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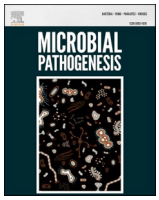

Induction of biofilm in extended-spectrum beta-lactamase *Staphylococcus aureus* with drugs commonly used in pharmacotherapy

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ABSTRACT

Staphylococcus aureus is a bacterial pathogen that causes bloodstream infections, pneumonia, and skin abscesses and is the primary pathogen responsible for medical devices associated with biofilm infections, accounting for approximately 70 % of cases. Therefore, the World Health Organization (WHO) has designated this microorganism as a top priority due to its role in causing over 20,000 bacteremia-related deaths in the US each year. The issue of pathogen resistance to antibiotics, mainly by a biofilm, further complicates these infections since biofilms render the bacterial colony impervious to antibiotics. However, many natural and synthetic substances also induce bacterial biofilm formation. Therefore, we investigated whether the most common active pharmaceutical ingredients (APIs) could induce biofilm formation in two clinical isolates of extended-spectrum beta-lactamase *Staphylococcus aureus,* one of them also methicillin-resistant (A2M) and two medical devices.

We detected biofilm inducers, inhibitors, and destabilizers. Microbial strain, medical devices, API structure, and concentration influenced the modulatory effects of biofilm. In all devices tested, including microplates, FR18 duodenal probe, and respiratory probe, the clinic isolate methicillin-resistant *S. aureus* A2M exhibited lower susceptibility to biofilm formation than *S. aureus* A1. The anti-inflammatory acetaminophen, the hypocholesterolemic lovastatin, and the diuretic hydrochlorothiazide all induced biofilm. However, verapamil, an antihypertensive, and cetirizine, an antihistamine, inhibited biofilm on *S. aureus* A2M, while propranolol, another antihypertensive, inhibited biofilm on *S. aureus* A1. Additionally, diclofenac, an analgesic, and cetirizine destabilized the biofilm, resulting in more free bacteria and possibly making them more susceptible to external agents such as antibiotics. Nonetheless, further epidemiologic analyses and *in vivo* assays are needed to confirm these findings and to establish a correlation between drug use, the onset of bacterial infections in patients, and the use of medical devices.

This work provides information about the probable clinical implications of drugs in patients using medical devices or undergoing surgical procedures. Inhibitory APIs could also be used as drug repurposing or templates to design new, more potent biofilm inhibitors.

1. Introduction

Staphylococcus aureus is a Gram(+) pathogen that can infect the bloodstream, skin, soft tissues, and respiratory tract. It is one of the main causative agents of nosocomial and community infections [1] associated with medical device invasion and severe diseases such as endocarditis and osteomyelitis [2]. Furthermore, it is a penicillin and methicillin-resistant bacteria (MRSA) with high mortality rates [3]. In the European Union, approximately 25,000 deaths have been reported due to resistant bacteria, including *S. aureus* MRSA [4]. It is also resistant to vancomycin, linezolid, and daptomycin. These antibiotics are the last choice for treating these infections [1]. Bacterial resistance is considered a public health problem worldwide because it increases the length of hospital stays for affected patients and costs to the healthcare system. Several factors can generate this resistance, one of the most important being the formation of a biofilm. The biofilm is a heterogeneous structure formed by an extracellular polymeric matrix composed of polysaccharides, proteins, enzymes, and bacterial DNA [5]. This structure

Abbreviations: API, Active Pharmaceutical Ingredients; ESBL, Extended-spectrum beta-lactamase; *S. aureus* A1, *Staphylococcus aureus* β-lactamase positive; *S.* aureus A2M, *Staphylococcus aureus* β-lactamase positive and methicillin-resistant; WHO, World Health Organization; MRSA, Methicillin-resistant *Staphylococcus aureus*; IPS, *Institución Prestadora de Salud*; CFU, Colony-forming unit.

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acts as a barrier that allows the pathogen to persist in host tissues or implanted materials such as catheters [6], probes, prostheses, and valves, among others, becoming unmanageable and challenging to eradicate [7].

Biofilm formation is part of microbial communication called Quorum Sensing, which is modulated in Gram-negative bacteria mainly by autoinducer peptides [8]. However, many natural and synthetic products have also been shown to induce this modulatory effect [9]. People are exposed to many types of chemicals in food, beverages, cosmetics, and even drugs that could induce biofilm and recurrent infections due to persister bacterial cells. All this could constitute a risk to human health, especially for elderly, immunosuppressed, or surgical patients. For example, ibuprofen and acetaminophen have already been reported to induce bacterial resistance [10]. Therefore, using two medical devices, we studied the biofilm-inducing potential of 12 common Active Pharmaceutical Ingredients (APIs) and mixtures in two clinical isolates of *S. aureus*-ESLB, one of them methicillin-resistant (A2M). Moreover, the presence of persister cells and biofilm morphology were also analyzed by microscopy.

