (Figure 2). Statistically significant decrease was determined in mono and dual-biofilm cells of *P. mirabilis* at 32 µg/ml concentration of ciprofloxacin.

In a previously published study concerning dual species biofilm formed by *Paeruginosa* and *P. mirabilis*, the inhibitory activity of *P. mirabilis* on *P. aeruginosa* has been reported. By the authors this inhibition was attributed to less biofilm biomass of *P. aeruginosa* in dual species biofilm than the single species biofilm [27]. The susceptibilities of *P. mirabilis* biofilm cells against eight different antimicrobial agents including amoxicillin, erythromycin, gentamicin, nitrofurantoin, ciprofloxacin, trimethoprim-sulfamethoxazole, ceftriaxone and chloramphenicol were tested by Wasfi et al. Among these agents ciprofloxacin and ceftriaxone both at 8 µg/ml concentration were found to be the most effective agents which removed up the pre-formed biofilms by 34-55% and 33-69%, respectively [28]. Another study determined the eradicating effect of ciprofloxacin against the biofilm forming isolates of *P. mirabilis* at four antibiotic concentrations, corresponding to 0.125 MIC, 0.25 MIC, 0.5 MIC and 1 MIC values [29].

Previously, it was shown that lactobacilli could break down cells of opportunistic pathogenic bacteria [30]. However, there was

limited information about the interactions between *Lactobacillus* and other bacteria that occurred during mixed-biofilm formation. Compared to *B. lactis*, *L. acidophilus* was found to be more effective on *P. mirabilis* cells both in single and dual-species biofilms than *E. faecalis*. Based on our findings, it can be concluded that probiotics may display distinct antimicrobial effects on individual species of mixed-biofilms. Rybalchenko et al. concluded that a probiotic strain, *L. fermentum* 97, suppressed the growth of *Staphylococcus* spp., Enterobacteriaceae and *C. albicans* biofilm cells [30]. In another study, the growth inhibition of *Streptococcus mutans* by *L. acidophilus* LA-5 was observed significantly [31].

In conclusion, the cells in dual-species biofilm may display different responses to antimicrobial agents than their counterparts in single-species biofilm. This study investigated the effects of microbial interactions in dual-species biofilms of *P. mirabilis* and *E. faecalis* on their responses to various antimicrobial agents in a flow-based biofilm reactor model. Single or dual-species biofilm models were successfully developed by *P. mirabilis* and *E. faecalis* which were frequently co-isolated from the samples of patients with CAUTI. Findings of this study demonstrated that glutaraldehyde and quaternary ammonium compounds are the agents that might be effectively used to eradicate biofilms in the hospital cleaning settings. Ampicillin and ciprofloxacin are antibiotics used to treat the urinary system infections caused by urinary pathogens such as *E. faecalis* and *P. mirabilis*. In the study, concentrations lower than 512 µg/ml were found to be insufficient to kill *E. faecalis* biofilm cells in single and dual-species biofilms for both antibiotics. However, ciprofloxacin was able to decrease the number of viable cells of *P. mirabilis* at 32 µg/ml both in mono and dual-species biofilms significantly. The presence of *E. faecalis* in the dual-species biofilm decreased the susceptibility of *P. mirabilis* to ciprofloxacin. Although, further studies are required, this study emphasized the importance of the microbial interactions in polymicrobial biofilms, especially in response to antimicrobials for the treatment of biofilm-related diseases. Although, the effect of probiotic supernatants were found to be dependant on the nature of biofilm strain, *L. acidophilus* was more successful in killing bacterial cells when grown alone.

Conflict of Interest: The author declares that she has no conflict of interest.

Ethical Issues: According to the Institutional Ethical Committee this study did not require ethics approval as it was conducted on reference stains and the data did not contain patient-specific information.

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