Enterococcus faecalis inhibits *Klebsiella pneumoniae* growth in polymicrobial biofilms in a glucose-enriched medium

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Abstract

Catheter-related urinary tract infections are one of the most common biofilm-associated diseases. Inside biofilms, bacteria cooperate, compete, or have neutral interactions. The aim was to study the interactions inside polymicrobial biofilms formed by *Klebsiella pneumoniae* and *Enterococcus faecalis*, two of the most common uropathogens.

Although *K. pneumoniae* was the most adherent strain, it was unable to maintain dominance in the polymicrobial biofilm due to the lactic acid produced by *E. faecalis* in a glucose-enriched medium. This result was supported using the *E. faecalis* V583 *ldh-1/ ldh-2* double mutant, which not inhibited the growth of *K. pneumoniae* since this mutant does not produce lactic acid. Lyophilized cell-free supernatants (L-CFS) obtained from *E. faecalis* biofilms also showed antimicrobial/antibiofilm activity against *K. pneumoniae*. Conversely, there were no significant differences in planktonic polymicrobial cultures.

In conclusion, *E. faecalis* modifies the pH by lactic acid production in polymicrobial biofilms, compromising the growth of *K. pneumoniae*.

Keywords: polymicrobial biofilms; interspecies interactions; competition; lyophilized cell-free supernatant, lactic acid.

Introduction

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular matrix which play an important role in a wide diversity of infections, including catheter-related infections (Dybowska-Sarapuk et al. 2017). In this context, although indwelling device-related urinary tract infections are one of the most common biofilm infections of the urinary system (Kirmusaoglu et al. 2017), it may not result in a high mortality rate. Nevertheless, they pose a challenge for the health care system by increasing morbidity and treatment costs (Frank et al. 2009).

The microorganisms that usually colonise indwelling urinary catheters and develop biofilms are *Staphylococcus epidermidis, E. faecalis, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, K. pneumoniae,* and other Gram-negative organisms (Sabir et al. 2017). Most research studies have focused on monomicrobial biofilms to understand the mechanisms that involve biofilm development (Lee et al. 2014); however, the majority of them appear in nature as a diverse community of microorganisms (Elias & Banin 2012) and the clinical field is no an exception. Recent reports have demonstrated that a large number of biofilms involved in catheter-associated urinary tract infections (CAUTI) are formed by polymicrobial communities (Azevedo et al. 2017). Galván *et al,* also reported that some of the dual-species associations showing higher prevalence in urine samples were *K. pneumoniae/E. coli, E. coli/E. faecalis, K. pneumoniae/E. faecalis,* and *K. pneumoniae/P. mirabilis* accounting for 26 %, 10 %, 8.5 %, and 7 % of the cases, respectively (Galván et al. 2016).

Inside biofilms, social interactions of cooperation or competition between cells occur, and could drive many changes or alterations in the community (Flemming et al. 2016). In fact, in contrast to liquid cultures, these interactions are allowed by high cell concentration and diffusion limitation (Rendueles & Ghigo 2012). The new technological developments have allowed the

study of the diversity of highly complex microbial communities. Nevertheless, there is a lack of knowledge about the implication of interspecies relationships that needs to be addressed (Røder et al. 2016).

The present study was focused on the interspecies interactions in polymicrobial biofilms formed by *K. pneumoniae* and *E. faecalis. K. pneumoniae* is a Gram-negative, encapsulated, non-motile, facultative anaerobe, and rod-shaped bacterium (Guentzel 1996). Different virulence factors are related to their biofilm formation, being the most important the capsular polysaccharides or the type 1- and type 3-fimbriae, which enhance the biofilm of these bacteria on urinary catheters (Bei et al. 2016). On the other hand, *E. faecalis* is a Gram-positive, non-motile, facultative anaerobe, and round-shape bacterium. Previous research has reported some virulence factors related to biofilm formation, including the *esp*, *gelE*, and *asa1* genes, which have been found to support cell adherence, colonization, aggregation or persistence in the urinary tract (Paganelli et al. 2012).

We considered the fact that these two pathogens are some of the most prevalent among urinary tract infections but information about their specific interaction inside biofilm is still scarce. Besides, *K. pneumoniae* is one of the species listed as "priority pathogens" by the World Health Organization to help in prioritizing the research and development of new and effective antibiotics (World Health Organization. 2017) and it requires our special attention.

Materials and methods

Bacterial strains

The strains used in this study are listed in Table 1. Clinical strains were isolated from midstream urine samples and belong to a collection of our research group. The main results of this study are focused on one *K. pneumoniae* (AT) and four *E. faecalis* (2, 3, 5, and V583) because they

are strong biofilm-forming strains. The results of the other *K. pneumoniae* strains (Kp ATCC 13883, Kp 188, Kp 529, Kp 725) analysed are presented in the supplementary material.

