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Research article

Proteus mirabilis outcompetes *Klebsiella pneumoniae* in artificial urine medium through secretion of ammonia and other volatile compounds

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ABSTRACT

Klebsiella pneumoniae and *Proteus mirabilis* form mixed biofilms in catheter-associated urinary tract infections. However, co-inoculation of *P. mirabilis* with *K. pneumoniae* in artificial urine medium (AUM) resulted in a drastic reduction of *K. pneumoniae* cells in both biofilm and planktonic growth. Here, the mechanism behind this competitive interaction was studied. Both pH and aqueous ammonia (NH_{3aq}) increased in mixed cultures (to 9.3 and 150 mM, respectively), while *K. pneumoniae* viable cells dramatically diminished over time (>6-log reduction, $p < 0.05$). Mixed cultures developed in either 2-(*N*-morpholino) ethanesulfonic acid (MES)-buffered AUM (pH 6.5) or AUM without urea did not show bacterial competition, evidencing that the increase in pH and/or NH_{3aq} concentration play a role in the competitive interaction. Viability of *K. pneumoniae* single-species cultures decreased 1.5-log in alkaline AUM containing 150 mM NH_{3aq} after 24 h inoculation, suggesting that ammonia is involved in this inter-species competition. Besides NH_{3aq}, additional antimicrobials should be present to get the whole competitive effect. Supernatants from *P. mirabilis*-containing cultures significantly diminished *K. pneumoniae* viability in planktonic cultures and affected biofilm biomass ($p < 0.05$). When subjected to evaporation, these supernatants lost their antimicrobial activity suggesting the volatile nature of the antimicrobial compounds. Exposure of *K. pneumoniae* to volatile compounds released by *P. mirabilis* significantly decreased cell viability in both planktonic and biofilm cultures ($p < 0.05$). The current investigation also evidenced a similar bactericidal effect of *P. mirabilis* volatiles over *Escherichia coli* and *Morganella morganii*. Altogether, these results evidence the secretion of ammonia and other volatile compounds by *P. mirabilis*, with antimicrobial activity against gram-negative uropathogens including *K. pneumoniae*. This investigation provides novel insight into competitive inter-species interactions that are mediated by production of volatile molecules.

1. Introduction

Catheter-associated urinary tract infections (CAUTIs) are among the most common nosocomial infectious diseases of humans, and significantly burden the healthcare system by increasing both morbidity and treatment costs [1]. The initial infections are usually caused by single bacterial species, such as uropathogenic *Escherichia coli* or *Enterococcus faecalis* [2]. Over time, a variety of organisms, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Morganella morganii* can colonize the urinary tract and form polymicrobial biofilms [3, 4].

P. mirabilis is commonly associated with polymicrobial CAUTIs [4, 5]. In this context, *P. mirabilis* co-infection with *K. pneumoniae* and *Providencia stuartii* and, in a lesser extent, with *E. faecalis*, *E. coli* and *M. morganii* has been reported [5, 6]. A high prevalence of *K. pneumoniae* and *P. mirabilis* co-isolation in catheter-associated polymicrobial bacteriuria was shown and the *in vitro* ability of these strains to establish mixed biofilms and planktonic cultures in artificial urine medium (AUM) were studied [7]. Unexpectedly, co-inoculation of *P. mirabilis* with *K. pneumoniae* resulted in a detrimental effect over *K. pneumoniae* in both biofilm and planktonic growth. The reason for the discrepancy between

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the *in vivo* co-occurrence of *P. mirabilis* and *K. pneumoniae* in patients with CAUTIs and the *in vitro* data showing that *P. mirabilis* is killing *K. pneumoniae* remains unclear. Moreover, the mechanisms behind the *in vitro* competitive interactions are still unknown.

Both *P. mirabilis* and *K. pneumoniae* are gram-negative bacteria present in the human fecal flora as innocuous commensal bacteria inhabiting the gastrointestinal tract. However, they are capable of causing a variety of opportunistic human infections including those of the wounds, respiratory tract, and urinary tract [5, 8]. *P. mirabilis* possesses a urea-inducible urease enzyme that hydrolyzes the urea present in urine to ammonia and carbon dioxide. This provides the bacteria an abundant nitrogen source while increases urine pH and causes the precipitation of otherwise soluble polyvalent anions and cations present in urine [5]. These crystals can result in blockage of the urinary catheter and also in the formation of bladder and kidney stones (urolithiasis) [5, 6]. In contrast to urease from *Proteus* spp., whose expression requires the presence of urea, the urease from *K. pneumoniae* is regulated solely by nitrogen limitation [9]. Besides urease, *P. mirabilis* possesses other virulence factors such as swarming motility, fimbriae expression and biofilm formation. Fimbriae expression and biofilm formation are also major virulence factors in *K. pneumoniae*, together with siderophores, capsular polysaccharide and lipopolysaccharide [8]. The emergence of extended-spectrum β-lactamase- and carbapenemase- producing *K. pneumoniae* strains is another cause of growing concern in the medical community [10]. In both *P. mirabilis* and *K. pneumoniae*, contact-dependent type VI secretion systems involved in inter-bacterial and bacteria-host interaction have been described [11, 12].

Competition between species appears to be the interaction that commonly predominate in microbial communities [13]. Microorganisms can display negative interactions after changing the environment by consuming resources and excreting metabolites. These changes to the environment influence the growth and survival of both the microbe that originally altered the environment as well as other microbial species that are present [14]. A very common environmental modification is a change of the environmental pH, because different species prefer different pH values [14]. In bacteria, alkaline stress has been reported to produce salt stress due to the significant increase in the Na^+ cytotoxicity as the pH rises [15]. Alkaline pH has interplay with cell wall stress, because of the alkali-vulnerability of the subset of cell wall biosynthetic enzymes that are exposed on the outside of the membrane has been described. Both events could contribute to cell death [15].

Another important competitive factor is the production of toxic molecules to exclude competitors [16]. In recent years, the role of volatile compounds in bacterial communication and competition has been revisited, and it was proposed that they could exert direct antimicrobial activity [17]. Bacteria produce and emit highly diverse organic and inorganic volatile compounds. Bacterial volatiles of organic origin include several chemical classes such as fatty acid derivatives (hydrocarbons, ketones, and alcohols), acids, sulfur and nitrogen-containing compounds, and terpenes [18]. Ammonia, nitric oxide, hydrogen sulfide, and hydrogen cyanide are among the inorganic volatiles released by bacteria [17]. The literature about volatile-mediated bacteria–bacteria competitive interactions is still scarce. It has been reported that members of the genus *Streptomyces* produce the sesquiterpenes albaflavenone and pentalenolactone, that are broad-range antibacterial volatiles [19, 20]. *Pseudomonas fluorescens* and *Serratia plymuthica* were described to emit volatiles, such as dimethyl disulphide, that strongly suppressed the growth of *Agrobacterium* sp. [21]. Other studies examined the effect of volatiles emitted by *Pseudomonas fluorescens* and *Bacillus* sp on the growth of *Ralstonia solanacearum* [22, 23]. Ammonia has been detected in volatile profiles of various bacterial species, including *Proteus* spp. [24, 25, 26]. Ammonia toxicity has been also described for several microorganisms, especially in an agronomic context [27]. It is well known that uncharged ammonia can cross easily biological membranes as compared to ions (NH_4^+). Inside microorganisms, the uncharged ammonia acts by increasing the internal pH to deleterious levels, so destroys cells by causing the dysfunction of the metabolism [28].

