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ORIGINAL ARTICLE

Broaden properties of ambroxol hydrochloride as an antibiofilm compound

María J. González[a], **Michela Lain**[b], **Victoria Iribarnegaray**[c], **Luciana Robino**[d], **Paola Scavone**[a,*]

[a] *Lab. De Biofilms Microbianos, Dept. de Microbiología, Inst. de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, Montevideo, Uruguay*
[b] *Università degli Studi di Camerino, Italy*
[c] *Dept. de Patobiología, Unidad de Microbiología, Facultad de Veterinaria, Univ. de la República, Montevideo, Uruguay*
[d] *Dept. de Bacteriología y Virología, Inst. de Higiene, Univ. de la Republica, Montevideo, Uruguay*

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Abstract Biofilm-associated microorganisms can cause many infections and are an important cause of resistance to several antimicrobials. The antibiotic crisis has led to a pressing need for new therapeutic tools. Ambroxol is frequently used as a mucolytic agent in respiratory diseases with increased mucus production. In addition, a wide range of properties has been described, including the effect on biofilms. In this work, we evaluate the anti-biofilm effect of ambroxol on four strains with clinical relevance: *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Acinetobacter baumannii*. In vitro, biofilm formation was assessed using the crystal violet quantification technique in microplate and glass coverslip. The inhibition of biofilm formation was evaluated by adding ambroxol at the initial time. Ambroxol hydrochloride was evaluated over the preformed biofilm and live/dead bacteria were quantified. The effect of ambroxol in the ethidium bromide efflux assay and the relative expression of the five major *P. mirabilis* efflux pump family genes were analyzed. Ambroxol inhibited biofilm formation in all the bacteria tested. Moreover, ambroxol significantly reduces both biofilm biomass and viable bacteria. Ambroxol was able to affect *P. mirabilis* efflux pumps depending on the concentration used and induced the overexpression of several efflux pump genes. In summary, ambroxol kills planktonic cells, reduce biofilm biomass as it increases cell death, and affect the expression of efflux pumps. Furthermore, it presents a viable alternative for the treatment of biofilm infection alone or in combination with antibiotic therapy.

* Corresponding author.
E-mail address: pscavone@gmail.com (P. Scavone).

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PALABRAS CLAVE
Ambroxol;
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Tratamiento antibiótico;
Patógenos

Nuevas propiedades del clorhidrato de ambroxol como compuesto antibiofilm

Resumen Los microorganismos en biopelículas pueden causar infecciones y contribuir a la resistencia antimicrobiana, lo que resalta la necesidad de nuevas terapias. Aunque el ambroxol se usa principalmente como mucolítico, se le atribuyen propiedades antibiofilm. En este trabajo evaluamos el efecto antibiofilm del ambroxol en 4 cepas de especies de relevancia clínica, como *Proteus mirabilis*, *Escherichia coli*, *Acinetobacter baumannii* y *Staphylococcus aureus*. La formación de biopelículas *in vitro* se evaluó mediante la técnica de cuantificación con cristal violeta en microplacas y cubreobjetos de vidrio. La inhibición de la formación de biopelículas se evaluó añadiendo ambroxol al inicio del ensayo. El clorhidrato de ambroxol se evaluó sobre la biopelícula preformada y se cuantificaron las bacterias vivas/muertas. Además, se analizó el efecto del ambroxol sobre las bombas de eflujo y la expresión relativa de los 5 genes principales de la familia de bombas de eflujo de *P. mirabilis*. El ambroxol inhibió la formación de biopelículas en todas las cepas bacterianas ensayadas. Además, redujo significativamente la biomasa de la biopelícula y las bacterias viables. El efecto de este fármaco sobre las bombas de eflujo de *P. mirabilis* dependió de su concentración. El ambroxol indujo la sobreexpresión de algunos de los genes de las bombas de eflujo. En conclusión, el ambroxol no solo mata células planctónicas y reduce la biomasa de biopelículas, sino que también afecta la expresión de bombas de eflujo. Esto sugiere que podría ser una adición valiosa al arsenal terapéutico contra infecciones causadas por bacterias formadoras de biopelícula.

Introduction

Bacterial biofilms offer protection to bacteria against adverse conditions such as desiccation, UV, antimicrobials, and chemical products. A biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum, interface, or each other and, exhibit an altered phenotype concerning growth rate and gene transcription while embedded in a matrix of EPS produced by themselves[14]. These structures are composed of bacterial cells and a matrix of hydrated extracellular polymeric substances (EPS), mainly extracellular DNA, polysaccharides, proteins, nucleic acids (DNA/RNA), and lipids[15].

In the medical field, biofilm-associated microorganisms cause a large number of infections. These infections include valve endocarditis, osteomyelitis, dental caries, middle ear infections, chronic lung infections in cystic fibrosis patients, urinary tract infections, and medical device-related infections[23]. Some of the most common bacteria causing this type of infection are *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Acinetobacter baumannii*. *P. mirabilis* infections are associated with urinary tract infections in patients undergoing urinary catheterization[22,33]. *E. coli* has also been the cause of various medical device-associated infections such as prosthetic grafts and joints, shunts, and urethral and intravascular catheters[38]. Moreover, uropathogenic *E. coli* can invade the bladder urothelium and enter the cells forming intracellular bacterial communities[24]. This ability makes treatment and bacterial elimination very difficult and contributes to re-infections. *S. aureus* forms biofilms on mechanical heart valves and the surrounding cardiovascular tissues, which can lead to serious diseases such as prosthetic valve endocarditis. They are also the causative agents of central venous catheter infections and ventilator-associated pneumonia[43]. *A. baumannii* is an emerging global antibiotic-resistant gram negative bacteria that most typically causes biofilm-associated infections such as ventilator-associated pneumonia and catheter-related infection, both of which are resistant to antibiotic therapy[17]. In this context, the search for new strategies to eradicate biofilms with medical implications is essential.