E. faecalis V583 wild type (wt) and its mutants with a deletion in *ldh-1* (*Δldh1*), *ldh-2* (*Δldh2*), or both genes (*Δldh1/Δldh2*) were used to complete our study on the influence of lactic acid production in the polymicrobial biofilms.

All the strains were previously identified by MALDI-TOF mass spectrometry, their antimicrobial profile was tested following the M100 guidelines (CLSI 2019) and their biofilm formation ability was characterized following the protocol of Stepanović *et al*, (Stepanović et al. 2007) where a cut-off value (ODc) was established as three standard deviations above the mean OD of the negative control. The interpretation was as follows: Optical density of the strain (OD) ≤ ODc, the strain was considered no biofilm producer; ODc < OD ≤ 2 X ODc it was considered weak biofilm producer; 2 X ODc < OD ≤ 4 X ODc it was considered moderate biofilm producer; 4 X ODc < OD, it was considered strong biofilm producer. The biofilm formation was measured in three technical and biological replicates.

[Table 1 near here]

Biofilm assays

Adhesion to abiotic surfaces

The adhesion to polystyrene plates was performed following the protocol described by (DiMartino et al. 2003) with some modifications. Briefly, flat-bottomed non-treated 6-well microtiter plates (VWR International) were filled with two mL of a suspension of 10^8 colony forming units (CFUs) per mL made in 1x phosphate-buffered saline (PBS) (pH 7.2) and incubated for 30 min, 1 h, 2 h and 3 h at 37 ºC. After the incubation, each well was washed with 1x PBS. Adherent bacteria were released by sonication for 30 seconds in an ultrasonic bath

(Branson 3510, Marshall Scientific) and quantified using 10-fold serial dilutions and conventional plating on Luria Bertani agar (Miller's LB AGAR, Condalab) for *K. pneumoniae* and BD Columbia agar with 5 % Sheep Blood (Becton Dickinson) for *E. faecalis.* Then, the plates were incubated at 37 ºC for 18 - 24 h. Bacterial adhesion was expressed as a percentage of the original inoculum adhering to the well. The strains were considered highly adherent to a surface when the percentage of adherent bacteria was superior to 1 % compared to the original inoculum. The experiment was carried out in three technical and biological replicates.

Development and quantification of mono- and polymicrobial biofilms

Development of the mono- and polymicrobial biofilms was performed using a modified protocol previously described by Makovcova *et al*. (Makovcova et al. 2017). Briefly, bacterial strains were grown in 10 mL of trypticase soy broth (TSB, Condalab) overnight at 37 °C with shaking at 180 rpm. Bacterial cells were then pelleted at 4000 g for 20 min, and the pellet was resuspended in 5 mL of fresh TSB supplemented with 1 % glucose. Optical densities (OD_{600nm}) of the bacterial suspensions were measured using the Ultrospec 10 cell density meter (Amersham Biosciences) and adjusted to a final concentration of ~ 1 x 10^7 CFU mL^{-1}. An equal volume of each strain was combined to make the polymicrobial culture ratio 1:1. Biofilms were grown in flat-bottomed non-treated 6-well microtiter plates (VWR International). A sterility control (culture medium without inoculum) was included. All plates were covered with adhesive lids to avoid evaporation and then incubated in static conditions at 37 °C for 30 min, 1, 2, 3, 4, 8, 24, 48, and 72 h. The media was replaced every 24 h with fresh supplemented TSB broth. The initial inoculum of each strain was confirmed by colony counting.

After incubation, biofilm production was quantified using a previously described protocol (Cepas et al. 2019). Briefly, the remaining culture was carefully removed and each well was gently rinsed with 1x PBS. Then, all the plates were dried at 65 °C to fix the biofilm to the

surface. Biofilms were stained with crystal violet (CV) (2 % v/v) and incubated for 10 min at room temperature. Afterwards, CV was removed, rinsed once with 1x PBS and dried at 65 °C for 60 min. Biofilm formation was quantified by eluting the CV fixed to the biofilm in 33 % glacial acetic acid and absorbance of each well was measured at 580 nm (OD_{580nm}) using a microplate spectrophotometer (EPOCH 2 microplate reader; BioTek, VT). The experiment was carried out in three technical and biological replicates.

Percentage of biofilm formation inhibition

Following the protocol described by Reece *et al*. (Reece et al. 2018), the percentage of biofilm formation inhibition was determined as the difference between the OD_{580nm} value of each mixed biofilm and the sum of the OD_{580nm} values of biofilm formed by the corresponding individual species expressed in percentage.

$$\text{Inhibition (\%)} = \left[\frac{(OD_1 + OD_2) - OD_3}{(OD_1 + OD_2)}\right] \cdot 100 \quad (1)$$