The present study is focused on the elucidation of the mechanisms involved in the competitive interactions observed *in vitro* between *P. mirabilis* and *K. pneumoniae* in mixed cultures developed in AUM. The presence of secreted antimicrobial and anti-biofilm compounds was evaluated. All together, the results obtained help to better understand the interactions between *P. mirabilis* and *K. pneumoniae* in mixed cultures in a medium mimicking the human urine. Importantly, this investigation revealed an important role of volatile compounds secreted by *P. mirabilis* as a competitive strategy between bacteria.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Klebsiella pneumoniae 04 (Kp04, Ap^R) and *Proteus mirabilis* 04 (Pm04, Tc^R) clinical strains were co-isolated from a urine sample of a patient undergoing long-term catheterization of the urinary tract and showing symptoms of CAUTI [7]. Isolates were maintained in the laboratory as frozen stocks (at –80 °C) in Luria-Bertani (LB) broth supplemented with 15% glycerol. Inocula for assays were prepared as follows. *K. pneumoniae* was freshly streaked in LB- 1.5% agar plates, whereas modified LB-agar plates containing 10-fold less NaCl were used for *P. mirabilis* to avoid its swarming [29]. Both bacteria were then grown overnight at 37 °C. Subsequently, individual colonies were used to inoculate LB broth and were incubated overnight at 37 °C and 200 rpm (311DS Shaking Incubator, Labnet International Inc., NJ, USA). Then, each inoculum was properly diluted in artificial urine medium (AUM) [30] to obtain 10^7 cells ml^{-1}. Inoculum cell number was determined by counting of colony forming units (CFU), as explained at the end of *2.2 section.* For dual-species assays, equal volumes of each single-species inoculum in AUM were mixed.

2.2. Planktonic growth in AUM

Planktonic growth was performed as previously described [7], using 12 ml polystyrene scew-cap tubes (DeltaLab, Barcelona, Spain). Briefly, a total of 3 ml of each single- and dual-species inocula was monitored for static planktonic growth at 37 °C over 36 h. To rule out any effect of residual LB on bacterial growth, experiments were performed with an additional washing step for inocula preparation [cultures developed in LB were centrifuged at 5,000 × *g* (Sorvall™ Legend™ XTR Centrifuge, Thermo Fisher Scientific, MA, USA), supernatants were discarded, bacteria were resuspended in AUM, then diluted to 10^7 cells ml^{-1} and monitored for growth at 37 °C], and similar results were obtained. When indicated, AUM was modified as follow: 1) MES-buffered AUM (pH 6.5) was obtained by adding 100 mM MES [2-(*N*-morpholino) ethanesulfonic acid] (Sigma-Aldrich, St Louis, MO, USA); 2) AUM without urea; 3) alkaline AUM (pH 8.0, 9.0 and 9.3) was obtained by addition of 100 mM TAPS [N-Tris((hidroxymethyl)methyl)-aminopropanesulfonic acid] (Sigma-Aldrich); 4) AUM with defined concentrations of NH_{3aq} was prepared as explained below. Cultures were sampled at the indicated time points to determine CFU ml^{-1} as previously described [7]. In brief, bacteria were serially diluted (1:10) and plated on LB-agar plates containing ampicillin (Ap, 30 μg ml^{-1}) for *K. pneumoniae* counts and LB-agar plates with 10-fold less NaCl and tetracycline (Tc) supplementation (10 μg ml^{-1}) for *P. mirabilis* counts.

2.3. Preparation of AUM containing defined concentrations of NH_{3aq}

It has been reported that at 37 °C and pH 9.1, 60% of the ammonium salt added to a solution is under the form of aqueous ammonia (NH_{3aq}) [31]. Culture media carrying defined NH_{3aq} concentrations were prepared as previously described by Koziel *et al.*, with minor modifications [32]. Briefly, AUM with neither urea nor NH_4Cl was buffered to pH 9.1

with 100 mM TAPS and then appropriate amounts of NH_4Cl were added. To achieve NH_{3aq} concentrations of 25, 50, 100, and 150 mM, the following NH_4Cl concentrations were added: 41, 83, 166, and 250 mM, respectively.

2.4. pH and NH_{3aq} determinations

The pH of the media was measured using a digital pH-meter (Orion 3 star Thermo Scientific, Beverly, MA, USA). Aqueous ammonia content of culture supernatants was determined by reaction with Cu^{2+} [33], with a calibration curve performed in AUM containing increasing NH_{3aq} concentrations (equation: $A_{700nm} = 0.003577\ NH_{3aq}$ concentration; $r^2 = 0.96$).

2.5. Biofilm formation assays

Bacterial inocula in AUM (2×10^7 cells ml^{-1}; 150 μl per well) were placed in 96-well polystyrene plates (DeltaLab, Barcelona, Spain) and incubated at 37 °C. Adhesion to polystyrene surface was allowed for 3 h and then AUM was replaced every 24 h, as already described [7]. At selected time points (1 to 5d), biofilms were washed three-times with sterile 0.9% NaCl and then assayed for biomass quantification by crystal violet staining [34] and cell viability by CFU counts after mechanical disruption [7].

2.6. Obtention of cell-free culture supernatants

Supernatants from 16-h-old planktonic cultures (single- or mixed-species) were collected and filtered through a 0.22 μm pore size (Millipore, Bedford, MA, USA). Supernatants were freshly prepared for each experiment, always keeping them in scew-cap tubes. When indicated, bacterial supernatants were allowed to evaporate for 3 h at room temperature in an open flask placed into a biological safety cabinet. This procedure diminished the original volume of the supernatants to their half. Then, supernatants were adjusted to their original volume with fresh AUM. NH_{3aq} concentration and pH was determined in both evaporated and non-evaporated supernatants, as explained in 2.4. When needed, the pH of evaporated supernatant from mixed cultures was adjusted to 9.3 by adding 100 mM TAPS pH 9.3.