2. Materials & methods

2.1. Chemical and reagents

The active pharmaceutical ingredients (APIs) naproxen, diclofenac, acetaminophen, ibuprofen, metformin, lovastatin, loratadine, propranolol, hydrochlorothiazide, verapamil, captopril, and cetirizine were obtained following procedures published by the United States Pharmacopeia [11] and identified by NMR and mass spectrometry.

2.2. Clinic bacterial isolates

Clinical isolates of *Staphylococcus aureus* were donated by the "Institución Prestadora de Salud" (IPS) Universitaria, Medellín, Colombia. They were cultured on Baird-Parker and nutrient agar, followed by incubation at 37 °C for 24 h. Bacterial inocula were prepared in sterile saline by superficial scraping with a microbiological loop, and the bacterial inoculum was quantified spectrophotometrically at a wavelength of 600 nm using sterile saline as a blank. The absorbance of the inoculum was adjusted to an optical density of 0.05 to give a final cell density of 1.0×10^6 CFU/ml.

The antibiotic resistance profile was determined using the automated VITEK 2 system (BioMérieux®, Marcy I'Etoile, France) at the Institución Prestadora de Salud (IPS), with a bacterial inoculum adjusted to the MacFarland scale (0.50–0.63). Antibiotics routinely used to determine the resistance profile included ceftaroline, ciprofloxacin, clindamycin, daptomycin, erythromycin, levofloxacin, linezolid, nitrofurantoin, oxacillin, penicillin G, rifampicin, tetracycline, trimethoprim/sulfamethoxazole, vancomycin, and detection of beta-lactamase, cefoxitin, and inducible clindamycin resistance.

2.3. Determination of the minimum inhibitory concentration of the APIs

Clinical isolates of *S. aureus* A1 and A2M were grown on Baird-Parker agar overnight at 37 °C. The standard broth dilution method determined minimum inhibitory concentrations (MICs) [12]. A culture of *S. aureus* at a cell density of 1.0×10^6 CFU/ml was exposed to different concentrations of drugs in 96-well microplates (Merck, St. Louis, Mo., USA) and then incubated at 37 °C for 20 h. Quantification was performed spectrophotometrically at a wavelength of 600 nm. The blank and growth control consisted of wells without bacteria or drugs.

2.4. Assessment of biofilm formation

Biofilm formation under static conditions was performed in 96-well plates and on 0.5 cm^2 FR18 duodenal and respiratory probes purchased from a local pharmacy. The plates contained sterile saline and LB medium containing the active ingredients of the drugs at concentrations of 50-5 μM. The plates were then incubated at 37 °C for 24 h. After incubation, the wells and medical probes were washed three times with sterile saline. The biofilm associated with the wells and medical probes was treated with 0.05 % crystal violet and eluted with 200 μl of ethanol. Crystal violet was quantified by spectrophotometry at a wavelength of 575 nm. Wells and duodenal probes without bacteria or drugs were used as blank and growth control, respectively. Results were normalized to the biofilm in the growth control, representing 100 %.

2.5. Effect of a mixture of APIs on S. aureus biofilm

A culture of *S. aureus* A1 and A2M at a cell density of 1.0×10^6 colony forming units (CFU/ml) was exposed to a mixture of drugs in 96-well plates. *S. aureus* A1 was incubated with a mixture of propranolol at 50 μM with hydrochlorothiazide, naproxen, metformin, and diclofenac at concentrations of 50-5 μM prepared in LB medium. On the other hand, for *S aureus* A2, the mixture consisted of diclofenac at 50 μM with loratadine, captopril, metformin, and acetaminophen at concentrations of 50-5 μM. The plates were incubated at 37 °C for 24 h. The wells were then washed three times with sterile saline. Biofilm was treated as described above.

2.6. Effect on S. aureus persister cells

Cruz et al., 2018 [13] followed the protocol previously described with some modifications. A bacterial inoculum of *S. aureus* A2M adjusted to a cell density of 1.0×10^6 CFU/ml, was cultured in LB broth in 96-well plates at 37 °C for 20 h. The supernatant was discarded, and the resulting biofilm was washed thrice with 200 μl of sterile saline. To the biofilm, 200 μl of drugs at a concentration of 25 μM dissolved in LB broth were added and incubated at 37 °C for 4 h. The supernatant was collected, and the bacteria in the biofilm were suspended in 200 μL of LB broth by vigorous pipetting and scraping of the surface. Both bacterial suspensions were transferred to a new 96-well microplate. In both cases, a tenfold serial dilution was made in sterile saline, and the dilutions were plated onto the surface of nutrient agar. Plates were incubated at 37 °C for 24 h, and colonies were counted. Assays were performed with two replicates of independent cultures.