OD_1 = OD *K. pneumoniae* individual specie biofilm

OD_2 = OD *E. faecalis* individual specie biofilm

OD_3 = OD mixed biofilm

Cultivable cells quantification

The number of cultivable cells from disrupted biofilms was obtained by colony counting. In brief, after the aspiration of supernatants, the wells were rinsed once with 1x PBS to remove non-attached cells. The plates were then sonicated at 40 kHz for 1 min, following the protocol described by Iñiguez-Moreno *et al.* (Iñiguez-Moreno et al. 2017). Later, the biofilms were scraped with a cell scraper (VWR international) and serially diluted for colony counting. In monomicrobial biofilms, *K. pneumoniae* and *E. faecalis* were plated on Luria Bertani agar

(Miller's LB AGAR, Condalab) and BD Columbia agar with 5 % Sheep Blood (Becton Dickinson), respectively. In polymicrobial biofilms, aliquots were plated both on selective media MacConkey II agar (Becton Dickinson) for selection of *K. pneumoniae* cells and on Enterococcosel agar (Becton Dickinson) for *E. faecalis*. Agar plates were incubated at 37 °C for 18 - 24 h. Assays were performed in triplicate.

Competitive index (CI)

The competitive index was calculated according to Macho *et al.* (Macho et al. 2007). Thus, the CI was defined as the *K. pneumoniae/E. faecalis* ratio within the output sample, divided by the corresponding ratio in the inoculum (input), where output and input samples were assessed after plating into selective media serial dilutions of the sample taken at fixed times. CI values were subjected to a Log transformation for normal distribution, and then interpreted as follows: a CI value equal to zero indicates no competition between the two species; a positive CI value indicates a competitive advantage for *K. pneumoniae,* and a negative CI value indicates a competitive advantage for *E. faecalis* (Magalhães et al. 2017).

$$\mathrm{CI} = \mathrm{Log}\left[\frac{(\mathrm{CFU\ mL^{-1}}\ K.pneumoniae)\ \mathrm{output}\ \cdot\ (\mathrm{CFU\ mL^{-1}}\ E.faecalis)\ \mathrm{input}}{(\mathrm{CFU\ mL^{-1}}\ E.faecalis)\ \mathrm{output}\ \cdot\ (\mathrm{CFU\ mL^{-1}}\ K.pneumoniae)\ \mathrm{input}}\right] \quad (2)$$

CI = 0: Equal competition between species.

CI > 0: Competitive advantage for *K. pneumoniae.*

CI < 0: Competitive advantage for *E. faecalis*.

Competition in planktonic cultures

The same volume of ~ 1 x 10^7 CFU mL^{-1} of each strain was mixed and incubated at 37 °C with shaking at 180 rpm. Aliquots were taken after specific time points (24, 48, and 72 h), serially

diluted 10-fold when needed and plated for colony counting as previously described. The cell count values were expressed as CFU mL^{-1}. Assays were performed in triplicate. After each cell count, the culture was centrifuged, the supernatant was discarded and replaced by fresh medium and the cells resuspended in the new one.

Supernatant analysis

Lyophilized cell-free supernatants collection (L-CFS)

The *E. faecalis* supernatants from biofilms were collected after 24 h of incubation. A portion of each supernatant was adjusted at pH 6.5 with sodium hydroxide (NaOH) 1 M. Then, the total volume collected was centrifuged for 15 min at 12,000 × g (at 4 °C) and passed through a 0.22 µm pore-size filter to remove bacteria, obtaining cell-free supernatants (CFS) (Wang et al. 2013). Afterwards, all the CFS were lyophilized in the CHRIST freeze dryer alpha 1-2 LD (Martin Christ Gefriertrocknungsanlagen GmbH) to get lyophilized cell-free supernatants (L-CFS).

Minimal inhibitory concentration (MIC)

The MIC values of the L-CFS were determined using the broth microdilution method (CLSI 2019). Two-fold serial dilutions in Mueller Hinton broth (MH) were carried out using round-bottom microtiter plates (Greiner bio-one 96 well, polystyrene, U-bottom). The final volume in each well was 100 µL. Bacterial 0.5 McFarland suspensions were diluted to obtain a final concentration of 5.10^5 CFU mL^{-1} per well. The MIC of DL-Lactic acid 85% (w/w), syrup (Sigma Aldrich) was also measured following the same protocol. To avoid evaporation, all plates were covered with adhesive foil lids and incubated in static conditions at 37 °C for 18 - 24 h and were visually read for the absence of turbidity. MIC values were defined as the lowest concentration of L-CFS that inhibited visible growth. The experiments were carried out in

triplicate.

Antibiofilm assays

The inhibition of biofilm formation was assessed following the procedure described by DosSantos Goncalves *et al.* (DosSantos Goncalves et al. 2014) with some modifications. Briefly, overnight cultures of *K. pneumoniae* were diluted to reach a 10^7 CFU mL^{-1} inoculum with fresh TSB supplemented with 1 % glucose. Biofilm formation assay was carried out in polystyrene flat bottomed microtiter plates (Nunc™ Edge 2.0 96-well plate, non-treated, with lid, VWR International). Each well, filled with the corresponding inoculum, contained ¼ (v/v) of *E. faecalis* supernatant extract at different concentrations. The microtiter plates were incubated at 37 °C for 24 h in static. Each well was rinsed once with sterile 1x PBS and the remaining biofilms were quantified following the CV staining procedure described previously.