2.7. Determination of antimicrobial activity of cell-free culture supernatants

To explore a potential inhibitory activity toward *K. pneumoniae* released by *P. mirabilis*-containing cultures, *K. pneumoniae* was inoculated in supernatants half-diluted with fresh AUM. For planktonic growth experiments, *K. pneumoniae* (1×10^7 cells ml^{-1}) was incubated at 37 °C and CFU ml^{-1} were determined after 24 h, as described at the end of *2.2 section*. The effect of bacterial supernatants on pre-formed *K. pneumoniae* biofilms was tested over 4-d-old biofilms, by adding 150 μl of the corresponding supernatant, or AUM as control, and performing crystal violet staining after 24 h static incubation at 37 °C. Because volatile compounds in the bacterial supernatants were suspected as antimicrobials, each supernatant was tested in a separated plate, sealed 4-times with parafilm. Parafilm efficacy to limit diffusion of *P. mirabilis* volatiles was experimentally determined, as indicated in Section 2.8.

2.8. Determination of antimicrobial activity of bacterial volatile compounds

To study the effect of *P. mirabilis* volatiles on the survival of *K. pneumoniae* in planktonic cultures, *K. pneumoniae* was inoculated in a small open tube (0.25 ml AUM containing 10^7 cells ml^{-1}). This small tube was placed inside a larger 15-ml screw-cap tube containing 1 ml of *P. mirabilis* in AUM (10^7 cells ml^{-1}). Both strains were physically separated, preventing the exchange of anything other than volatile compounds between organisms. Colony numbers in the inner and outer compartments were quantified after 24 h at 37 °C, as described above. As control, similar experiments were done without *P. mirabilis* inoculation in the larger tube (instead, only AUM was added in the outer compartment). This system was also used to test the efficiency of parafilm in preventing gas diffusion between compartments. Briefly, the open small tube containing *K. pneumoniae* was wrapped 4-times with parafilm to avoid volatiles of *P. mirabilis* culture to become in contact with *K. pneumoniae* cells. In contrast to the bactericidal effect observed over *K. pneumoniae* in the absence of parafilm, when this film was covering the inner tube inoculated with *K. pneumoniae* no detrimental effect on bacterial viability was observed. This finding supported the use of parafilm to limit volatiles diffusion [35].

Another strategy utilized to evaluate the putative antimicrobial activity of *P. mirabilis* volatiles over *K. pneumoniae* was a 2-Petri-dish assay, performed as described by Farag *et al.* [35], with minor modifications. Briefly, an uncovered 3.5 cm Petri dish was aseptically placed inside a 9.5 cm Petri dish and both the internal Petri dish and the resulting external ring were filled with AUM-1.5% agar. *K. pneumoniae* and *P. mirabilis* were spotted on the internal agar dish and the external agar ring, respectively (15 spots of each bacterial species, with each spot corresponding to 10 μl of a bacterial suspension containing 1×10^7 CFU ml^{-1}). The large Petri dish was then closed, sealed with parafilm, and incubated for 48 h at 37 °C. Quantification of bacterial growth after exposure to volatile compounds was monitored by CFU counts after disrupting the bacterial colonies in sterile 0.9 % NaCl. Similar 2-Petri-dish assays were performed to test the antimicrobial activity of *P. mirabilis* volatiles over the following CAUTI's clinical strains: *Escherichia coli* (Ec01), *Morganella morganii* (Mm05), and *Enterococcus faecalis* (Ef02) [7].

Effect of *P. mirabilis* volatiles on pre-formed *K. pneumoniae* biofilms was tested over 3-d-old biofilms. To this aim, *K. pneumoniae* biofilms were developed in 16 wells placed in the middle of a 96-well plate. At day 3, *P. mirabilis* was inoculated in the 48 wells surrounding *K. pneumoniae* biofilms (200 μl per well of a bacterial suspension containing 2×10^7 CFU ml^{-1}). As control, 200 μl of AUM was placed in the 48 wells surrounding *K. pneumoniae* biofilms. The plates were sealed with parafilm and incubated for 24 h at 37 °C. *K. pneumoniae* biofilms were evaluated for both biofilm biomass by crystal violet staining and cell viability by CFU counts after mechanical disruption.

2.9. Statistical analysis

Statistical significance was assessed using either the Student's t test or the one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. A p-value <0.05 was considered significant. Analyses were performed using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Competitive interactions of P. mirabilis toward K. pneumoniae in AUM occurred with a concomitant increase in both aqueous ammonia and pH

In a previous work, a robust competitive effect of *P. mirabilis* over *K. pneumoniae* in mixed cultures developed in AUM has been reported [7]. Here, the goal of this study was to investigate the mechanisms behind this competitive interaction. It is known that *P. mirabilis* possess a urea-inducible urease that hydrolyze urea into ammonia and carbon dioxide thus increasing the medium pH [5]. Both pH and aqueous ammonia (NH_{3aq}) content were measured in mixed and single-species cultures developed in AUM, a medium that contained 170 mM urea [30]. Mixed and single-species *P. mirabilis* cultures showed a gradual pH increase over time (Figure 1A, B). The highest pH value (pH 9.3) was reached 12 h after inoculation and it was maintained until the end of the experiment (36 h). Determination of NH_{3aq} concentration also showed an increase over