Ambroxol [trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanole] hydrochloride (AMB) is a synthetic derivative of vasicine, the active principle extracted from the plant *Adhato davasica*[40]. AMB has antioxidant and anti-inflammatory activities[39]. Usually, it is used for patients who suffer from asthma and chronic bronchitis as a mucolytic and expectorant agent[41]. Cataldi et al. showed that AMB could inhibit biofilms at various stages when used alone and could enhance the killing effect of *Staphylococcus epidermidis* biofilms in combination with vancomycin[9,42]. Furthermore, AMB has antibacterial and antibiofilm activity against *Serratia marcescens*, *Pseudomonas aeruginosa*, *Candida albicans*, and oral bacteria[2,8,10,26].

It is well known that efflux pumps are widely implicated in antibiotic resistance; however, several studies suggest they play a role in a range of bacterial behaviour, such as biofilm formation, quorum sensing (QS), pathogenicity, and virulence[5]. There are five superfamilies of efflux pumps associated with antimicrobial export: multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), major facilitator superfamily (MFS), ATP-binding cassette (ABC), and resistance-nodulation-division (RND)[5]. The effect of AMB on the efflux pumps in the biofilms has not been evaluated previously.

As mentioned above, AMB hydrochloride is a compound with a very promising outlook. First, it is already used in humans where the intake dose is 6 mg/ml. Second, AMB has shown anti-biofilm properties in the bacterial species evaluated, among other useful activities.

In this work, we tested AMB hydrochloride properties such as biofilm inhibition and eradication in *P. mirabilis*, *E. coli*, *S. aureus*, and *A. baumannii* in vitro. Additionally, we studied the mechanisms behind these properties, focusing on *P. mirabilis*.

Materials and methods

Bacterial strains and culture conditions

Four strains capable of forming biofilm were studied. *E. coli* U144 and *P. mirabilis* 2921 were isolated from urine samples from patients with UTI and previously characterized in our laboratory[18,44]. *A. baumannii* C100 was isolated from a tracheal secretion from a patient admitted to the Intensive Care Unit at Hospital de Clínicas, Montevideo, Uruguay, and previously characterized[6,13]. *S. aureus* ATCC 6538 was included as a gram positive microorganism that could also form biofilms. All strains were stored in glycerol at −80 °C except *A. baumannii* and kept at −20 °C. Bacteria were grown in either Luria-Bertani (LB) broth or Luria-Bertani agar (LA) at 37 °C.

Ambroxol hydrochloride MIC assay

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) standard method with some modifications[11]. Standardized bacteria suspensions were prepared from overnight cultures in LB (optical density OD 600 nm = 1.0). From the overnight suspension, we inoculated 20 μl into 180 μl of LB with a serial dilution of AMB (Sigma) in a microtiter plate. Strains were grown for 24 h at 37 °C. The MIC was calculated as the lowest concentration of AMB that inhibited the visible growth in the wells.

Inhibition of biofilm formation assays

Biofilm formation assays were performed as previously described with some modifications[34]. Briefly, aliquots of 20 μl from overnight cultures in LB were inoculated into 180 μl of LB with 1 mg/ml of AMB (Sigma) in 96 flat-bottomed well, polystyrene microtiter plates (Deltalab) and incubated for 24 and 48 h at 37 °C without shaking. For bacterial growth, the OD were read at λ = 600 nm. Planktonic bacteria were removed and the wells were washed with phosphate-buffered saline (PBS) pH 7.4 three times and stained with 200 μl of 1% crystal violet for 15 min at room temperature. Then, the plates were washed to remove the dye excess and crystal violet (CV) was solubilized with 200 μl of 95% ethanol. The ODs were read at λ = 590 nm for stained biofilms using a Microplate Reader (Varioskan, Thermo Scientific). All the measurements were performed in triplicate for all strains and with two biological repetitions. The means and standard error were calculated for all experiments. Subsequently, an inhibition assay was performed in a glass coverslip, where we incubated the coverslip in LB with or without subinhibitory concentration of AMB (0.3 or 0.5 mg/ml) for 48 h at 37 °C without shaking. After that, the coverslips were washed to remove all the planktonic cells with PBS and the biofilm was stained with CV for macroscopic visualization.

Eradication of biofilm assay

Biofilm formation assays were performed as previously described[18]. After 48 h of biofilm formation, planktonic bacteria were removed and fresh LB was added to each well with (0.1, 0.6, 0.8, and 1 mg/ml) or without AMB. The biofilms were incubated for another 24 h, and the CV procedure for biofilm quantification was done as mentioned before. All the measurements were performed in triplicate for all the strains and with two biological repetitions. The means and standard error were calculated for all experiments.

Live/dead assay

We have adapted a procedure to evaluate the percentage of viable bacteria in the biofilm. This assay involved performing the same 96-well microtiter plate inhibition experiment as described before but with the addition of live/dead staining and quantification. After the incubation with AMB, various bacterial suspensions of known ratios of live:dead cells were prepared to quantify the live:dead bacteria with two fluorophores. Syto9 stains all bacteria in the green channel while propidium iodide (PI) stains the dead cells in the red channel. Before adding the dye, the medium containing AMB was removed and washed with PBS, leaving the biofilm attached to the surface. 100 μl of the dye solution (Syto9 0.01 mM, PI 3 μg/ml) was added to the wells, including those with the curve ratios, and mixed. The OD was measured using different wavelengths. In order to measure the number of total cells, the excitation wavelength was set to 485 nm and the fluorescent intensity emission wavelength to 530 nm, while the reading of dead cells required the fluorescent intensity emission wavelength to be changed to 630 nm. To obtain the percentage of live cells in the biofilm, the percentage of dead cells was subtracted from the total fluorescence. The percentage of live bacteria was plotted against the ratio of live:dead bacteria and calculated from the curve values. The formula $y = mx + c$ was used to calculate the rate of live bacteria (x) in the rest of the wells. All the measurements were performed in triplicate for all the strains and with two biological repetitions.

