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 (NH₃aq) 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 NH₃aq 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 NH₃aq after 24 h inoculation, suggesting that ammonia is involved in this inter-species competition. Besides NH₃aq, 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 \textit{P. mirabilis} and \textit{K. pneumoniae} in patients with CAUTIs and the in vitro data showing that \textit{P. mirabilis} is killing \textit{K. pneumoniae} remains unclear. Moreover, the mechanisms behind the in vitro competitive interactions are still unknown.

Both \textit{P. mirabilis} and \textit{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 \cite{5, 8}. \textit{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 \cite{5}. These crystals can result in blockage of the urinary catheter and also in the formation of bladder and kidney stones (urolithiasis) \cite{5, 6}. In contrast to urease from \textit{Proteus} spp., whose expression requires the presence of urea, the urease from \textit{K. pneumoniae} is regulated solely by nitrogen limitation \cite{9}. Besides urease, \textit{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 \textit{K. pneumoniae}, together with siderophores, capsular polysaccharide and lipopolysaccharide \cite{8}. The emergence of extended-spectrum \(\beta\)-lactamase- and carbapenemase-producing \textit{K. pneumoniae} strains is another cause of growing concern in the medical community \cite{10}. In both \textit{P. mirabilis} and \textit{K. pneumoniae}, contact-dependent type VI secretion systems involved in inter-bacterial and bacteria-host interaction have been described \cite{11, 12}.

Competition between species appears to be the interaction that commonly predominate in microbial communities \cite{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 \cite{14}. A very common environmental modification is a change of the environmental pH, because different species prefer different pH values \cite{15}. In bacteria, alkaline stress has been reported to produce salt stress due to the significant increase in the Na\textsuperscript{+} cytotoxicity as the pH rises \cite{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 \cite{15}.

Another important competitive factor is the production of toxic molecules to exclude competitors \cite{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 \cite{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 \cite{18}. Ammonia, nitric oxide, hydrogen sulfide, and hydrogen cyanide are among the inorganic volatiles released by bacteria \cite{17}. The literature about volatile-mediated bacteria–bacteria competitive interactions is still scarce. It has been reported that members of the genus \textit{Streptomyces} produces the sesquiterpenes albaflavenone and pentalenolactone, that are broad-range antibacterial volatiles \cite{19, 20}. \textit{Pseudomonas fluorescens} and \textit{Serratia plymuthica} were described to emit volatile compounds, that strongly suppressed the growth of \textit{Agrobacterium} sp. \cite{21}. Other studies examined the effect of volatiles emitted by \textit{Pseudomonas fluorescens} and \textit{Bacillus} sp on the growth of \textit{Ralstonia solanacearum} \cite{22, 23}. Ammonia has been detected in volatile profiles of various bacterial species, including \textit{Proteus} spp. \cite{24, 25, 26}. Ammonia toxicity has been also described for several microorganisms, especially in an agronomic context \cite{27}. It is well known that uncharged ammonia can cross easily biological membranes as compared to ions (NH\textsubscript{4}\textsuperscript{+}). Inside microorganisms, the uncharged ammonia acts by increasing the internal pH to deleterious levels, so destroys cells by causing the dysfunction of the metabolism \cite{28}.

The present study is focused on the elucidation of the mechanisms involved in the competitive interactions observed in vitro between \textit{P. mirabilis} and \textit{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 \textit{P. mirabilis} and \textit{K. pneumoniae} in mixed cultures in a medium mimicking the human urine. Importantly, this investigation revealed an important role of volatile compounds secreted by \textit{P. mirabilis} as a competitive strategy between bacteria.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