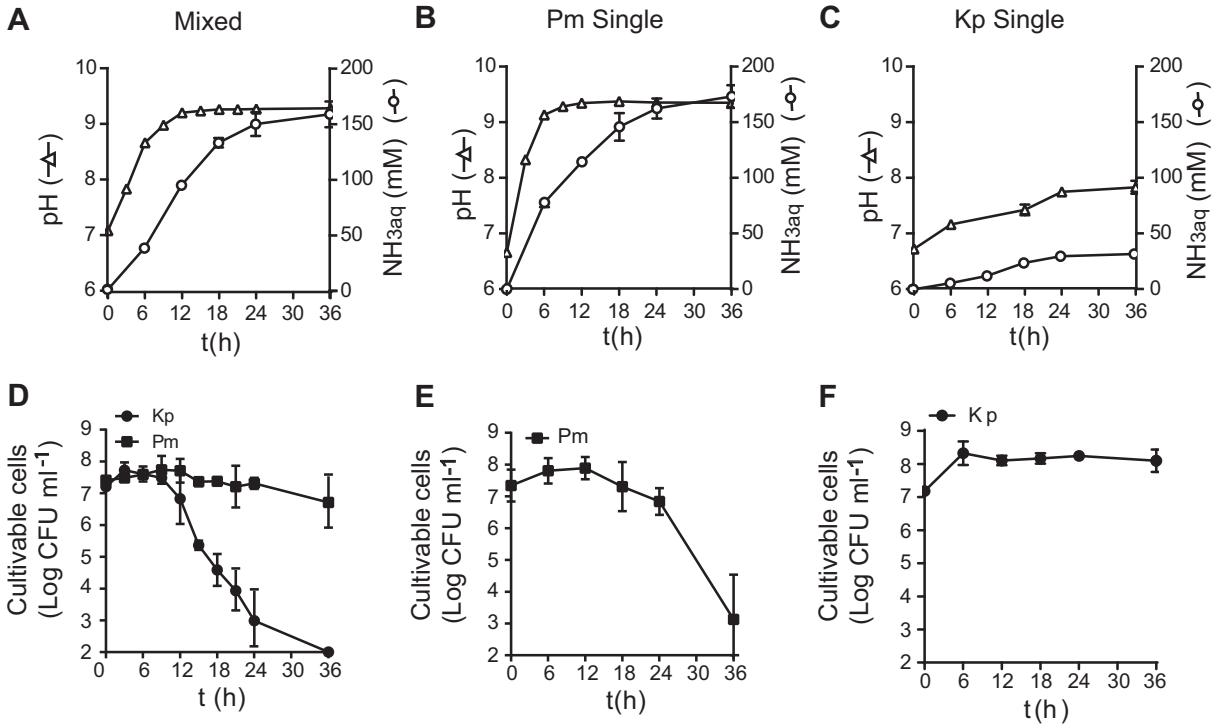

Figure 1. Time-course of medium pH, aqueous ammonia concentration, and bacterial cell number of *P. mirabilis* and *K. pneumoniae* mixed and single-species planktonic cultures in AUM. *P. mirabilis* (Pm) and *K. pneumoniae* (Kp) were inoculated, alone or mixed, in AUM and incubated at 37 °C. At different time-points both pH and NH_{3aq} levels were measured (A-C) and the number of cultivable cells was assessed (D-F). Each point represents the mean ± SDs of at least three independent experiments.

time, reaching ~150 mM at 24 h post-inoculation and maintaining similar levels up to 36 h (Figure 1A, B). In contrast, single-species *K. pneumoniae* cultures showed a moderate increase in both pH (to 7.6) and NH_{3aq} (to 24 mM) (Figure 1C). The data presented here are consistent with previously reported studies in which *P. mirabilis* grown in urine raised medium pH to values ≥ 9.3 [36,37]. These results indicate that *P. mirabilis* is driving the changes in both pH and NH_{3aq} concentration.

K. pneumoniae viability began to decrease 12 h after co-inoculation with *P. mirabilis*, showing a 4-log reduction in *K. pneumoniae* cell number at 24 h, and >6-log reduction (below detection level) at 36 h (Figure 1D). On the contrary, *P. mirabilis* viability was not significantly modified over time in mixed cultures ($p > 0.05$). In single-species cultures, both *K. pneumoniae* and *P. mirabilis* cell numbers increased after 6 h cultivation, reaching approx 10^8 cells per ml (Figure 1E, F). In contrast to *K. pneumoniae*, *P. mirabilis* viability moderately decreased after 24 h culture to 10^7 cells per ml, followed by a drastic reduction in viable cell counts at 36 h post-inoculation (10^3 cells per ml) ($p < 0.05$).

When lower bacterial inocula were assayed (10^5 cells per ml), *K. pneumoniae* cell counts in mixed cultures reached 2×10^7 cells per ml at 6 h post-inoculation, and thereafter its viability gradually decreased to 4×10^4 cells per ml at 18 h post-inoculation (Figure S1). After 21 h co-culture, no viable *K. pneumoniae* cells were detected (<10^2 cells per ml). *P. mirabilis* in mixed cultures reached approx 10^8 cells per ml 9 h after inoculation and its viability did not significantly changed up to 24 h ($p > 0.05$). On the other hand, both single-species cultures showed an increase in cell numbers over time, reaching their maximum of approx 10^8 cells per ml 6 h after inoculation (Figure S1). Then, whereas *K. pneumoniae* single-species cultures stayed at 10^8 cells per ml up to 24 h post-inoculation, *P. mirabilis* single-species cultures showed a reduction in viable cells to 6×10^6 cells per ml after 24 h culture.

A decline in *P. mirabilis* viable cell number when grown in AUM and pooled human urine has been previously reported [36, 38] and this effect was attributed to the urease activity in urine (that causes a raising of both NH_{3aq} concentration and pH) since no reduction in cell numbers was observed for a *P. mirabilis* mutant strain producing an inactive urease [36, 38]. Notably, co-culture with *K. pneumoniae* enhanced *P. mirabilis* survival in AUM, compared to single-species culture. Likely, in mixed cultures *K. pneumoniae* cell death provided nutrients into the medium that could be used by *P. mirabilis*.

To evaluate whether the changes in pH and NH_{3aq} concentration are involved in the detrimental effect of *P. mirabilis* over *K. pneumoniae*, mixed cultures were developed in conditions impairing those changes, such as MES-buffered AUM (pH 6.5) and AUM without urea. As shown in Figure 2A, B, both modified AUMs maintained the pH and NH_{3aq} concentration below 7.3 and 1 mM, respectively, after 24 h of mixed inoculation. Under these conditions, *K. pneumoniae* cell counts did not decrease; instead, both bacterial species showed a slightly better growth compared to unmodified AUM (Figure 2C, D). Similar results were obtained for single-species cultures in modified AUMs (Figure S2). These results evidenced the importance of the increase in pH and/or NH_{3aq} concentration for the competitive effect of *P. mirabilis* over *K. pneumoniae*.

3.2. Aqueous ammonia, but not alkaline pH, affected K. pneumoniae viability

It is noteworthy that the loss of *K. pneumoniae* viability in mixed cultures in AUM occurred at the same time the highest pH value was reached (pH = 9.3) (Figure 1A, D). To evaluate whether medium alkalinization affects single-species *K. pneumoniae* growth and viability, bacterial cell counts were determined in cultures developed in alkaline AUM (pH 8–9.3), after 24 h of bacterial inoculation (Figure 3). A statistically significant inhibition of bacterial growth was observed at all the alkaline conditions tested ($p < 0.05$), with the highest inhibition observed at pH 9.3 (~0.7-log decrease compared to control AUM). Noticeably, *K. pneumoniae* cells survived when inoculated in AUM buffered to pH of 9.3. This result indicates that the alkaline pH reached by mixed cultures, *per se,* could not cause *K. pneumoniae* cell death. It is