To visualize the effect of ambroxol over the biofilm, confocal scanning laser microscopy (CSLM) was performed. Briefly, bacteria were inoculated in small plates containing a coverslip covered with LB during 48 h. After that, the media was removed and replaced with LB + AMB (1 mg/ml), washed for another 24 h. Finally, planktonic bacteria were removed with PBS, and stained for 15 min in an opaque chamber with the fluorophore mixture (5 μg/ml propidium iodide, and 10 μM Syto9 (Thermo Fisher Scientific)). Bacteria were then fixed with paraformaldehyde 4% in a light-isolated chamber. Lastly, the coverslips were washed with PBS, mounted on slides, and sealed with enamel. CSLM images were acquired

using a LSM800 ZEISS, software Zen Blue 2.3, oil immersion 100X objective (NA 1.4).

Efflux inhibition assay

Assays for inhibition of efflux were modified from a protocol previously described[12]. Overnight LB broth cultures were re-suspended in fresh broth at 1:100 dilution, and grown for a further 1.5 h with shaking. The suspensions were adjusted to an OD600 of 0.6 and dispensed into a 96-well plate (50/well) containing 50 μl LB supplemented with glucose (40 mM), ethidium bromide (EtBr; 10 μg/ml) as an efflux substrate, and 0.3 or 0.5 mg/ml of AMB. Controls included LB with AMB without bacteria, cell suspensions with EtBr, and bacteria suspension with AMB. Thioridazine (THR) was used as a positive control for efflux inhibition (100 μg/ml)[30]. Plates were incubated at 37 °C with gentle rocking for 2 h. Following incubation, fluorescence was measured at 540 nm excitation and 600 nm emission wavelength and the percentage of accumulation of EtBr (indicative of efflux inhibition) was calculated as: (Fluorescence in treated cells × 100)/fluorescence in control without AMB. The percentage was calculated relative to the control without AMB.

Ambroxol effect on *P. mirabilis* efflux pump expression in biofilm

To evaluate the effect of AMB on the expression of efflux pumps in the *P. mirabilis* biofilm, we performed a quantitative real-time PCR. Specific primers were designed to amplify *P. mirabilis* efflux pump genes using Primer Blast (Table 1) and *rpoA* RNA polymerase as housekeeping gene control[21,37]. *P. mirabilis* 2921 was grown in 5 ml of LB broth in six-well flat-bottom plates (Greiner CELLSTAR®) under two conditions for 48 h at 37 °C to allow biofilm formation. The conditions assayed were biofilm formation in LB and LB with 0.6 mg/ml of AMB. Planktonic cells were removed and placed in RNA later® (Ambion) until processing. Then, 1 ml of RNA later® (Ambion) was added to the biofilm, and RNA extraction was performed using the PureLink® RNA mini kit (Ambion) according to the manufacturer's recommendations. Total RNA was treated with DNAse I (Invitrogen) to remove genomic DNA contamination. After that, first-strand cDNA was synthesized using random primers and M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. Syber Green (Invitrogen) fluorescence was used for quantitative real-time PCR with the Biorad Detection System. The amplification program consisted of one cycle of 2 min at 50 °C, 15 min at 95 °C and 40 cycles of 15 s to 94 °C, 30 s at 60 °C and 30 s at 72 °C. Data were normalized to *rpoA* (and analyzed by the threshold cycle ($2-\Delta\Delta CT$) method as described by Livak and Schmittgen[27]. Experiments were performed in triplicate.

P. mirabilis swarming and swimming motility assays

The swarming migration distance assay was performed as described previously for *P. mirabilis*[32]. Briefly, an overnight bacterial culture (5 μl) was inoculated centrally onto the surface of dry LB swarming agar (2%, w/v) plates with or without AMB (0.5 mg/ml), which were then incubated at 37 °C for 24 h. The swarming migration distance was measured from the center of the inocula to the edge of migration.

The swimming migration distance assay was performed by inoculating the bacterial suspension by puncture into LB supplemented with 0.3% agar[20]. Assays were performed six times and motility areas were measured and compared using the Student's *t*-test.

Statistical analyses

The differences between the treatment groups were first assessed using the Kruskal–Wallis test, and the differences between pairs of groups were further evaluated using the Mann–Whitney *U* test. In the live/dead experiments, the differences were assessed with one-way ANOVA. The differences between the treatment groups for each gene were assessed using the two-way ANOVA test, and Bonferroni's multiple comparisons test. All the assays have three replicates and two biological repetitions.

Results

Ambroxol MICs

E. coli 144 and *A. baumannii* C100 had a MIC of 0.625 mg/ml. *P. mirabilis* 2921 and *S. aureus* ATCC had a MIC of 1.25 mg/ml.

Inhibition and eradication of biofilm by ambroxol hydrochloride

The inhibition of biofilm formation was tested in vitro by adding AMB (1 mg/ml) to bacterial suspensions of *P. mirabilis*, *E. coli*, *A. baumannii*, and *S. aureus*, and incubating for 24 and 48 h. The two time points were chosen because some bacteria form mature biofilms in 48 h, while others do so in 24 h in this microtiter plate model. AMB significantly reduced the biofilm biomass after 24 and 48 h of incubation of the four strains tested compared with the control without AMB (Fig. 1A). *P. mirabilis* biofilm was reduced by 88.3% at 24 h and 80% at 48 h (*p*-value 0.006 and 0.00006). *E. coli* biofilm showed a reduction of 99.5% at 24 h and 98.1% at 48 h (*p*-value 0.005 and 0.003). *A. baumannii* biofilm showed a similar reduction at 24 and 48 h, 96.9% and 91.9%, respectively (*p*-value 0.003 and 0.002). For *S. aureus*, the reduction in the biofilm biomass was 96.9% and from 91.9% at 24 and 48 h, respectively (*p*-value 0.010 and 0.002). In all the biofilms, the highest inhibition was observed at 24 h, and the biomass decrease ranged from 80% to 98.9%, confirming that AMB could inhibit biofilm formation.