\textit{Klebsiella pneumoniae} 04 (Kp04, Ap\textsuperscript{8}) and \textit{Proteus mirabilis} 04 (Pm04, Tc\textsuperscript{8}) 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 \cite{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. \textit{K. pneumoniae} was freshly streaked in LB-1.5% agar plates, whereas modified LB-agar plates containing 10-fold less NaCl were used for \textit{P. mirabilis} to avoid its swelling \cite{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) \cite{30} to obtain \(10^7\) cells ml\textsuperscript{-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 \cite{7}, using 12 ml polystyrene scw-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 \(\times\) g (Avanti™ LegendTM XTR Centrifuge, Thermo Fisher Scientific, MA, USA), supernatants were discarded, bacteria were resuspended in AUM, then diluted to \(10^3\) cells ml\textsuperscript{-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(hydroxymethyl)methyl]-amino propane sulfonic acid] (Sigma-Aldrich); 4) AUM with defined concentrations of NH\textsubscript{3}aq was prepared as explained below. Cultures were sampled at the indicated time points to determine CFU ml\textsuperscript{-1} as previously described \cite{7}. In brief, bacteria were serially diluted (1:10) and plated on LB-agar plates containing ampicillin (Ap, 30 \(\mu\)g ml\textsuperscript{-1}) for \textit{K. pneumoniae} counts and LB-agar plates with 10-fold less NaCl and tetracycline (Tc) supplementation (10 \(\mu\)g ml\textsuperscript{-1}) for \textit{P. mirabilis} counts.

2.3. Preparation of AUM containing defined concentrations of NH\textsubscript{3}aq

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\textsubscript{3}aq) \cite{31}. Culture media carrying defined NH\textsubscript{3}aq concentrations were prepared as previously described by Koziel \textit{et al.}, with minor modifications \cite{32}. Briefly, AUM with neither urea nor NH\textsubscript{4}Cl was buffered to pH 9.1 with 0.1 M MES buffer (pH 6.5) and diluted 10-fold with AUM (pH 7) for dual-species assays.
with 100 mM TAPS and then appropriate amounts of NH₄Cl were added. To achieve NH₃aq concentrations of 25, 50, 100, and 150 mM, the following NH₄Cl concentrations were added: 41, 83, 166, and 250 mM, respectively.

2.4. pH and NH₃aq 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 Cu²⁺ [33], with a calibration curve performed in AUM containing increasing NH₃aq concentrations (equation: \( \text{A} = 0.003577 \times \text{NH}_3\text{aq concentration} \times r^2 = 0.96 \)).

2.5. Biofilm formation assays

Bacterial inocula in AUM (2 × 10⁷ 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 freshly prepared for each experiment, always keeping them in screw-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 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 screw-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. NH₃aq 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 × 10⁷ cells ml⁻¹) was inoculated 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 10⁷ cells ml⁻¹). This small tube was placed inside a larger 15-ml screw-cap tube containing 1 ml of P. mirabilis in AUM (10⁷ 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 incubated 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 × 10⁷ 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 CAUTIs 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 × 10⁷ 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 (NH₃aq) 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 NH₃aq concentration also showed an increase over
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\textsubscript{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\textsubscript{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\textsuperscript{8} cells per ml (Figure 1E, F). In contrast to K. pneumoniae, P. mirabilis viability moderately decreased after 24 h culture to 10\textsuperscript{7} cells per ml, followed by a drastic reduction in viable cell counts at 36 h post-inoculation (10\textsuperscript{5} cells per ml) (p < 0.05).

When lower bacterial inocula were assayed (10\textsuperscript{5} cells per ml), K. pneumoniae cell counts in mixed cultures reached 2 × 10\textsuperscript{7} cells per ml at 6 h post-inoculation, and thereafter its viability gradually decreased to 4 × 10\textsuperscript{4} cells per ml at 18 h post-inoculation (Figure S1). After 21 h co-culture, no viable K. pneumoniae cells were detected (<10\textsuperscript{2} cells per ml). P. mirabilis in mixed cultures reached approx 10\textsuperscript{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\textsuperscript{8} cells per ml 6 h after inoculation (Figure S1). Then, whereas K. pneumoniae single-species cultures stayed at 10\textsuperscript{8} cells per ml up to 24 h post-inoculation, P. mirabilis single-species cultures showed a reduction in viable cells to 6 × 10\textsuperscript{6} cells per ml 24 h after 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 alkalization 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
known that Enterobacteriaceae, including *K. pneumoniae*, are neutrophiles 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$_3$q conc. (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$_3$q and no significantly higher detrimental effect were produced by NH$_3$q concentrations up to 150 mM (p > 0.05). Because at the conditions assayed (pH 9.1 and 37 °C) around 60% of the NH$_4$Cl 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
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 NH₃aq 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 NH₃aq 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 NH₃aq 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 removeNH₃aq. 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 NH₃aq 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 10² CFU ml⁻¹) (p < 0.05) whereas P. mirabilis cell counts were 10³ 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 morgani and Enterococcus faecalis (Figure 5D). Both E. coli and M. morgani drastically reduced their cells
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 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 as control. (C-D) Pm volatile mediated antimicrobial activity determined by 2-Petri-dish assays. (A) untreated biofilms, (B) evaporated, (C) adjusted to pH 9.3.**p**< 0.001 and (*) 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
vivo 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 vol-
atile 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
these volatiles have been previously reported to possess antibacterial activity [17,
41]. Interestingly, the increase in NH3aq 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 NH3aq 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 NH3aq, reaching values of 9.3 and 150 mM, respect-
ively. 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 NH3aq 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.
Sporanants from P. mirabilis-containing cultures accounted for the
whole competitive effect over K. pneumoniae observed in mixed cul-
tures. 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 anti-
microbial 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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References