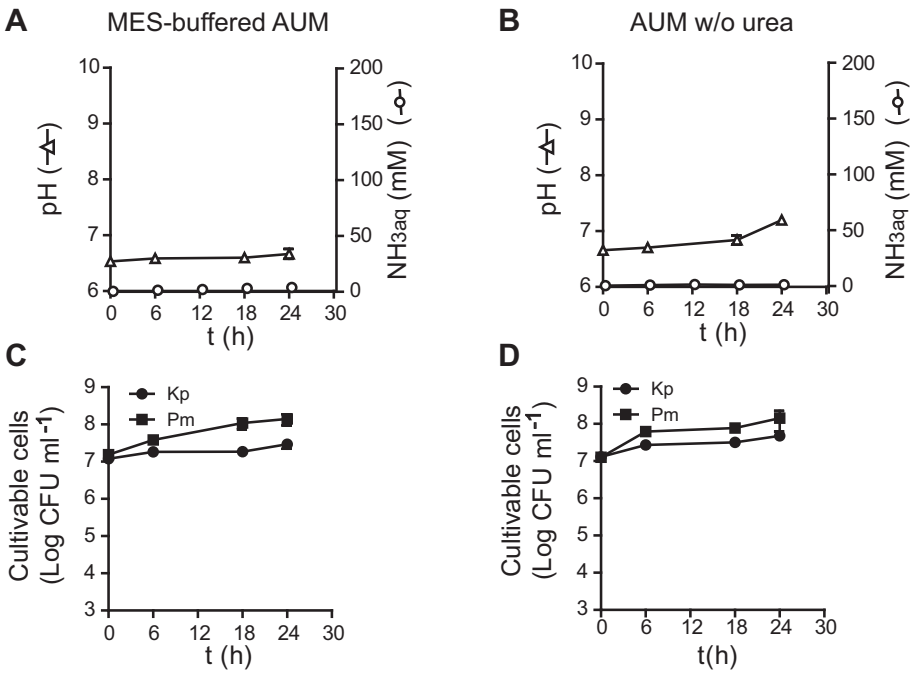

Figure 2. Effect of AUM modifications on the development of mixed *P. mirabilis* and *K. pneumoniae* planktonic cultures. MES-buffered AUM (pH 6.5) (A, C) or AUM without urea (B, D) were co-inoculated with *P. mirabilis* (Pm) and *K. pneumoniae* (Kp) and incubated at 37 °C. At different time-points both pH and NH_{3aq} levels were measured (A-B) and the number of cultivable cells was assessed (C-D). Each point represents the mean ± SDs of at least three independent experiments.

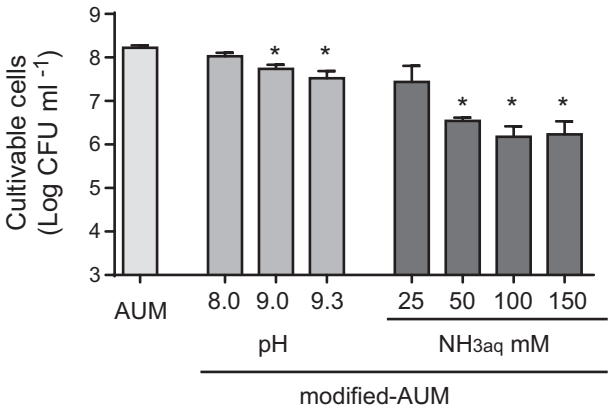

Figure 3. Survival of *K. pneumoniae* in AUM modified by alkalinization and increased aqueous ammonia concentration. *K. pneumoniae* was inoculated in AUM and modified-AUMs and the number of viable cells was assessed at 24 h post-inoculation. Modified AUMs having alkaline pH (8.0, 9.0 and 9.3) and increasing NH_{3aq} concentrations (25–150 mM at pH 9.1) were prepared as described in Material and Methods. Each bar represents the mean ± SDs of at least three independent experiments. (*) $p < 0.01$ compared to control AUM by ANOVA with Dunnett's post test.

known that Enterobacteriaceae, including *K. pneumoniae*, are neutralophiles and grow optimally in a pH range within 5.5–8.5 [15]. Outside this pH range, bacteria have strategies for surviving without growth and pH homeostasis under alkaline conditions is reached by active transport of protons inward, which usually involves activation and transcriptional up-regulation of key cation/proton antiporters [15]. *K. pneumoniae* inoculated in AUM buffered to pH of 9.3 might activate this pH homeostasis mechanism.

Next, the viability of *K. pneumoniae* in the presence of increasing NH_{3aq} concentrations (25–150 mM) was tested after 24 h of bacterial inoculation (Figure 3). A 1.5-log decrease in *K. pneumoniae* cell counts, compared to initial inoculums, was observed at 50 mM NH_{3aq}; and no significantly higher detrimental effect were produced by NH_{3aq} concentrations up to 150 mM ($p > 0.05$). Because at the conditions assayed (pH 9.1 and 37 °C) around 60% of the NH_4Cl was converted to ammonia and the rest remains as NH_4^+, toxicity of this ion over *K. pneumoniae* was tested. No detrimental effect on bacterial growth was observed up to 350 mM NH_4^+ (Figure S3). While ammonia is an important source of nitrogen, its toxicity has been described for several microorganisms [32]. The results presented here suggest that increased levels of ammonia could be in part responsible for the loss of *K. pneumoniae* viability in mixed cultures. Nevertheless, other/s competitive mechanisms must be taking place to get the whole competitive effect.

3.3. Secreted *P. mirabilis* compounds reduced both planktonic cell viability and biofilms biomass of *K. pneumoniae*

To investigate whether the whole antagonistic effect of *P. mirabilis* on *K. pneumoniae* population was the result of secreted effectors, the antimicrobial activity of bacterial supernatants was tested on planktonic *K. pneumoniae* cultures. Supernatants from 16 h-old cultures were chosen for these assays because at this time-point a significant loss of *K. pneumoniae* viability was observed in mixed cultures (4-log decrease in cell counts) ($p < 0.05$) (Figure 1D). Prior to bacterial inoculation, supernatants were diluted 1:1 with fresh AUM to ensure nutrients availability. Figure 4A shows *K. pneumoniae* cell counts after 24 h challenge with supernatants. As expected, supernatant from *K. pneumoniae* did not affect bacterial growth. Supernatants from both single-species *P. mirabilis* and mixed cultures drastically reduced *K. pneumoniae* viability below the detection limit (>6-log reduction) ($p < 0.05$). In comparison to the 36 h of growth in direct co-culture needed to obtain a similar effect (Figure 1D), *P. mirabilis* and mixed culture supernatants showed stronger antimicrobial activity against *K. pneumoniae*. It is likely that in direct co-culture antimicrobial compounds were gradually secreted over time whereas in supernatants collected after 16 h culture these molecules were already present from the beginning of *K. pneumoniae* inoculation. Overall, these results evidenced the presence of secreted compounds with bactericidal activity against *K. pneumoniae* in the media from *P. mirabilis*-containing cultures. These diluted supernatants contained ~50 mM