The eradication of a 48 h preformed biofilm was evaluated by adding AMB to the preformed biofilm and incubating it for another 24 h. The AMB concentrations tested were 1.0, 0.8, 0.6, and 0.1 mg/ml. All the concentrations used, except 0.1 mg/ml, were able to significantly reduce the biofilm biomass (Fig. 1B). The treatment with AMB on the *P. mirabilis* biofilm decreased the biofilm biomass by 84.3%, 57%, and 57.3% with 1.0, 0.8, and 0.6 mg/ml of AMB, respec-

Table 1 Specific primers were designed to amplify bacterial efflux pump genes using Primer Blast.

Gene	PMI	Name/function	Primer forward	Primer reverse	TM	Pb	Reference
MFS							
MFS-1	PMI_RS04070	Bcr/CflA multidrug efflux transporter	CCTGGTGGCCAAGTTCAGAT	GCGCTCGATGCTAATGCAAA	59.96 °C	146	This work
MFS-2	PMI_RS05875	AEC family transporter	ACACAGTGACTCGGTTGCTT	CCCCGCCATTAGCTCAGAAT	59.84 °C	173	This work
MFS-3	PMI_RS00920	MFS transporter glucose 6-P receptor	AAACGCAGCAGGTACGGTAA	AGTCCAACCATAATGCGCCA	60 °C	106	This work
MFS-4	PMI_RS08255	Multidrug MFS mdgT	ACCTGCGGTACAAACGCTTA	CGCCCATTAACGGTCCAGTA	59.88 °C	116	This work
MATE							
MATE-1	PMI_RS00720	MATE family efflux transporter	CGCGATAAAGCCAGTCAAGC	GTTGACCATGCAACACCCAC	59.97 °C	350	This work
SMR							
SMR-1	PMI_RS13360	QacE quaternary ammonium efflux transporter	GCCAGCGGGATCGTCATTAT	CTCCGTCGTTTCTTCTGGCT	60.32 °C	72	This work
SMR-2	PMI_RS17830	SugE	ACGCCATTTGGACCGGTATT	TGGCGGATTCACCAAAAACG	60.03 °C	72	This work
RND							
RND-1	PMI_RS13340	MexH adaptor	GCACTGTGCAAGTACAAGCC	ACGGTATCCCCATAGGCAGT	60.04 °C	151	This work
RND-2	PMI_RS13345	MexW/MexI	TCAACCGCAGTCGCTTATGT	CCAGTTGTTGTCCGCCAAAG	60.04 °C	145	This work
RND-3	PMI_RS00640	MexE	CAGTACGCTCAAGCTGTTGC	CCAGAAATTGGTGCGGTGAC	59.84 °C	101	This work
ABC							
ABC-1	PMI_rs00600	Multidrug ABC transporter permease/ATP-binding protein	GCGGGGAAAAGCACTTTGTT	TCAGGGCGTCCTAAGGCTAT	60 °C	191	This work
ABC-2	PMI_RS17270	LPS export ABC transporter permease LptF	GAAGGTACGGCGGTACTACG	ACTCAGGCTCTGTTGAGTGC	59.90 °C	145	This work
ABC-3	PMI_RS00605	Multidrug ABC transporter permease/ATP-binding protein	TCGCCACTTTGTCGCTTTTG	GAGCAGTGACGCCCTAAAGT	60 °C	228	This work

ABC: ATP-binding cassette; MSF: major facilitator superfamily; MATE: multidrug and toxic compound extrusion; SMR: small multidrug resistance; RND: resistance-nodulation-division family.

tively (p-value 0.002, 0.021, and 0.005). *E. coli* biofilm showed similar behavior, being reduced to 84.1% at the highest AMB concentration, to 70.1% and 56.2% with 0.8 and 0.6 mg/ml, respectively (p-value 0.016, 0.024, and 0.0237). *A. baumannii* biofilm showed the greatest decrease (98.4%) with 1.0 mg/ml of AMB (p-value 0.005). The reduction was 71.4% and 64.5% at the lowest AMB concentrations assayed (p-value 0.023 and 0.015). The *S. aureus* biofilm was reduced by 94.5%, 81.8%, and 60.8% at the above mentioned concentrations (p-value 0.029, 0.041, and 0.050). The observed effect is dose-dependent, where the greatest reduction was at the highest AMB concentration.

The effect on the biofilms formed in the glass coverslip surfaces was also observed. We compared biofilm formation with and without subinhibitory concentrations of AMB. We observed in the liquid–air interphase the accumulation of biofilm biomass in the control slides without AMB. *E. coli*, *A. baumannii*, and *S. aureus* biofilms were visibly reduced with