2.7. Biofilm analysis by scanning electron microscopy (SEM)

Small pieces (0.5 cm^2) of duodenal and respiratory probes were placed in the wells of a 96-well microplate. Then, 100 μL of bacterial inoculum with a final cell density of 1.0×10^6 CFU/ml of *S. aureus* A2M and 100 μL of drugs prepared in LB broth at defined concentrations were added and incubated at 37 °C for 24 h. The probes were then rinsed three times with 200 μL of sterile saline and placed in 1.5 ml Eppendorf tubes for fixation with 1 mL of 2.5 % glutaraldehyde. After fixation, they were dried with CO2 and coated with gold for visualization by scanning electron microscopy (JEOL JSM-6490 L variable-pressure SEM, Akishima Tokyo, Japan). Probes with bacterial inoculum, no treatment, and probes without bacterial inoculum were used as controls.

2.8. Statistical analysis

Biofilm formation data were expressed as mean and standard deviation (SD). All results showed a normal distribution and homoscedasticity, so differences were evaluated by one-way ANOVA. The experiments with persister cells were assessed using an unpaired two-tailed *t*-test. A significance level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Minimum inhibitory concentration

The results indicated that loratadine was the only API that inhibited the growth of *S. aureus* A1, with a MIC <25 μM. Conversely, the majority of drugs induced the growth of *S. aureus* A2M, with naproxen, diclofenac, acetaminophen, and ibuprofen at 50 μM showing significant growth induction (p-value <0.0001) of 53 %, 63 %, 42 %, and 36 %, respectively, while the growth of *S. aureus* A1 was unaffected by APIs in the 50-5 μM concentration range, with bacterial growth rates varying from 85 to 115 %. At the same concentration, lovastatin increased growth by 58 %, propranolol by 32 %, and loratadine by 41 % at 5 μM. The antibiogram revealed the presence of β-lactamase in both clinical isolates. However, *S. aureus* A1 exhibited higher antibiotic susceptibility and was only resistant to penicillin G. In contrast, *S. aureus* A2M was resistant to methicillin, benzylpenicillin, erythromycin, oxacillin, and tetracycline and tested positive for cefoxitin (Supplementary Material #1).

3.2. Effect of APIs on S. aureus biofilm formation in 96-well plates

In microplate wells, most APIs increased biofilm formation on the clinical isolate *S. aureus* A1. So, loratadine exhibited a strong induction of biofilm formation (109 %) at 5 μM, while ibuprofen showed an induction of 74 % at the same concentration. Acetaminophen promoted biofilm formation by up to 57 % at all concentrations tested (Fig. 1).

Only four active pharmaceutical ingredients (APIs) (Fig. 2) induced the biofilm of the clinic isolate *S. aureus* A2M. Diclofenac significantly increased the biofilm by 119 % at 50 μM, while naproxen induced a 61 % increase at the same concentration. Acetaminophen increased the biofilm at all concentrations up to 44 %.

3.3. Effect of drugs on S. aureus biofilm formation in the duodenal probe

The biofilm of *S. aureus* A1 isolate formed in duodenal probe FR18 was promoted by lovastatin, loratadine, verapamil, and captopril, finding induction effects up to 80 %, only naproxen decreased the biofilm by 33 % at 5 μM (Fig. 3).

Ibuprofen, lovastatin, hydrochlorothiazide, and verapamil induced the biofilm of *S. aureus* A2M formed in the duodenal probe FR18, resulting in effects of up to 92 % (Fig. 4).

3.4. Effect of drugs on the formation of S. aureus biofilm in respiratory probes

Acetaminophen and hydrochlorothiazide promoted the formation of *S. aureus* A1 biofilm on the respiratory probes by 48 % and 54 %, respectively, at a concentration of 50 μM. In contrast, propranolol inhibited biofilm formation by up to 36 % at all concentrations evaluated (Fig. 5).

The biofilm formed by *S. aureus* A2M in the respiratory probe was enhanced by 31 % with a 5 μM acetaminophen concentration, while captopril was promoted by 38 % at 50 μM. Verapamil and cetirizine showed inhibitory effects, with a 21 % and 40 % reduction in biofilm, respectively (see Fig. 6).