To evaluate the effects of L-CFS on pre-formed *K. pneumoniae* biofilms, the following method was carried on. After 24 h of incubation at 37 ºC in static conditions, each well containing the established biofilm was carefully rinsed once with sterile 1x PBS and treated with L-CFS at different concentrations. The microtiter plates were then incubated at 37 °C for another 24 h in static, and quantified using the CV staining procedure.

In both assays, a negative control (culture medium without inoculum) and positive control (culture medium with bacterial inoculum) were included in each plate.

Both inhibitory and eradication capacities of DL-Lactic acid 85% towards *K. pneumoniae* biofilms were also measured following the same protocol. The experiments were carried out in three technical and biological replicates.

Determination of lactic acid

Quantitative detection of lactic acid in cell-free supernatants was performed using the L-lactic acid Kit (BioSystems S.A.). The method is based on lactic acid oxidation. L-lactic acid in the sample generates, using the reaction described below, NADH, which can be measured by spectrophotometry. Measurements were made on the Analyser Y15 (BioSystems S.A.).

$$\text{L-lactate} + NAD^{+} \xrightarrow{\text{L-LDH}} \text{Pyruvate} + NADH \quad (3)$$

Biofilm development using lactic acid E. faecalis mutant strains

E. faecalis V583 wt and its mutant strains with deletions in either *ldh-1*, *ldh-2*, or both genes were used to assess the inhibition caused by lactic acid production. Development and quantification of mono- and polymicrobial biofilms using counting of bacterial CFUs were performed as described above. The pH of the supernatants was also measured.

Interspecies interaction using pooled human urine

To evaluate the interactions between some of the strains, human urine was collected from six healthy volunteers of both sexes who had no history of urinary tract infection. Urinalysis showed normal parameters (glucose, ketones, nitrites, leukocyte esterase, bilirubin, urobilinogen, blood, and proteins). The urine was pooled, filter sterilized and stored at 4 ºC. Urine pH was 6.5 at the beginning of the analysis. Mono and polymicrobial biofilms were developed using the pooled human urine with or without glucose 1 %, and quantified by counting of bacterial CFUs as previously described. The competitive index was also calculated.

Data plotting and statistical analysis

All statistical analyses were performed using GraphPad Prism v8.0.2 software (La Jolla, California, USA). Graphs were created using GraphPad Prism v8.0.2 software and Tableau

Software (Seattle, USA). The data are expressed as mean ± SD (standard deviation). The percentage of inhibition in biofilm formation was evaluated via Student t-test, comparing the OD_{580nm} value of polymicrobial biofilms and the sum of OD_{580nm} of each monomicrobial biofilm. One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests were used to analyse the quantification of the cultivable cells, competition in planktonic cultures, the antibiofilm capacities by the L-CFS of *E. faecalis*. One-way ANOVA followed by *post hoc* Tukey's multiple comparisons test was used to analyse adhesion to abiotic surfaces. Confirmation of inhibition by lactic acid production, using *E. faecalis* V583 wt and its mutants, was analysed by confidence intervals on the difference between means. Tests with *P* values < 0.05 were considered significant.

Results

Biofilm assays

Adhesion to abiotic surfaces

The time-dependent adhesion to polystyrene plates was measured by conventional plating (Figure 1 and Figure S1). For all these strains, the number of adherent bacteria increased during the incubation period. *K. pneumoniae* AT was the most adherent strain, with an increase of 2.42 % after 60 min of incubation compared to the initial inoculum. Among the *E. faecalis* strains, Ef 2 increased its adhesion in a 1.20 % after 60 min of incubation, and Ef 3, Ef 5, and Ef V583 reached a percentage of 1.50 %, 1.51 %, and 1.18 % respectively, after 120 min of incubation. One-way ANOVA showed statistically significant differences when the percentage of adherence of *K. pneumoniae* AT after 60 minutes of incubation was compared with the percentages of adherence of all *E. faecalis* strains tested at the same incubation time. ($p < 0.0001$).

[Figure 1 near here]

Percentage of biofilm formation inhibition

When assessing the interaction between the two pathogens within the biofilm, the reduction of the total biomass of polymicrobial biofilms formed by *K. pneumoniae* and *E. faecalis* compared to the sum of the total biomass of monomicrobial biofilms of each strain was statistically significant ($p < 0.001$) in all comparisons, being the inhibition observed expressed in percentages in Table 2. The same effect was observed using the other *K. pneumoniae* strains (Table S1). These results suggest that the co-cultivation of *K. pneumoniae* and *E. faecalis* in a polymicrobial biofilm significantly compromised their biomass formation compared to those formed individually.