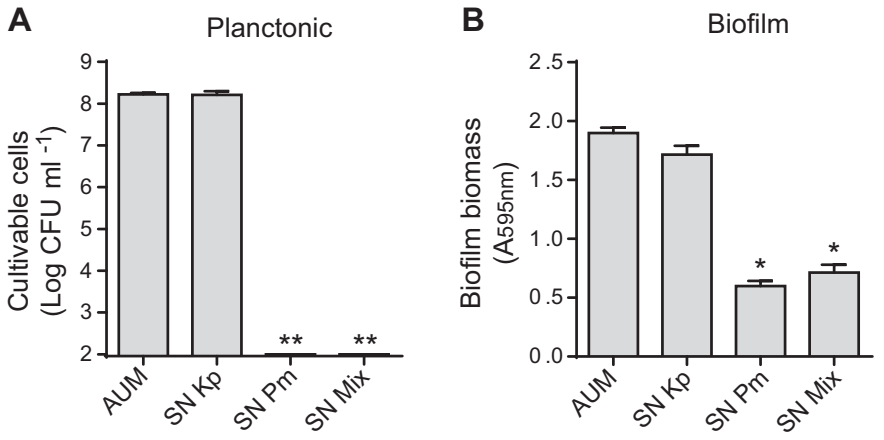

Figure 4. Effect of bacterial supernatants on *K. pneumoniae* planktonic and biofilm growth. Cell-free supernatants (SN) from 16-h-old single-species *K. pneumoniae* (Kp), *P. mirabilis* (Pm), and mixed (Mix) planktonic cultures were analyzed. (A) The number of cultivable *K. pneumoniae* cells per ml was determined after planktonic growth in AUM containing SN at a ratio 1:1, for 24 h. (B) *K. pneumoniae* pre-formed biofilms (4-days-old) were challenged with AUM containing SN at a ratio 1:1. After 24 h, biofilm biomass was assessed by crystal violet staining (A_{595nm}). In both panels, each bar represents the mean ± SDs of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.001$ compared to control AUM by ANOVA with Dunnett's post test.

NH_{3aq} and had a pH of 9.3 (similar values were measured both at the beginning and at the end of the experiment). The current study already showed that 50 mM NH_{3aq} would account for a 1.5-log decrease in *K. pneumoniae* cell counts (Figure 3). The dramatic killing effect over *K. pneumoniae* (>6-log reduction) achieved by *P. mirabilis*-containing supernatants could not be explained by their NH_{3aq} content, indicating the existence of additional secreted factors responsible for *K. pneumoniae* cell death.

Next, the anti-biofilm activity of bacterial supernatants on pre-formed *K. pneumoniae* biofilms was explored. For this purpose, development of *K. pneumoniae* biofilms in AUM was monitored over time to determine a time-point where a large amount of biofilm was produced. A substantial amount of biofilm biomass was observed after 4 d of growth, as determined by crystal violet (Figure S4). Then, 4-d-old biofilms were challenged with bacterial supernatants for 24 h. Supernatants from *P. mirabilis* single-species and mixed cultures produced approx. 60 % decrease in *K. pneumoniae* biofilm biomass, whereas no effect was observed when *K. pneumoniae* supernatants were assayed (Figure 4B). This result suggests the presence of molecules secreted by *P. mirabilis*-containing planktonic cultures that can partially remove pre-established *K. pneumoniae* biofilms. It is possible that these compounds secreted by *P. mirabilis* play a role in the competitive effect previously shown in mixed biofilms [7].

Additional experiments evidenced that *P. mirabilis* can adhere to a pre-formed *K. pneumoniae* biofilm, and thereafter outcompete this bacteria in the mixed biofilm (Figure S5). A 5-log decrease in *K. pneumoniae* cell counts was observed after 24 h *P. mirabilis* adhesion over a pre-formed *K. pneumoniae* biofilm, compared to a control single-species biofilm. On the contrary, 24 h incubation of pre-formed *K. pneumoniae* biofilms with AUM buffered to pH 9.3 did not affect cell viability. Concerning *P. mirabilis*, viable cells showed similar numbers in mixed than in single-species biofilms. This result supports the idea of a significant competitive effect of *P. mirabilis* toward *K. pneumoniae* in AUM.

3.4. Volatile nature of P. mirabilis secreted antimicrobial compounds

As shown above (Figure 4A), supernatants from *P. mirabilis*-containing cultures displayed an important bactericidal activity against *K. pneumoniae* planktonic cultures, causing more than 6-log reduction in viable cell counts after 24 h incubation. The NH_{3aq} content of these supernatants (~50 mM) would account for a 1.5-log decrease in *K. pneumoniae* cell counts. To investigate antimicrobial molecules other than ammonia secreted by *P. mirabilis*, supernatants were subjected to evaporation to removeNH_{3aq}. Supernatants from mixed cultures were partially evaporated in an open flask at room temperature during 3 h and then reconstituted to its original volume with fresh AUM. After evaporation, the NH_{3aq} content was reduced to 15 mM and the pH to 8.0. Surprisingly, this evaporated supernatant did not show any antimicrobial activity against *K. pneumoniae* after 24 h incubation (Figure 5A). This result could be due to the requirement of a pH of 9.3 (like the pH originally reached by *P. mirabilis*-containing cultures) for the antimicrobial activity. To analyze this possibility, evaporated supernatants were buffered to pH 9.3 and then incubated with *K. pneumoniae* during 24 h. Under this condition, only a 1-log decrease in cell counts was observed, compared to control AUM. This effect was similar to the decrease observed when *K. pneumoniae* was grown in AUM buffered to pH 9.3. These results suggest that the whole antimicrobial activity observed in mixed cultures supernatants are mediated by volatile compound/s soluble in the aqueous supernatants, including ammonia.

To get additional evidence of the putative bactericidal effect of *P. mirabilis* volatiles on *K. pneumoniae* planktonic cultures, an experimental design consisting of a small open tube containing *K. pneumoniae* in AUM placed inside a larger screw-cap tube containing *P. mirabilis* in AUM was used (Figure 5B). This design allowed both strains to be physically separated, preventing the exchange of anything other than volatile compounds between organisms. Quantification of the number of viable cells in the two physically separated compartments after 24 h showed a drastic reduction in *K. pneumoniae* cells (bellow the detection limit of 10^2 CFU ml^{-1}) ($p < 0.05$) whereas *P. mirabilis* cell counts were 10^7 CFU ml^{-1} (Figure 5B). On the contrary, *K. pneumoniae* viability in the inner tube was not affected when only AUM was placed in the larger tube instead of *P. mirabilis*. The decrease in *K. pneumoniae* viability when cells were exposed to *P. mirabilis* volatile compounds was similar to the effect observed with *P. mirabilis* cell-free supernatants.