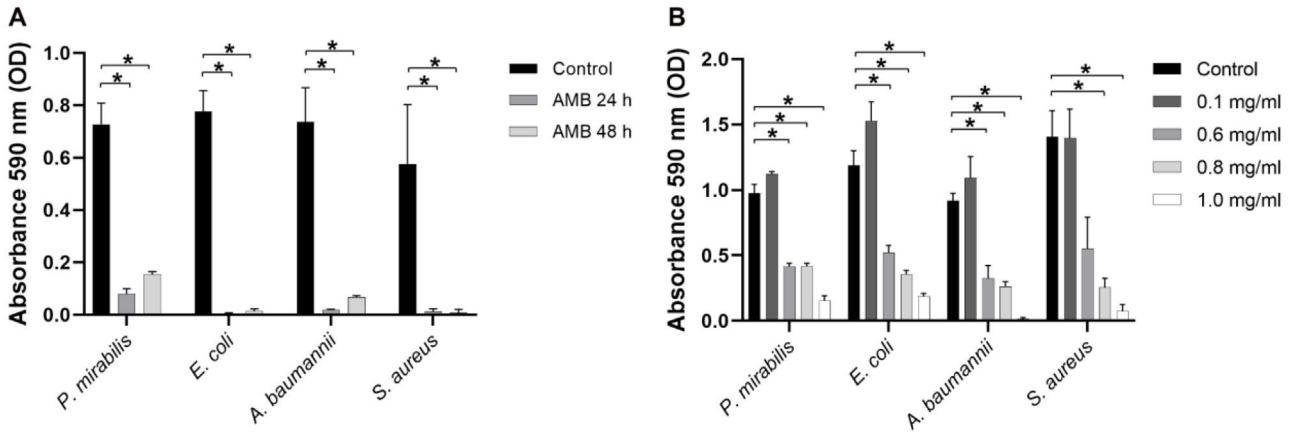

Figure 1 Effect of ambroxol (AMB) on *P. mirabilis*, *E. coli*, *A. baumannii*, and *S. aureus* biofilm formation. (A) Inhibition of biofilm formation after 24 h and 48 h of incubation in LB broth with (grey bars) and without (black bars) 1 mg/ml of ambroxol. (B) Eradication of preformed biofilm. Biofilms were cultured for 48 h and then AMB was added for another 24 h. Biofilm biomass was quantified using crystal violet. *Differences were considered significant compared with control when $p \leq 0.05$ using the Mann–Whitney *U*. The bars show the mean OD values ± standard error.

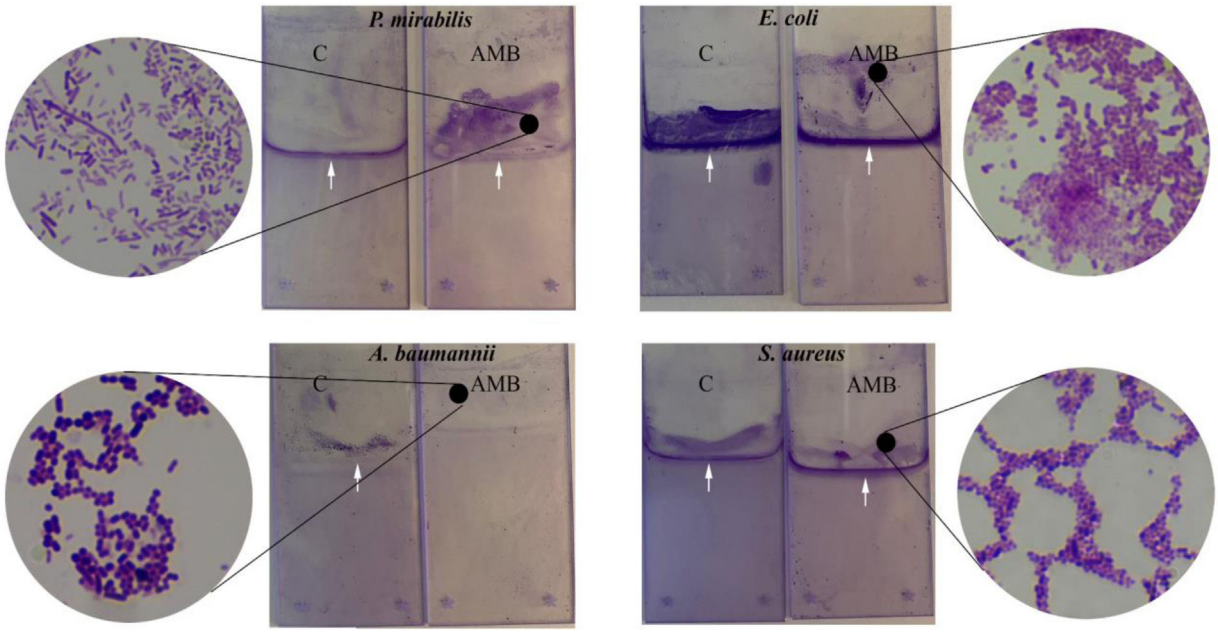

Figure 2 Biofilm formation inhibition on the glass surface. The coverslips were incubated with each strain and with or without ambroxol (AMB). The AMB-free group was the biofilm formation control (C). The subinhibitory concentrations used were 0.3 mg/ml for *E. coli* and *A. baumannii*, and 0.5 mg/ml for *P. mirabilis* and *S. aureus*. The biofilm formed on the glass was stained with crystal violet. The white arrows show the liquid–air interface zone. All strains present a higher biofilm formation compared to the AMB treatment. Representative microscope images (100× magnification) from this area are depicted on each glass.

AMB compared to the control. In the case of *P. mirabilis*, even the biomass in the interphase was lower (the violet line was reduced), interestingly bacterial migration was observed upward in the glass, and the CV stain above the interphase line (Fig. 2).

Bacterial survival after ambroxol

To quantify the percentage of viable bacteria within the biofilms treated with or without AMB, we adapted a live/dead assay. We observed that the percentage of live bacteria in the biofilm was significantly reduced with the AMB treatment (Fig. 3).

The addition of 1.0 mg/ml of AMB into the *P. mirabilis* biofilm significantly reduced viable cells by 73.9 ± 1.06% (*p*-value 0.006). Furthermore, this occurred with the *A. baumannii* biofilm, where the reduction percentage was 71.3 ± 1.38% (*p*-value 0.019). *E. coli* biofilm showed a significant reduction in viable cells of 38.3 ± 3.2%, and *S. aureus* biofilm was significantly reduced at 56.5 ± 0.9%. Representative CSLM images were taken to observe the effect of