3.5. The effect of drug mixtures on biofilm formation of S. aureus A1 on different medical devices

The drug combination enhanced the biofilm of both bacterial clinic isolates. Specifically, the mixture of propranolol at 50 μM and hydrochlorothiazide increased the biofilm formed in 96-well plates by up to 263 %. Additionally, naproxen and diclofenac showed a significant increase of 115 % and 68 %, respectively (Fig. 7).

The hydrochlorothiazide and naproxen mixture's inductive effect was maintained at 35 % on FR18 duodenal probes, which was lower

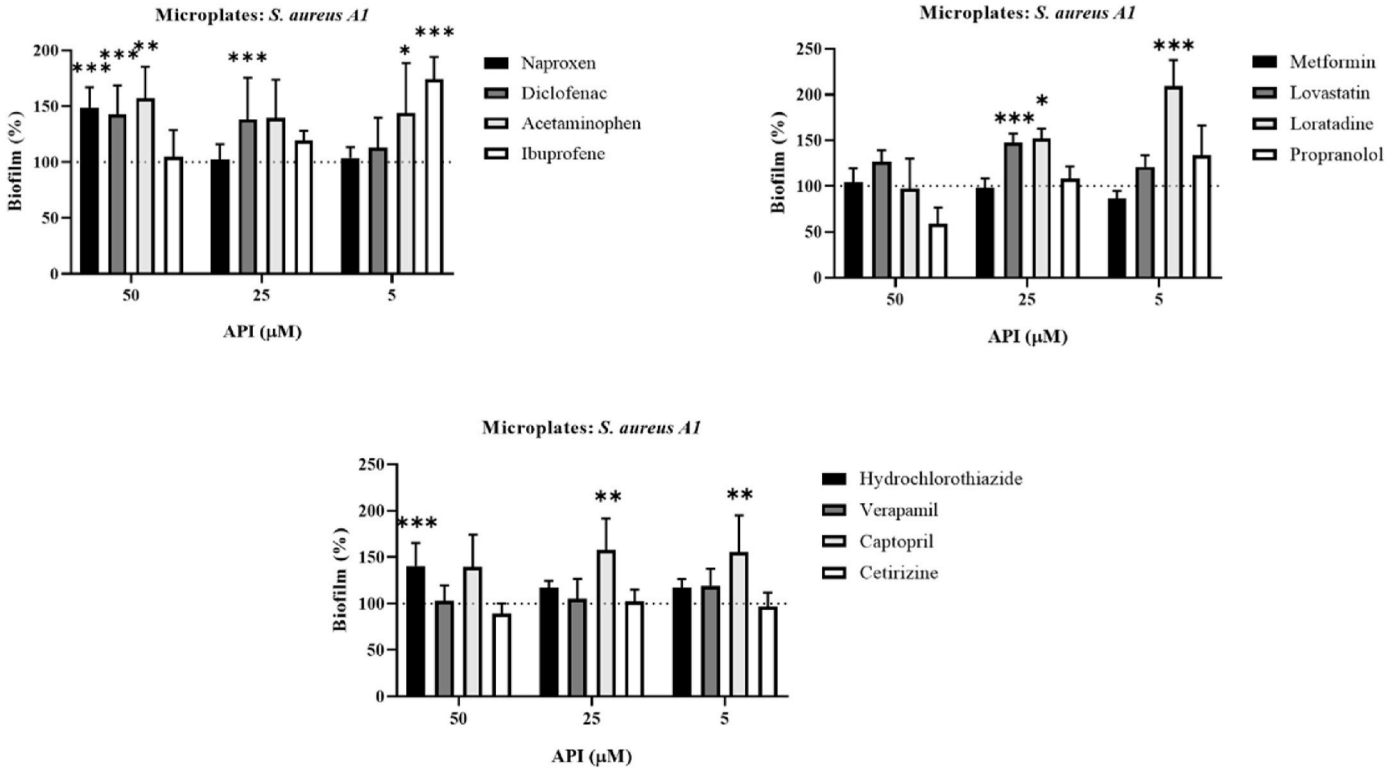

Fig. 1. Biofilm formation of *S. aureus* A1 in 96-well plates treated with different APIs at 50, 25, and 5 μM concentrations. Data represent the mean and standard deviation (±SD), with statistical significance indicated by asterisks: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