[1] K. Clarke, C.L. Hall, Z. Wiley, S.C. Tejedor, J.S. Kim, L. Reif, L. Witt, J.T. Jacob,
Catheter-associated urinary tract infections in adults: diagnosis, treatment, and
prevention, J. Hosp. Med. 14 (2019) E1–E5.

[2] N. Sabir, A. Brain, G. Zaman, L. Satti, A. Gardezi, A. Ahmed, P. Ahmed, Bacterial
biofilm-based catheter-associated urinary tract infections: causative pathogens and
antibiotic resistance, Am. J. Infect. Contr. (2017).

[3] A.S. Azevedo, C. Almeida, L.F. Melo, N.F. Azevedo, Impact of polymicrobial biofilms
in catheter-associated urinary tract infections, Crit. Rev. Microbiol. 43 (2017)
423–439.

[4] I. Kotsavera, H. Oberc, B. Malisova, V. Videnka, B. Zivkova, T. Peroutkova,
M. Dvorackova, P. Kunstal, P. Trojan, F. Rusicka, V. Hla, T. Freiberger, Molecular
techniques complement culture-based assessment of bacteria composition in mixed
biofilms of urinary tract catheter-related samples, Front. Microbiol. 10 (2019) 462.

[5] C.E. Armbruster, H.L.T. Moley, M.M. Pearson, Pathogenesis of Proteus mirabilis
infection, Ecodul Plus 8 (2018).

[6] S.M. Macleod, D.J. Stickler, Species interactions in mixed-community crystalline
biofilms on urinary catheters, J. Med. Microbiol. 56 (2007) 1549–1557.

[7] E.M. Galvan, C. Mateyca, L. Ielpi, Role of interspecies interactions in dual-species
biofilms developed in vitro by uropathogens isolated from polymicrobial urinary
catheter-associated bacteriuria, Biofouling 32 (2016) 1067–1077.

[8] R.M. Martin, M.A. Bachman, Colonization, infection, and the accessory genome of
Klebsiella pneumoniae, Front. Cell. Infect. Microbiol. 8 (2018) 4.

[9] Q. Liu, R.A. Bender, Complex regulation of urease formation from the two
promoters of the ure operon of Klebsiella pneumoniae, J. Bacteriol. 189 (2007)
7593–7599.

[10] K.L. Wyres, K.E. Holt, Klebsiella pneumoniae as a key trafficier of drug resistance
genes from environmental to clinically important bacteria, Curr. Opin. Microbiol.
45 (2018) 131–139.

[11] C.J. Alteri, S.D. Himpel, K. Zhu, H.L. Hershey, N. Musili, J.E. Miller, H.L.T. Moley,
Subtle variation within conserved effector operon gene products contributes to
TSSS-mediated killing and immunity, PLoS Pathog. 13 (2017), e1006729.

[12] L. Liu, M. Ye, X. Li, J. Li, Z. Deng, Y.-F. Yao, H.-Y. Ou, Identification and
characterization of an antibacterial type VI secretion system in the carbapenem-
resistant strain Klebsiella pneumoniae HS11286, Front. Cell. Infect. Microbiol. 7
(2017) 442.

[13] E.T. Gramato, T.A. Meiller-Legrand, K.R. Foster, The evolution and ecology of
bacterial warfare, Curr. Biol. 29 (2019) R521–R537.