[Table 2 near here]

Cultivable cells quantification

Results on the cultivable bacterial quantification after polymicrobial biofilm growth at different time points are presented in Figure 2 and Figure S2. In general, cell counts of *K. pneumoniae* in the polymicrobial biofilm decreased when compared to monocultures. *E. faecalis* maintain similar growth in polymicrobial biofilms compared with monocultures and it is the prevailing specie over *K. pneumoniae* in co-culture. This predominance was not statistically significant after 30 min, 1, 2, 3, 4, and 8 h of incubation. A statistically significant reduction of CFUs was observed in *K. pneumoniae* after 24, 48, and 72 h ($p <0.001$).

[Figure 2 near here]

Competitive index (CI)

The CI value allows us to compare the differences among growth curves of polymicrobial biofilms and explains which of the pathogens present in the co-culture has a predominant behaviour within the biofilm. Negative CI values in polymicrobial biofilms at most of the incubation times tested were observed (Figure 3 and Figure S3), agreeing with the colony count

results. This could mean a clear advantage for *E. faecalis* over *K. pneumoniae.*

[Figure 3 near here]

Competition in planktonic cultures

The competition between both species in planktonic cultures was also assessed. Although a reduction in the CFUs of *K. pneumoniae* was observed when it was co-cultured with different *E. faecalis* strains in biofilm growth, no statistically significant reduction of CFUs of any of the involved species in planktonic co-cultures was observed at any time point tested ($P>0.05$) compared to monocultures. Therefore, neutral interactions were evident between the involved species in the planktonic state. (Figure S4)

Supernatant analysis

pH measurement

The pH values of supernatants of mono- and polymicrobial cultures (biofilms and planktonic) were measured throughout the incubation time. A pH decrease over time is observed in both mono and co-cultures (Figure 4).

In biofilms, it is worth noting that the pH of the *E. faecalis* supernatant was lower in monomicrobial than in polymicrobial. Nevertheless, when co-cultured, pH decreases enough to impair *K. pneumoniae* growth according to the colony count observed in figure 2.

This *K. pneumoniae* colony count changes depending on the *E. faecalis* strain used in the polymicrobial biofilm, and it may be the reflection of nutrient competition and different growth rates of the strains. In the same way, inherent characteristics of each strain such as virulence factors and their expression, as well as the physical interactions and other metabolites exchanges between the strains involved could produce these differences.

On the other hand, the pH in polymicrobial planktonic cultures was not low enough to affect the *K. pneumoniae* growth, which would explain why the colony count of none of the species was affected.

[Figure 4 near here]

Antimicrobial and antibiofilm effect

All non-pH adjusted L-CFS collected from *E. faecalis* biofilms showed antimicrobial and antibiofilm activity against *K. pneumoniae*. Thus, the MIC value in planktonic growth was 32 mg mL^{-1}. The minimal biofilm inhibitory concentration (MBIC), defined as the last well in which no visible growth was observed after incubation in the presence of biofilm and antimicrobial agents (LaPlante & Mermel 2009), was 64 mg mL^{-1} (Figure 5a and Figure S5). The minimal biofilm eradication concentration (MBEC), defined as the lowest concentration that an antimicrobial agent required to eradicate biofilm (Perumal & Mahmud 2013), was 256 mg mL^{-1}, although a complete eradication of the mature biofilm was not observed with *K. pneumoniae* AT (Figure 5b and Figure S6).

MBIC and MBEC were measured in three biological and technical replicates and were statistically significant when compared to the control *(K. pneumoniae* biofilm without L-CFS) ($P<0.001$). No antimicrobial or antibiofilm activity was observed when the pH of L-CFS collected from biofilms was adjusted to a pH of 6.5 with sodium hydroxide (NaOH) 1M.

[Figure 5 near here]

The antibacterial and antibiofilm effects of commercial lactic acid were also measured against *K. pneumoniae*, being the MIC value = 1.25 mg mL^{-1}, MBIC value = 4 mg mL^{-1}, and MBEC value was > 256 mg mL^{-1}.

Determination of lactic acid in supernatants

To confirm that the decrease in pH was due to the production of organic acids, the lactic acid concentration of supernatants collected from biofilms was measured. An important concentration of lactic acid was detected in supernatants (Table 3 and Table S2), which may confer the observed antibacterial and antibiofilm activities against *K. pneumoniae*. These results are consistent with the MIC values obtained with commercial lactic acid, where a concentration of 1.25 mg mL^{-1} inhibited *K. pneumoniae* growth. As well as the pH, lactic acid of the *E. faecalis* supernatant was lower in monomicrobial than in polymicrobial, because, in the second one, two kinds of species with different growth rates are competing for nutrients, and *Klebsiella pneumoniae*, which has a higher growth rate than *E. faecalis*, also use up the glucose and *E. faecalis* has not enough to produce the same lactic acid than produced when it grows in monomicrobial biofilms.