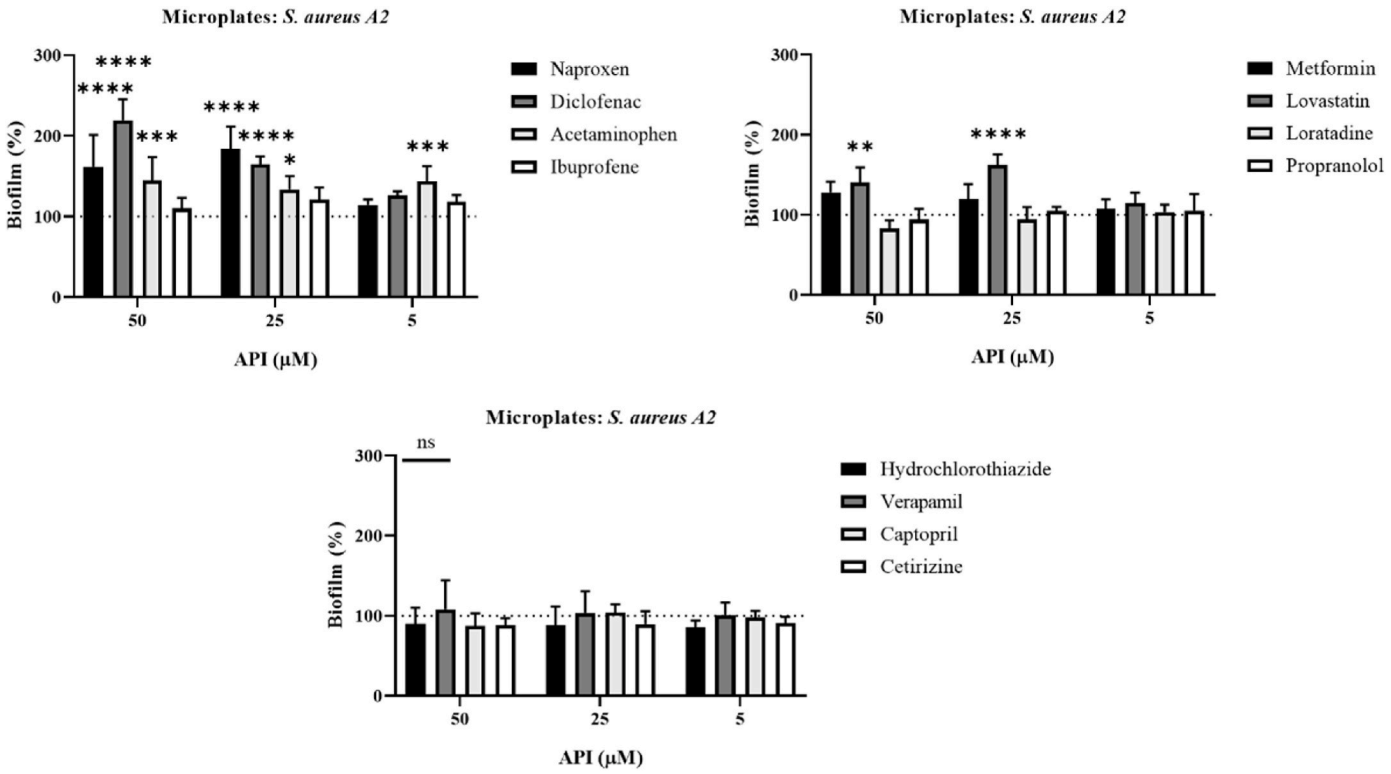

Fig. 2. Biofilm formation in *S. aureus* A2M in 96-well plates treated with different APIs at 50, 25, and 5 μM concentrations. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