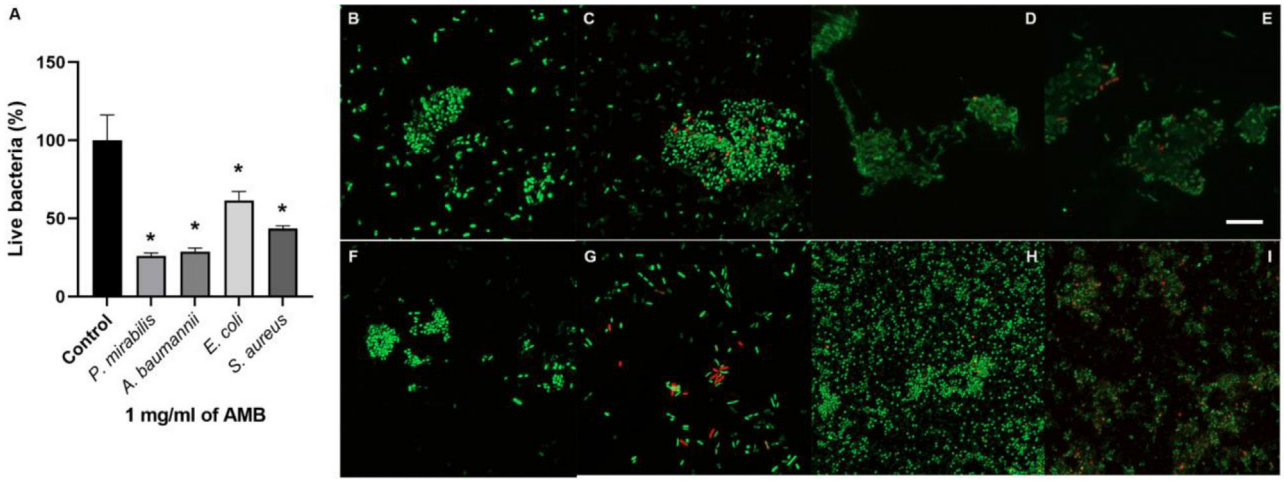

Figure 3 (A) Ambroxol (AMB) effect on bacteria viability in the biofilm quantified through live/dead fluorescent staining. AMB reduces the viable cells by more than 50% at 1 mg/ml except in *E. coli* (55%). *Differences were considered statistically significant with $p \leq 0.05$ using a one-way ANOVA test. The bars show the mean percentage values ± standard error. The panel shows images of total bacteria in green (Syto9 staining) and dead bacteria in red (propidium iodide staining). (B) *Proteus mirabilis* biofilm. (C) *P. mirabilis* biofilm with AMB. (D) *Acinetobacter baumannii* biofilm. (E) *A. baumannii* biofilm with AMB. (F) *Escherichia coli* biofilm. (G) *E. coli* biofilm with AMB. (H) *Staphylococcus aureus* biofilm. (I) *S. aureus* biofilm with AMB. Scale bar 10 μm.

ambroxol and this is depicted in Figure 3 panel. Images correlate with the result of the quantification as dead bacteria appeared when 1.0 mg/ml of AMB were added to the biofilm compared with the control without AMB. Biofilms were stained with Syto9/PI, all bacteria were observed in green color while dead bacteria appeared in red.

Ambroxol effect on efflux pumps

The effect of AMB on the efflux pumps was assayed using an EtBr accumulation assay. THR was used as an efflux pump inhibitor (EPI). The AMB concentrations tested were 0.3 and 0.5 mg/ml. AMB at 0.3 mg/ml generated an increase in cellular fluorescence accumulation suggesting a possible EPI role in this concentration, similar to the positive control. On the other hand, at a higher AMB concentration, there was an increase in the efflux transport to outside the cells that was observed as a low accumulation of EtBr inside the cells (Fig. 4A).

The effect of AMB in the expression of the efflux pumps gene in the *P. mirabilis* biofilm formation was evaluated by adding 0.6 mg/ml of AMB at the initial time. After 48 h of incubation, we removed the planktonic cells and extracted the RNA material from the biofilm. We performed a real-time PCR to quantify the gene expression of the efflux pumps, compared to the gene expression in the biofilm without AMB. We found that all tested genes were expressed in the biofilm with AMB, and there was no inhibition of gene expression. Additionally, some genes were upregulated, and the expression level was significantly increased compared to the control without AMB (Fig. 4B). This significantly increased expression was found mainly in the ABC (ABC-2: $p = 0.0413$), MSF (MSF-2: $p < 0.0001$, MSF-3: $p < 0.0001$) and RND efflux pumps family (RND-1: $p < 0.0001$, RND-2: $p = 0.0006$, and RND-3: $p = 0.0062$). In the case of other genes that encode for MATE and SMR efflux pumps, we did not observe significant variations.

Ambroxol hydrochloride effect on *P. mirabilis* swarming and swimming motility

Swimming motility was assayed by inoculating a bacterial suspension in the middle of an LB soft agar plate and incubating for 5 and 24 h. The migration distance was measured as the diameter of the bacterial growth and was compared to the control without AMB. The distance of bacterial migration with 0.5 mg/ml AMB was significantly lower compared to the control in both time points suggesting that AMB affected swimming (Fig. 5A).

Swarming motility was tested on *P. mirabilis*, adding 0.5 mg/ml of AMB to the agar. The results showed that AMB completely inhibits swarming motility (Fig. 5B).

Discussion

The search for new strategies to inhibit and eradicate microbial biofilms is on the rise. One interesting approach is the use of old compounds with potential new activities. One example of this is the repositioning of AMB, which has been widely studied for clinical use to assess its effectiveness in respiratory diseases, cystic fibrosis, hyaline membrane disease in newborns, bronchial asthma, and spastic bronchitis. Additionally, its use in antioxidant therapy, and more recently in COVID-19 treatment, is being studied[7,30]. AMB anti-biofilm properties were previously studied in *P. aeruginosa*, *C. albicans*, *S. marcescens*, *P. mirabilis*, *S. epidermidis*, and *Cryptococcus*[2,4,25,29,36,42]. AMB mechanism to reduce and eradicate bacterial biofilms still needs to be established. However, this drug affects the reversible and irreversible attachment and maturation of biofilms[9]. In addition, AMB interferes with prodigiosin production important