[Table 3 near here]

K. pneumoniae biofilm development at different pH conditions

To define the influence of pH in the growth and the subsequent biofilm development of *K. pneumoniae*, TSB medium was adjusted with NaOH 1M at pH ranging from 3.5 to 7.0, with intervals of 0.5. Biofilms were established following the protocol of development and quantification of biofilms and then incubated in static conditions at 37 °C for 24 h. After incubation, biofilm production was quantified using CV staining. The results of OD 580 nm showed that the lowest pH at which *K. pneumoniae* AT can form biofilm was 5.0, being 7.0 the optimal pH value to develop a strong biofilm. This condition corresponds to the pH used in conventional culture media (Figure 6 and Figure S7).

[Figure 6 near here]

Biofilm development using lactic acid E. faecalis mutant strains

E. faecalis possesses two cytosolic L - (+) - lactate dehydrogenases encoded by the *ldh-1* and *ldh-2* genes. Most of the activity is associated with LDH-1, and LDH-2 plays only a minor role (Fatima Rana et al. 2013). Therefore, polymicrobial biofilms formed by *E. faecalis* V583 wt or V583 *Δldh-2* displayed the same inhibitory effect over *K. pneumoniae* observed previously with the other *E. faecalis* clinical strains tested in this study. However, when *E. faecalis* V583 *Δldh-1* or *Δldh-1/Δldh-2* double mutant were analysed, the colony count of *K. pneumoniae* was not statistically affected when compared to monocultures (Figure 7). Confidence intervals on the difference between means showed statistically significant differences between means of *K. pneumoniae* AT monomicrobial and *K. pneumoniae* AT co-cultured with *E. faecalis* V583 wt or *E. faecalis* V583 *Δldh-2*. The difference between means of *K. pneumoniae* AT monomicrobial and *K.pneumoniae* AT co-cultured with *E. faecalis* V583 *Δldh-1* or *E. faecalis* V583 *Δldh-1/Δldh-2* was not statistically significant. The same effect was observed using the other *K. pneumoniae* strains (Figure S8). The competitive index showed an advantage of *E. faecalis* over *K.pneumoniae* with all the strains used, but the difference of the obtained values when *E. faecalis* V583 or *E. faecalis* V583 *Δldh-2* were used (-3.63 and -2.52 respectively after 24 h of incubation), is higher than the obtained when *E. faecalis* V583 *Δldh-1* or *E. faecalis* V583 *Δldh-1/Δldh-2* were in the polymicrobial biofilm (-0.74 and -0.59 respectively after 24 h of incubation). In the same way, the pH decrease in the polymicrobial cultures using *E. faecalis* V583 or *E. faecalis* V583 *Δldh-2* was enough to inhibit the *K. pneumoniae* growth. Although the decrease in the pH could be done by other organic acids produced, the loss of lactic acid production in these *E. faecalis* mutant strains (V583 *Δldh-1* or *Δldh-1/Δldh-2* double mutant) made these values not as lower as the wt V583 or the V583 *Δldh-2* strain, causing less alteration on *K. pneumoniae* growth (Table S3).

[Figure 7 near here]

Interspecies interaction using pooled human urine

Using pooled human urine with and without glucose, the urine conditions of diabetic and non-diabetic patients were simulated. Neutral interactions between the strains were found when the urine without glucose was used. However, the same inhibitory effect of *E. faecalis* over *K. pneumoniae* was observed when the pooled human urine was supplemented with glucose 1 %, similarly, as the results obtained when TSB supplemented with glucose was used (Figure 8 and Figure S9). The reduction of CFUs was statistically significant in *K. pneumoniae* after 24, 48, and 72 h of incubation (p <0.001).

[Figure 8 near here]

Discussion

It is widely known that the interactions established in polymicrobial biofilms imply cell-to-cell communication, typically via quorum sensing (Thornhill & McLean 2018). These interactions may promote synergism, in which the involved species cooperate between them by increasing biofilm formation and, therefore, their resistance to antibiotics, compared to monomicrobial biofilms (Schwering et al. 2013; Makovcova et al. 2017). However, these interactions can lead to a benefit for one of the species involved, based on nutrient competition or by inhibiting the proper growth of their counterparts, a mechanism known as antagonism (Harrison 2007). Thus, the co-culture of different bacteria in the biofilm state can lead to an increase or decrease in their biomass. The third scenario is in which neither synergism nor antagonism is evident among the species involved. Therefore, in this case, their interaction is classified as neutral.

Considering that *K. pneumoniae* and *E. faecalis* are common uropathogens, and biofilm formation is an important trait in their pathogenesis, the study of their interspecies interactions within biofilms seems mandatory. This approach could help identify possible targets or new antimicrobial compounds, mainly produced by predominant strains, with therapeutic activity.