[14] C. Ratcliffe, J. Gore, Modifying and reacting to the environmental pH can drive
bacterial interactions, PLoS Biol. 16 (2018), e2004248.
Production of ammonia as a low-cost and long-distance antibiotic strategy by Agrobacterium tumefaciens, Biochemistry 45 (2006) 6118–6127.

B. Zhao, X. Lin, L. Lei, D.C. Lamb, S.L. Kelly, M.R. Waterman, D.E. Cane, C.N. Tetzlaff, Z. You, D.E. Cane, S. Takamatsu, S. Omura, H. Ikeda, A gene cluster for biosynthesis of the sesquiterpenoid antibiotic pastalenoactone in Streptomyces avermectin, Biochemistry 45 (2006) 6179–6186.

M. Avalos, P. Garbeva, J.M. Raaijmakers, G.P. van Wezel, Healthy scents: microbial volatiles as new frontier in antibiotic research? Curr. Opin. Microbiol. 45 (2018) 84–91.

M.C. Lemfack, S.R. Ravella, N. Lorenz, M. Kai, K. Jung, S. Schulz, B. Piechulla, Inactivation of bacteria and viruses in digestate of animal carcasses: proof-of-concept, PloS One 12 (2017), e0176825.

R. Blinn, F. Gunther, Determination of ammonia with cupric carbonate, Anal. Chem. 29 (1957) 1882–1883.

J.A. Koziel, T.S. Frana, H. Ahn, T.D. Glanville, L.T. Nguyen, J.H. van Leeuwen, Efficacy of NH3 as a secondary barrier treatment for inactivation of Salmonella Typhimurium and methicillin-resistant Staphylococcus aureus in digestate of animal carcasses: proof-of-concept, PloS One 12 (2017), e0176825.

J. C. Armbruster, S.N. Smith, A. Yep, H.L.T. Mobley, Increased incidence of urolithiasis and bacteremia during Proteus mirabilis and Providencia stuartii coinfection due to synergistic induction of urosecretion activity, J. Infect. Dis. 209 (2014) 1524–1532.

A. Torzew ska, K. Bednarska, A. Rozalski, Influence of various uropathogens on crystallization of urine mineral components caused by Proteus mirabilis, Res. Microbiol. 170 (2019) 80–85.

J.N. Schaffer, A.N. Nosworthy, T.T. Sun, M.M. Pearson, Proteus mirabilis fimбриae and uroseptic-related clusters assemble in an extracellular niche to initiate bladder stone formation, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 4494–4499.

F.I. Rajer, H. Wu, Y. Xie, S. Xie, W. Raza, H.A.S. Tahir, X. Gao, Volatile organic compounds produced by Pseudomonas fluorescens WR-1 restrict the growth and virulence traits of Ralstonia solanacearum, Microbiol. Res. 192 (2016) 103–113.

H.A.S. Tahir, Q. Gu, H. Wu, Y. Niu, R. Hao, X. Gao, Bacillus volatiles adversely affect the physiology and ultra-structure of Ralstonia solanacearum and induce systemic resistance in tobacco against bacterial wilt, Sci. Rep. 7 (2017) 40481.

M. Avalos, P. Garbeva, J.M. Raaijmakers, G.P. van Wezel, Production of ammonia as a low-cost and long-distance antibiotic strategy by Streptomyces species, ISME J. (2019).

F. Zhou, L. Xu, S. Wang, B. Wang, Q. Lou, M. Lu, J. Sun, Bacterial volatile ammonia regulates the consumption sequence of d-pinitol and d-glucose in a fungus associated with an invasive bark beetle, ISME J. 11 (2017) 2809–2820.

M.K. Storer, K. Hibbard-Melles, B. Davis, J. Scotter, Detection of volatile compounds produced by microbial growth in urine by selected ion ow tube mass spectrometry (SIFT-MS), J. Microbiol. Methods 87 (2011) 111–113.

C.G. Park, F. Díez-Gonzalez, Utilization of carbonate and ammonia-based treatments to eliminate Escherichia coli 0157:H7 and Salmonella Typhimurium DT104 from cattle manure, J. Appl. Microbiol. 94 (2003) 675–685.

B. Vinneras, A. Nordin, C. Niwagaba, K. Nyberg, Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate, Water Res. 42 (2008) 4607–4614.