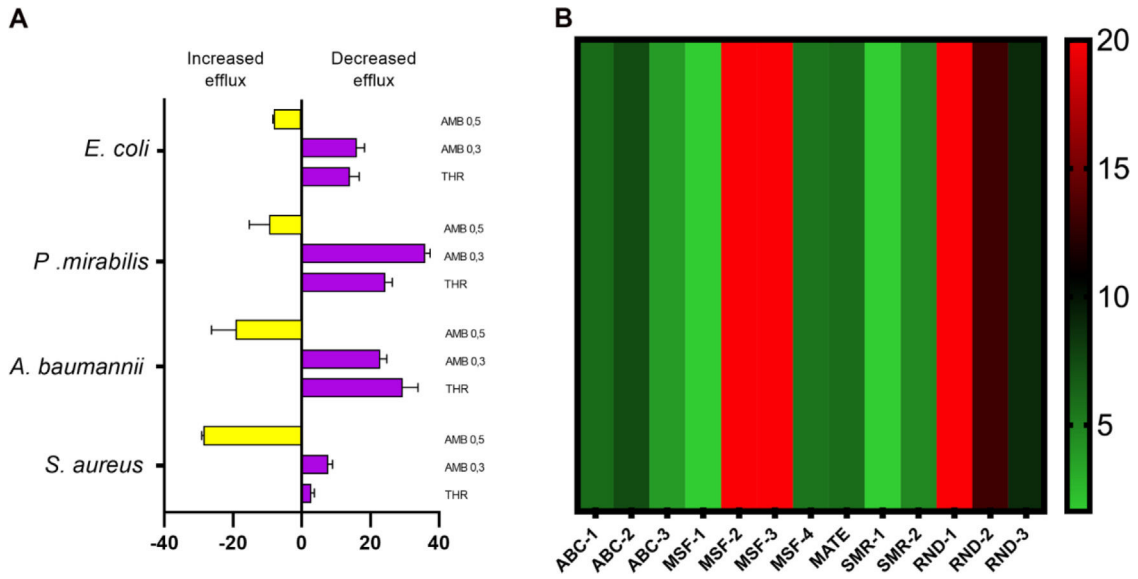

Figure 4 Effect of ambroxol (AMB) on the function and expression of efflux pumps. (A) Ethidium bromide (EtBr) accumulation assay. The percentage of EtBr accumulation inside the cells was calculated relative to the control without AMB. Thioridazine (THR) was used as a positive control of efflux inhibition. A low percentage of EtBr accumulation (yellow bars) means an increased efflux outside the bacteria. Inversely, a high percentage of accumulation inside the cells (violet bars) means a decreased efflux and a possible block in efflux pumps. (B) Expression heat map illustrating the relative expression levels of efflux pumps. Each colored cell in the heat map represents the standardized relative gene expression value for the treatment of ambroxol (0.6 mg/ml) compared with control without ambroxol. The color scale represents how much the fold change increases. The light green color shows the same gene expression with respect to the control, and the red the maximum fold change increased. ATP-binding cassette (ABC): ABC-1, ABC-2, ABC-3; major facilitator superfamily (MFS): MSF-1, MSF-2, MSF-3, MSF-4; multidrug and toxic compound extrusion (MATE); small multidrug resistance (SMR): SMR-1, SMR-2; resistance-nodulation-division family (RND): RND-1, RND-2, RND-3.

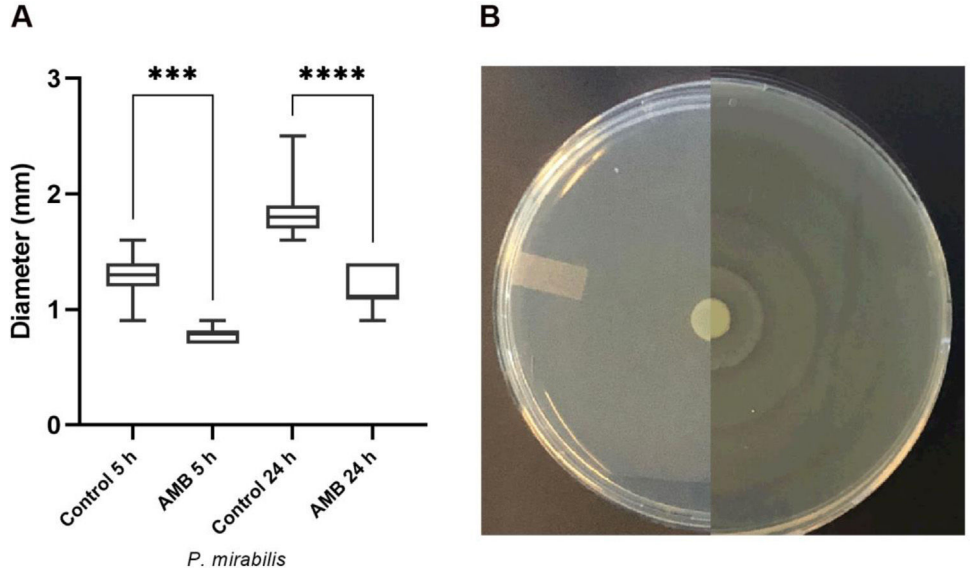

Figure 5 Ambroxol (AMB) effect on *P. mirabilis* swimming and swarming. (A) Effect over swimming motility with a subinhibitory AMB concentration at 5 and 24 h. AMB concentration tested was 0.5 mg/ml for *P. mirabilis*. The control included bacteria without AMB. ***The *p*-value was 0.0002. ****The *p*-value was <0.0001. The boxplot compares the swimming diameter values ± standard error. (B) Representative images of the *P. mirabilis* swarming assay. On the left side of the image, we showed the total inhibition of swarming motility by 0.5 mg/ml of AMB. On the right side is the normal swarming motility pattern.

in *S. marcescens* quorum sensing[2]. Cheng et al. found that AMB decreases alginate content, reducing the expression of *algD*, *algR*, and *algU* genes, and promoting alginate synthesis in *P. aeruginosa* biofilm[26]. These results showed that AMB also influences early-stage biofilms, inhibiting the synthesis of exopolysaccharides. In this regard, we found a strong and significant reduction in biofilm biomass quantification when we tested AMB activity in the inhibition of biofilm

formation. We observed an 80% decrease in biofilm biomass. These results are in accordance with the reports by Abbas et al., where AMB was able to inhibit *P. mirabilis* biofilms isolated from diabetic foot ulcers in a dose-dependent manner, the reduction percentage being more than 70%[4]. The AMB concentration used in this work was lower than the minimum inhibitory concentration (MIC) found for *P. mirabilis* and *S. aureus* (1.25 mg/ml) and higher for *E. coli* and *A. baumannii* (0.625 mg/ml). Other authors reported the MIC as 1.875 mg/ml for *P. aeruginosa*, 3.75 mg/ml for *S. marcescens*, and over 0.9 mg/ml for *P. mirabilis*[2–4]. In addition, the MIC was 1 mg/ml for *C. albicans* and 15 mg/ml for *Candida parapsilosis*[35,36]. For *P. aeruginosa* PAO1 the MIC was 0.5 mg/ml and for oral bacteria indicators such as *Streptococcus mutans* was also 0.5 mg/ml[8,28]. The above suggests that the MIC varies between bacterial genera and species.