406 However, research in this specific interaction is currently scarce.

407 According to our study, when the CV assay and the percentage of inhibition were carried out,
408 the sum of their separate monomicrobial biomasses did not correlate with the total biomass of
409 polymicrobial biofilms, which was frequently much lower. Therefore, these results point a
410 competitive interaction between the involved species, also supported by their quantification in
411 agar plates, where a predominance of *E. faecalis* over *K. pneumoniae* was observed. In
412 polymicrobial biofilms, the CFU cm^{-2} values of *E. faecalis* continued almost unaltered through
413 all set times tested, while *K. pneumoniae* concentration decreased over time in contrast to the
414 monomicrobial biofilm. In the same way, the negative CI values obtained during the time
415 specifies an inhibitory effect of all *E. faecalis* over *K. pneumoniae* suggesting a more
416 competitive rather than cooperative interaction between species. It is important to note that this
417 competitive interaction was not observed in the planktonic state, although nutrient content of
418 growth media, pH, and temperature were initially the same in both experiments. It was because
419 the oxygen availability among cells differs in planktonic and biofilm state. Thus, as observed
420 in the biofilm assay, limited airing leads to an increase in lactic acid production by *E. faecalis*
421 compared to planktonic cultures.

422 A previous study performed by Galván *et al.* observed neutral interactions between *K.*
423 *pneumoniae* - *E. faecalis* regarding biofilm formation and viable cell-counts were similar when
424 mixed *vs* pure cultures were compared (Galván et al. 2016). Our findings contrast with this
425 previous work, showing a decrease in the biofilm population of *K. pneumoniae* and an
426 advantage over time of *E. faecalis* in biofilm co-culture. Such differences observed between
427 studies could be attributed to the use of artificial urine medium without glucose, which, when
428 tested in our laboratory, did not allow an adequate growth of our *E. faecalis* strains. Therefore,
429 we preferred to use pooled human urine supplemented with glucose, observing the same
430 inhibitory effect as when TSB broth was used. It should be noted that, as was observed by

Galvan et al, neutral interactions between the species were observed when the pooled human urine without glucose was used, which makes a difference in the interspecies interactions that may be going on diabetic and non-diabetic patients. In patients with CAUTI and risk factors such as diabetes, less virulent pathogens than Enterobacteriaceae, such as *Candida* spp. and *Enterococcus* spp. become more predominant (Tandogdu & Wagenlehner 2016), and several studies have shown that urinary tract infection by Enterococcus species is often polymicrobial (Giannakopoulos et al. 2019), confirming our findings. Although the data obtained in this study has the limitation of no using a continuous flow system, future research could be focused on a comparison between the static and dynamic models.

Nadell *et al* (Nadell et al. 2016) affirmed that when several strains and species come into contact with others, predominance is expected of the most competitive phenotypes, as an act of natural selection to favour genetic lineages that may be helpful to themselves more than they are to the others. In this way, even though *K. pneumoniae* has a shorter generation time than *E. faecalis*, the first one is not capable to maintain dominance in the biofilm. Indeed, Schluter *et al* (Schluter et al. 2015), stated that in polymicrobial biofilms predominate the most adherent genotype, but the obtained results indicate that, although *K. pneumoniae* AT has a stronger capacity to adhesion than *E. faecalis* in monomicrobial biofilms, this characteristic is not key in the further development of the polymicrobial biofilm with *E. faecalis*, where the production of substances like lactic acid affects the growth of *K. pneumoniae*. So, not only the adhesion to the abiotic surface but also the production of inhibitory substances should be taken into account when these interactions are analysed.

Several bacteria generate different compounds that interfere with the growth of their counterparts, like hydrogen peroxide, different organic acids or bacteriocins (Mariam et al. 2017). Bacteriocins have stability at different pH concentrations and possess other biological and physicochemical properties, some of which are related to their capacity to eradicate

biofilms (Mathur et al. 2018). However, in some cases, one of the involved species produces hydrogen peroxide or organic acids as metabolic waste, causing a change in the pH of the surrounding medium that harms the other species growing into the biofilm (Makovcova et al. 2017). Thus, the producing strain could benefit itself or not be affected by its waste product, but accidentally it could affect the normal growth of the other species (Nadell et al. 2016). For example, vaginal *Lactobacilli* spp. produces lactic acid that causes a decrease in environmental pH interfering in the growth of species such as *Neisseria gonorrhoeae* (Graver & Wade 2011). *E. faecalis* is also considered a lactic acid bacteria, and some of the mechanisms that involve in the inhibition of different pathogens comprise competition for nutrients, production of different organic acids and secretion of antimicrobial substances (Mariam et al. 2017). Lactic acid is the main product of *Enterococcus* fermentation under conditions of excess glucose and limited oxygen. It is generated by the reduction of pyruvate to regenerate NAD^+ for ongoing glycolysis and is the most important in all lactic acid bacteria (Ramsey et al. 2014). However, certain strains produce ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin (DeVuyst & Leroy 2007).