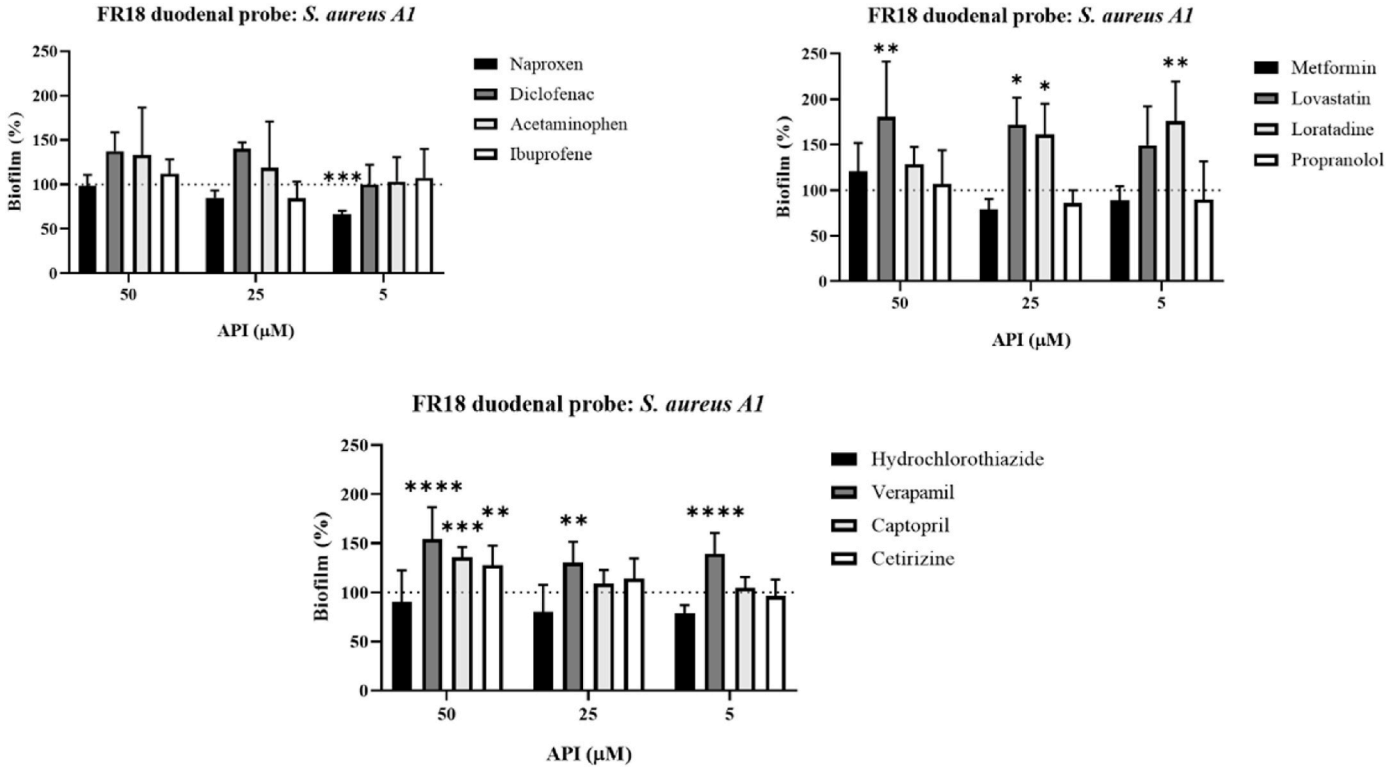

Fig. 3. Biofilm formation in *S. aureus* A1 in duodenal probe FR18 treated with different APIs at 50, 25, and 5 μM concentrations. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