Next, a 2-Petri-dish assay was performed to confirm that volatile molecule(s) released from *P. mirabilis* growing in AUM affected *K. pneumoniae* viability (Figure 5C). Each bacterial species was spotted in physically separated areas of the AUM-agar 2-Petri-dish system, sharing the same aerial environment, and incubated for 48 h at 37 °C. As control, only *K. pneumoniae* was spotted in a similar system. Formation of *P. mirabilis* and *K. pneumoniae* bacterial colonies was observed in all tested conditions, demonstrating that bacteria were able to grow. Quantification of viable cells in the bacterial colonies evidenced a significantly lower number of viable *K. pneumoniae* cells when exposed to *P. mirabilis* volatiles than when exposed to their own volatile molecules ($p < 0.05$). Three other co-incident bacterial species reported in polymicrobial CAUTIs [7] were tested with *P. mirabilis* in the 2-Petri-dish assay: *Escherichia coli*, *Morganella morganii* and *Enterococcus faecalis* (Figure 5D). Both *E. coli* and *M. morganii* drastically reduced their cells

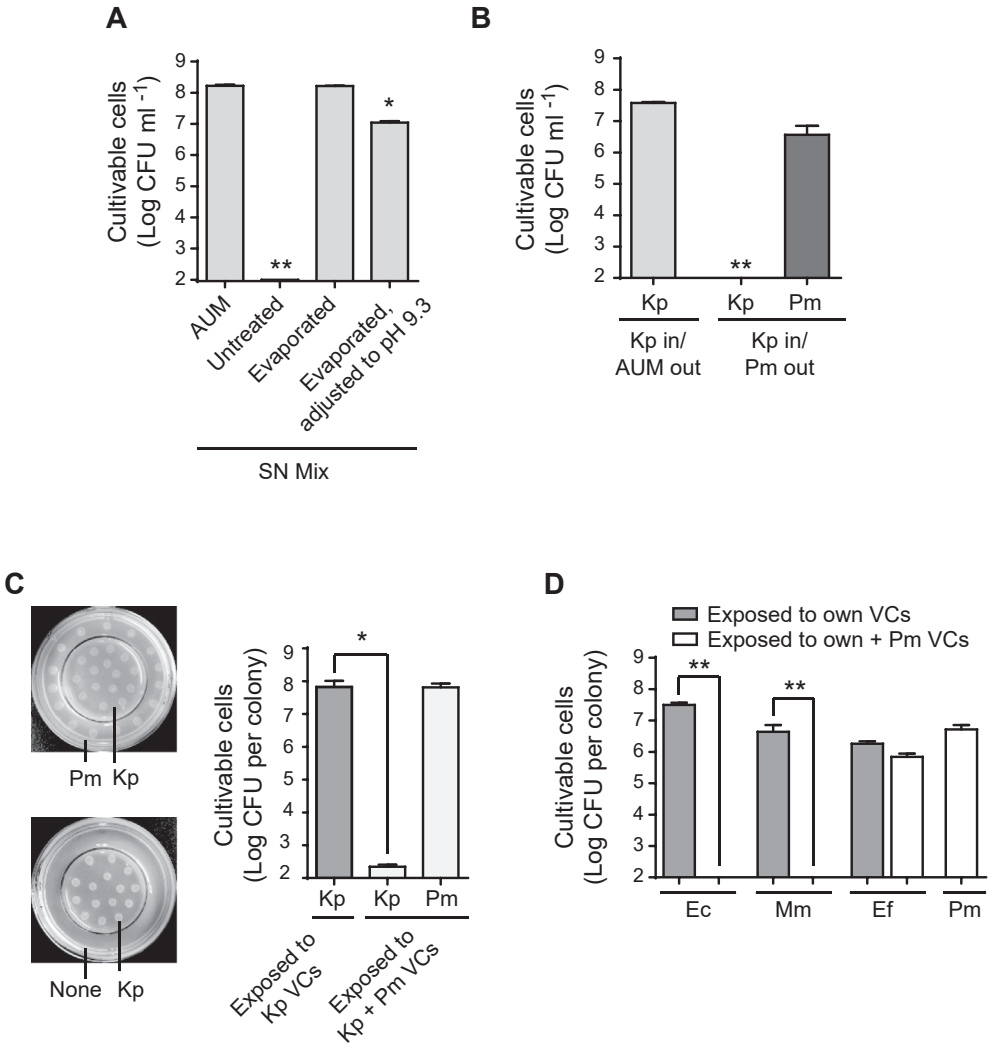

Figure 5. Bactericidal activity of *P. mirabilis* volatiles over *K. pneumoniae* cultures. (A) Effect of evaporation on the antimicrobial activity of supernatants from mixed cultures (SN Mix; see legend of Figure 4). SN without any treatment (untreated), after evaporation (evaporated), or adjusted to pH 9.3 after evaporation were evaluated as described in legend of Figure 4. Each bar represents the mean ± SDs of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.001$ compared to control AUM by ANOVA with Dunnett's post test. (B) Effect of *P. mirabilis* (Pm) volatiles on the viability of *K. pneumoniae* (Kp) in planktonic cultures in AUM. Kp was inoculated in a small open tube placed inside a larger tube containing Pm (Kp in/Pm out). Cell numbers in both compartments were quantified after 24 h at 37 °C. Kp in/AUM out was performed ss control. (C-D) Pm volatile mediated antimicrobial activity determined by 2-Petri-dish assays. (C) A small lidless Petri dish was placed inside a larger one, closed by its lid. Kp were spotted on the central small AUM-agar Petri dish (left panels) and Pm, on the external AUM-agar ring (upper left panel), and incubated for 48 h at 37 °C. As control, no Pm were spotted on the external AUM-agar ring (lower left panel). Quantification of bacterial viability in each colony by CFU counts (right panel). VCs: volatile compounds. (D) 2-Petri-dish assays performed as described in (C) to test Pm VCs over *Escherichia coli* (Ec), *Morganella morganii* (Mm), and *Enterococcus faecalis* (Ef). In panels B–D, each bar represents the mean ± SDs of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.001$ by Student's t test.

counts when exposed to *P. mirabilis* volatiles ($p < 0.05$), whereas *E. faecalis* viability was not significantly affected by *P. mirabilis* ($p > 0.05$). In all assays, *P. mirabilis* cell counts reached 10^7 cells per colony. These results suggest a broad-acting antimicrobial activity of *P. mirabilis* volatiles over gram-negative bacteria.