Antibacterial and antibiofilm effects were also observed when assessing the activity of L-CFS extracted from *E. faecalis* biofilms over *K. pneumoniae*. Since the inhibitory effect was only observed when testing the non-pH adjusted L-CFS, it was probably caused by the production of organic acids and may not be related to bacteriocin production. So, when lactic acid in supernatants was measured, the concentration was enough to inhibit the growth and biofilm development of *K. pneumoniae*, which was also supported by the commercial lactic acid MIC and MBIC results.

In different environments, *E. faecalis* has been found inhibiting the growth of other bacteria. In root canals, *Streptococcus gordonii* was completely inhibited when it was co-cultured with *E. faecalis* (Gao et al. 2016). Also, other studies showed that the presence of *E. faecalis* limited

the presence of *Listeria monocytogenes* in polymicrobial biofilms at 39 ºC due to the competition for nutrients and production of toxic metabolites. (DaSilva Fernandes et al. 2015).

Studies of Alakomi *et al* (Alakomi et al 2000) confirmed that 5 mM (pH 4.0) of lactic acid causes the permeabilization of Gram-negative bacteria by disrupting the outer membrane. The average pH found in the *E. faecalis* biofilm supernatants after 24 h was 4.18 and the lactic acid concentration was 2.37 g L^{-1}. Besides, when testing the ability of *K. pneumoniae* to form a biofilm in a range of pH between 3.5 and 7.0, biofilm biomass production increased along with the pH of the media, finding pH 7.0 as the most favourable condition to form the biofilm. On the other hand, at pH values below 5.0, bacteria lose this ability. Other researchers have also demonstrated the effect of pH in biofilm production, establishing that biofilm production in *K. pneumoniae* increased 151–319 % at pH 8.5 and 111–177 % at pH 7.5 compared with the biofilm formed under pH 5.5 (Hoštacká et al. 2010).

Additionally, the analysis carried out with *E. faecalis* V583 wt and V583 mutant strains with deletions in *ldh-1*, *ldh-2*, or both genes, confirm also the hypothesis related to inhibition caused by lactic acid production. The inhibition was found when the polymicrobial biofilms were formed by *E. faecalis* V583 wt or the *E. faecalis Δldh2* strains. On the other hand, no statistically significant decrease in the colony counts of *K. pneumoniae* when *E. faecalis V583 Δldh-1* or *Δldh-1/Δldh-2* mutant strains were involved. This is because the majority of the lactic acid production is conferred to the *ldh-1* gene, where *ldh-2* plays a minor role in this process. (Jönsson et al. 2009; Fatima Rana et al. 2013), concluding that the antibiofilm effect by *E. faecalis* over *K. pneumoniae* is mainly due to the production of lactic acid and the consequent reduction of pH.

This suggests that, in CAUTIs of diabetic patients, lactic acid production could confer an advantage to *E. faecalis* over *K. pneumoniae* or other species, because some *E. faecalis* strains

505 can resist and adapt to different pH ranges growing in highly acid conditions (pH 2.9) (Rince
506 et al. 2000; Mubarak & Soraya 2018). Moreover, it is well known that growth inhibition of
507 different Gram-negative pathogens in urine occurs at pH 5.0 and below (Kaye 1968). Different
508 researchers explain how some lactic acid bacteria can be used as candidates to develop probiotic
509 microorganisms that could inhibit uropathogens. In female adults and children, probiotics have
510 been studied and used for urogenital tract health (Reid & Bruce 2001; Lim et al. 2009; Akgül
511 & Karakan 2018). *E. faecalis* has been proposed as well as a probiotic due to its adherence to
512 intestinal cells and reinforcement of the epithelial barrier (Baccouri et al. 2019). Additionally,
513 the study of Manohar et al found *Enterococcus* spp. on virtually all the catheters studied
514 regardless of duration, but was not associated with UTI development during follow-up
515 (Manohar et al. 2020), which could shed light on the use of *Enterococcus* as uropathogens
516 inhibitor due to lactic acid production when is adhered to catheters, avoiding their adhesion by
517 competition or by reducing available nutrients, which should be explored further in future
518 research. All these results make us continue the study of potential lactic acid bacteria as
519 biocontrol agents to tackle the problematic emergence of antibiotic resistance and, in this case,
520 against biofilm formation on indwelling devices related to urinary tract infections.

521 **Conclusions**

522 *K. pneumoniae* and *E. faecalis* interact competitively when grown in biofilms in a rich glucose
523 environment. Both microorganisms produce more biomass in monomicrobial than in
524 polymicrobial biofilms. *E. faecalis* has shown to exhibit inhibitory activity against *K.*
525 *pneumoniae*, modifying the pH as a result of lactic acid production, which originates deleterious
526 effects over *K. pneumoniae* but without compromising their growth. However, the complex
527 network of interspecies interaction between this polymicrobial biofilm and others needs further
528 investigation.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Supplementary material

Results obtained using other *K. pneumoniae* clinical strains are compiled in the supplementary tables and figures.

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