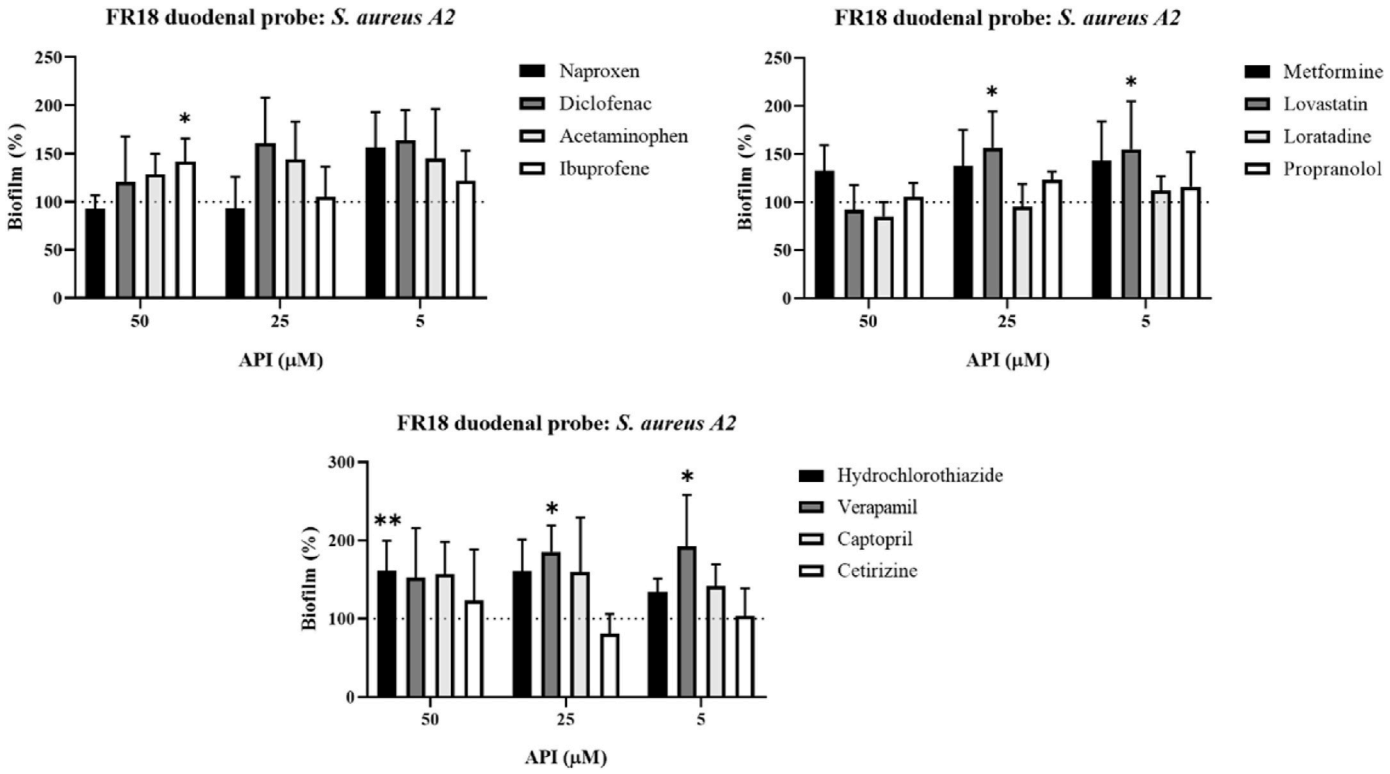

Fig. 4. Biofilm formation of *S. aureus* A2M in duodenal probe FR18 treated with different APIs at 50, 25, and 5 μM concentrations. Data represent the mean and standard deviation ± SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