The ability of AMB to inhibit biofilm formation can be used to prevent surface colonization. Hafez et al. found that AMB decreased the adhesion to biotic surfaces, such as Vero and Hep-2 cells[19]. The minimum concentration for inhibiting bacterial adherence to cells was 2.5 ng/ml. AMB effectively inhibited adhesion when the eukaryotic cells were previously exposed to the drug, whereas it was ineffective when bacteria were pretreated with it[19]. Therefore, AMB could interact with a cell membrane receptor preventing bacterial adhesion.

AMB has been described as a biofilm disruptor, able to destroy the structure of mature biofilms. For example, a seven-day-old biofilm of *P. aeruginosa* was disrupted with 3.75 mg/ml of AMB[26]. In our work, AMB was able to disrupt the preformed biofilm even at lower concentrations such as 0.8 and 0.6 mg/ml in 24 h. Generally, the minimal biofilm eradication concentration (MBEC) is greater than the MIC. The MBEC is defined as the least concentration of AMB that showed no growth when the bacteria in the biofilm are scrapped and inoculated in TSA[1]. For example, Abbas et al. found that the MBEC for *P. mirabilis*, E. *coli*, and *A. baumannii* was 0.94 mg/ml, while the MIC was 0.47 mg/ml in 24 h mature biofilms[1]. For *S. aureus* the MIC and the MBEC had the same value, being 0.47 mg/ml[1]. Our results showed that MBEC was lower than MIC, with a complete eradication of the biofilm with 1 mg/ml of AMB. Moreover, AMB reduced biofilm biomass in a dose-dependent manner, as it was reported in previous works[2,8,36]. Moreover, AMB can reduce the viability of at least 50% of the bacteria inside the biofilm. This suggests that AMB can penetrate the biofilm and kill the bacteria inside. However, considering the differences in the reported MIC values and that viable bacteria could escape the biofilm to colonize another surface, concentrations should be carefully selected at least to kill planktonic cells and eradicate biofilms.

One of the resistance mechanisms to antimicrobials is the overexpression of efflux pumps. Bacterial efflux pumps are membrane proteins that have the function to export substances outside bacteria. The efflux pumps are also involved in biofilm formation[5]. In this work, we found that all the efflux pump genes tested in *P. mirabilis* were expressed in the biofilm with AMB, and there was no inhibition of gene expression. Even more, we found an overexpression in some of those genes that encode efflux pumps from *P. mirabilis*, compared to the expression in the biofilm without AMB. This suggests that in response to AMB presence, bacteria increase ABC, MFS, and RND efflux pump gene expression. This differs from Pulcrano et al. who found a significant decrease in MDR and CDR efflux pump gene expression in *C. parapsilosis* biofilm, when treating the biofilms with AMB, compared to the biofilm without AMB[35]. Even though AMB inhibits the gene expression of these efflux pumps, this system can be different between a fungus and gram negative bacteria. The increase in gene expression could be a mechanism to export different molecules that contribute to biofilm formation, in response to the presence of AMB. This effect can also be explained by the AMB concentration used in this work. One of the most astonishing efflux pump families is RND as they can extrude a wide range of substrates from antibiotics, dyes, detergents, and metals, to bacterial metabolites[31]. The common feature of the substrates of this RND pump is the amphiphilic nature of the compounds. Interestingly, ambroxol has been reported as an amphiphilic compound[16]. Taking into consideration the chemical nature of AMB and our results, we can strongly suggest that ambroxol is the substrate of RND efflux pumps at least in *P. mirabilis*.

We found that in all the strains evaluated, a concentration of 0.5 mg/ml stimulated the efflux of EtBr outside bacteria. This can be related to the overexpression of efflux pump genes. On the other hand, when we use a lower concentration of 0.3 mg/ml, the effect is the opposite, and the efflux is blocked by the AMB, so at this concentration it could have an EPI role. The effect over the efflux pumps appears to be dual and dose-dependent. At low concentrations AMB functions as an EPI and at higher concentrations it can activate efflux pumps.

Bacterial motility, such as swarming and swimming, is relevant for biofilm formation and virulence in several pathogens. Motility and particularly swarming are considered to be important in the initial colonization of surfaces and the early stages of biofilm formation. In this work, we found that AMB blocks the swarming and swimming motilities of *P. mirabilis*, as also found by Abbas et al. in the isolation of *P. mirabilis* from a diabetic foot infection[4].

Conclusion

As mentioned earlier, all the above suggests that AMB is capable of disrupting several biofilm stages. First, it can interfere with bacterial adhesion to surfaces and inhibit swarming and swimming motility. It can also reduce the total biomass, probably affecting the extracellular matrix, alter efflux pump expression, and kill bacteria inside the biofilm.

Several authors show the synergistic effect of AMB when used with a clinical antimicrobial, such as ciprofloxacin or vancomycin, enhancing the antibiotic effect on the biofilm. The use of AMB in conjunction with other antimicrobials is a possible strategy to improve the effectiveness of antibiotics. However, the concentration of AMB should be taken into consideration.

CRediT authorship contribution statement

M.J. González: Investigation, visualization, writing-original draft. M. Lain: Investigation. V. Iribarnegaray: Investigation, visualization. L. Robino: Conceptualization, writing-review

& editing. P. Scavone: Conceptualization, writing-review & editing.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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