The effect of *P. mirabilis* volatiles over *K. pneumoniae* pre-formed biofilms was studied. As shown in Figure 6A, the experimental design consisted in development of *K. pneumoniae* biofilms in the center of a 96-well plate for 3 d, and then their exposure to the volatiles produced by *P. mirabilis* cultures located in the wells surrounding *K. pneumoniae* biofilms during 24 h. Compared to control biofilms exposed to AUM volatiles, *P. mirabilis* volatile compounds significantly decreased *K. pneumoniae* viability in biofilms (~3-log lower cell counts) ($p < 0.05$) (Figure 6B). Also a significant reduction in biomass was observed in *K. pneumoniae* biofilms exposed to *P. mirabilis* volatile compounds (Figure 6C) ($p < 0.05$).

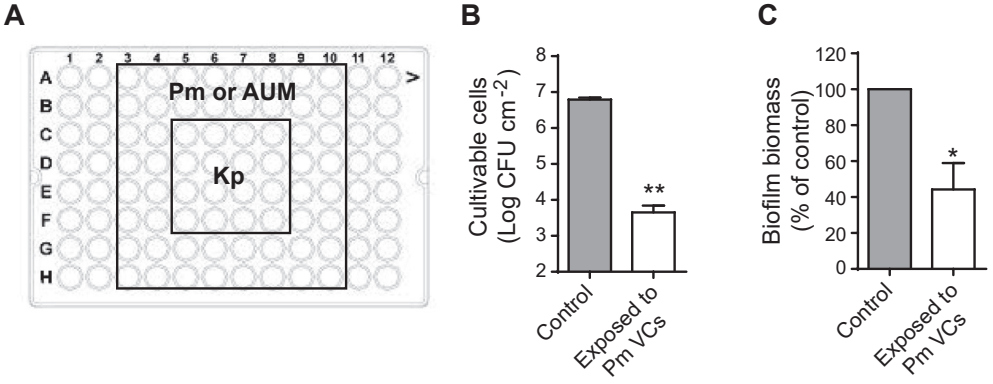

Figure 6. Detrimental effect of *P. mirabilis* volatiles over *K. pneumoniae* pre-formed biofilms. (A) *K. pneumoniae* (Kp) biofilms were developed in 16 wells placed in the middle of a 96-well plate and, at day 3, *P. mirabilis* (Pm) was inoculated in the 48 wells surrounding Kp biofilms. This condition corresponds to biofilms exposed to Pm volatile compounds (VCs). As control, AUM was placed in the wells surrounding Kp biofilms (control biofilms). The plates were then incubated for additional 24 h at 37 °C. Kp biofilms were evaluated for both cell viability by CFU counts after mechanically disruption (B) and biofilm biomass by crystal violet staining (C). Each bar represents the mean ± SDs of three independent experiments. (**) $p < 0.001$ by Student's t test.

The reason for the discrepancy between the *in vivo* co-occurrence of *P. mirabilis* and *K. pneumoniae* in patients with CAUTIs and the *in vitro* data showing that *P. mirabilis* is killing *K. pneumoniae* remains unclear. It is possible that the *in vitro* experiments performed in batch (medium changing every 24 h) allowed an enhanced antimicrobial effect due to a higher accumulation of antimicrobials secreted by *P. mirabilis*, whereas the *in vivo* urine flow would diminish the local concentration of these antimicrobials. The contribution of host factors to *in vivo* modulation of inter-species interactions in polymicrobial CAUTIs involving *P. mirabilis* and *K. pneumoniae* remains to be established.

Bacteria produce and emit highly diverse inorganic and organic volatile compounds [17, 18]. Microbial volatiles can have a significant role in antagonistic interactions between microorganisms occupying the same ecological niche [39, 40]. The amount and composition of volatiles produced by microorganisms can vary according to culturing condition. It was reported that the main volatile compounds detected when *Proteus* sp. was grown in urine were ammonia, hydrogen sulfide, trimethylamine, dimethylsulfide, formaldehyde, and methylmercaptan [26]. Some of them have been previously reported to possess antibacterial activity [17, 41]. Interestingly, the increase in NH_{3aq} and pH in the culture media was shown to be needed to get the dramatic decrease in *K. pneumoniae* viability when co-inoculated with *P. mirabilis* (Figure 2). These changes in NH_{3aq} and pH were driven by the activity of *P. mirabilis* urease, whose expression is induced in AUM by the presence of urea [5]. These results suggest a critical role of this enzyme to favor the generation and/or the activity of the antimicrobial volatile compounds secreted by *P. mirabilis*. Further investigation is needed to identify and to quantify by GC-MS the volatile compounds produced by *P. mirabilis* in AUM. Future studies will investigate the relative contributions of the volatile(s) and ammonia in terms of the competitive interaction of *P. mirabilis* over *K. pneumoniae* and other gram-negative bacteria.

4. Conclusions

A previous study reported a robust competitive effect of *P. mirabilis* over *K. pneumoniae* in mixed cultures developed in AUM [7]. Here, it was evidenced that growth of *P. mirabilis* in AUM promoted an increase in both pH and NH_{3aq}, reaching values of 9.3 and 150 mM, respectively. These changes in the culture media were needed to get the dramatic decrease in *K. pneumoniae* viability when inoculated together with *P. mirabilis*. *K. pneumoniae* inoculated in AUM buffered to pH of 9.3 did not show any decrease in cell viability, whereas a medium containing 150 mM NH_{3aq} partially diminished *K. pneumoniae* viable cell counts. This result strongly suggests the presence of additional antimicrobial factors/mechanisms, besides ammonia, that contribute to *K. pneumoniae* cell death in mixed cultures with *P. mirabilis*. Supernatants from *P. mirabilis*-containing cultures accounted for the whole competitive effect over *K. pneumoniae* observed in mixed cultures. Additional experiments evidenced the volatile nature of these secreted antimicrobial compounds (ammonia and others). Moreover, the volatile compounds secreted by *P. mirabilis* cultures developed in AUM were able to partially disrupt *K. pneumoniae* pre-formed biofilms and significantly reduce their cell viability. In addition, a broad antimicrobial effect of *P. mirabilis* volatile compounds over other gram-negative uropathogens, such as *E. coli* and *M. morganii*, was observed.

The results presented here highlight the need to better understand the role of bacterial volatile compounds as antimicrobials, which would help in the search for novel bioactive molecules to counteract antibiotic resistance. Future studies must elucidate the mechanisms associated with the specific effects of secreted *P. mirabilis* compounds on *K. pneumoniae* growth both planktonically and as biofilm. It will be interesting to evaluate the toxicity of these antimicrobial compounds in eukaryotic cells and next to study their effectiveness in a host.

Declarations

Author contribution statement

Guillermo E. Juarez, Celeste Mateyca: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Estela M. Galvan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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