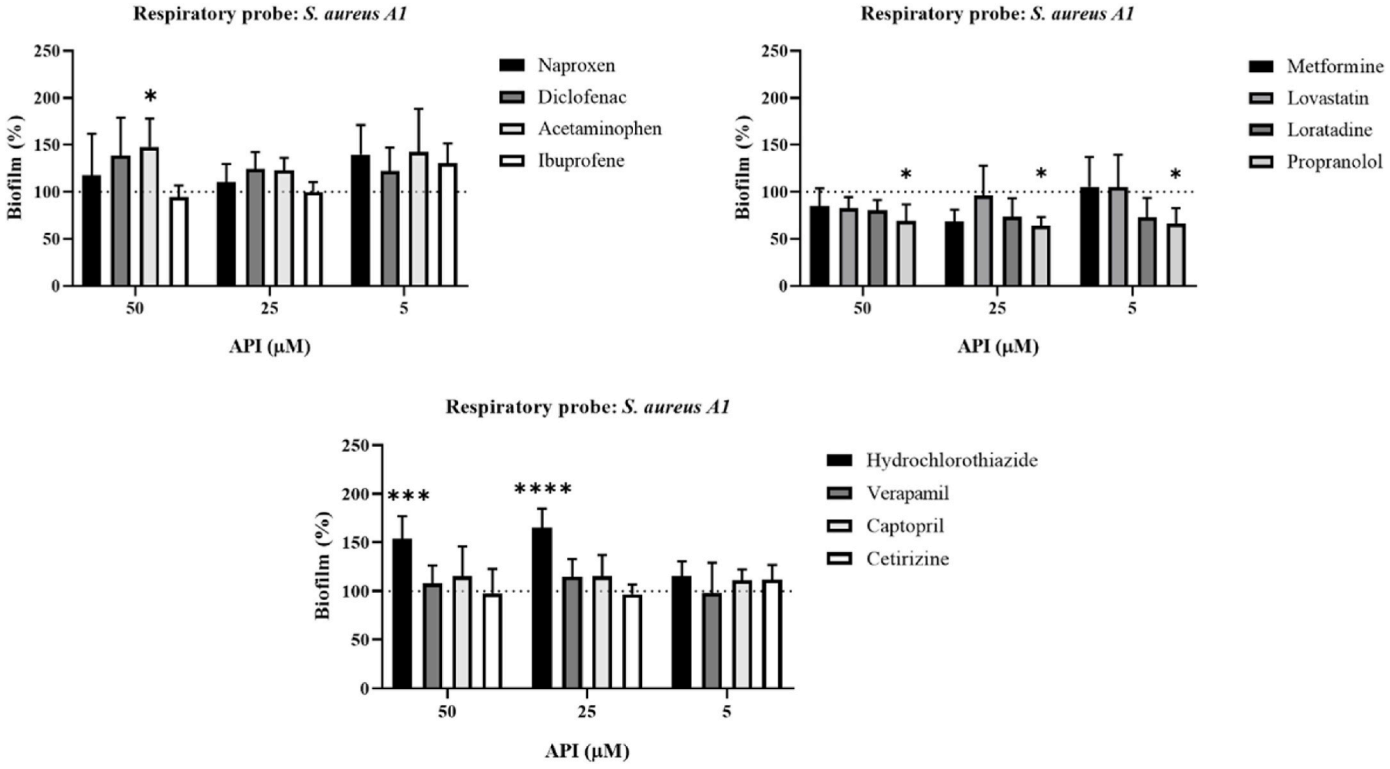

Fig. 5. Biofilm of *S. aureus* A1 formed in a respiratory probe treated with different APIs at 50, 25, and 5 μM. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

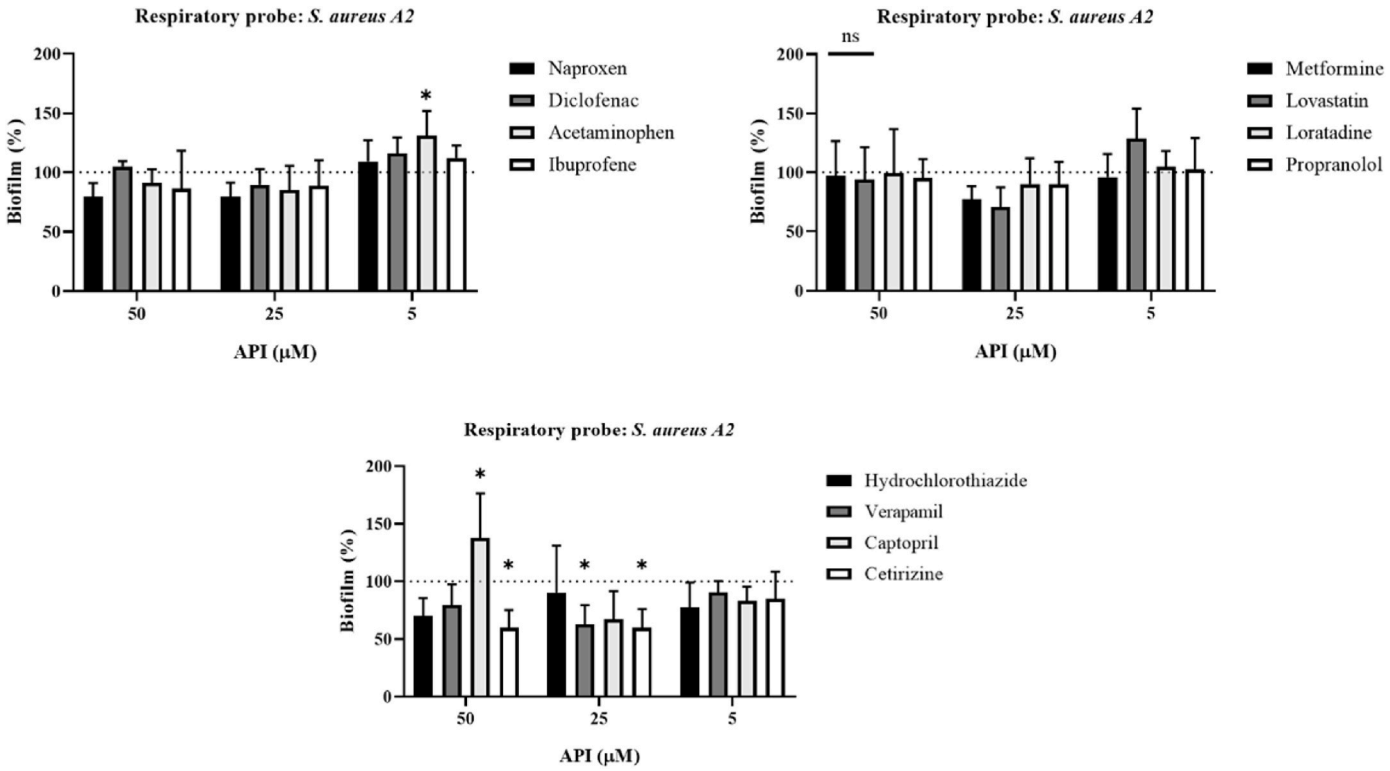

Fig. 6. *S. aureus* A2M biofilm formed on respiratory probes with APIS at 50, 25, and 5 μM concentrations. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

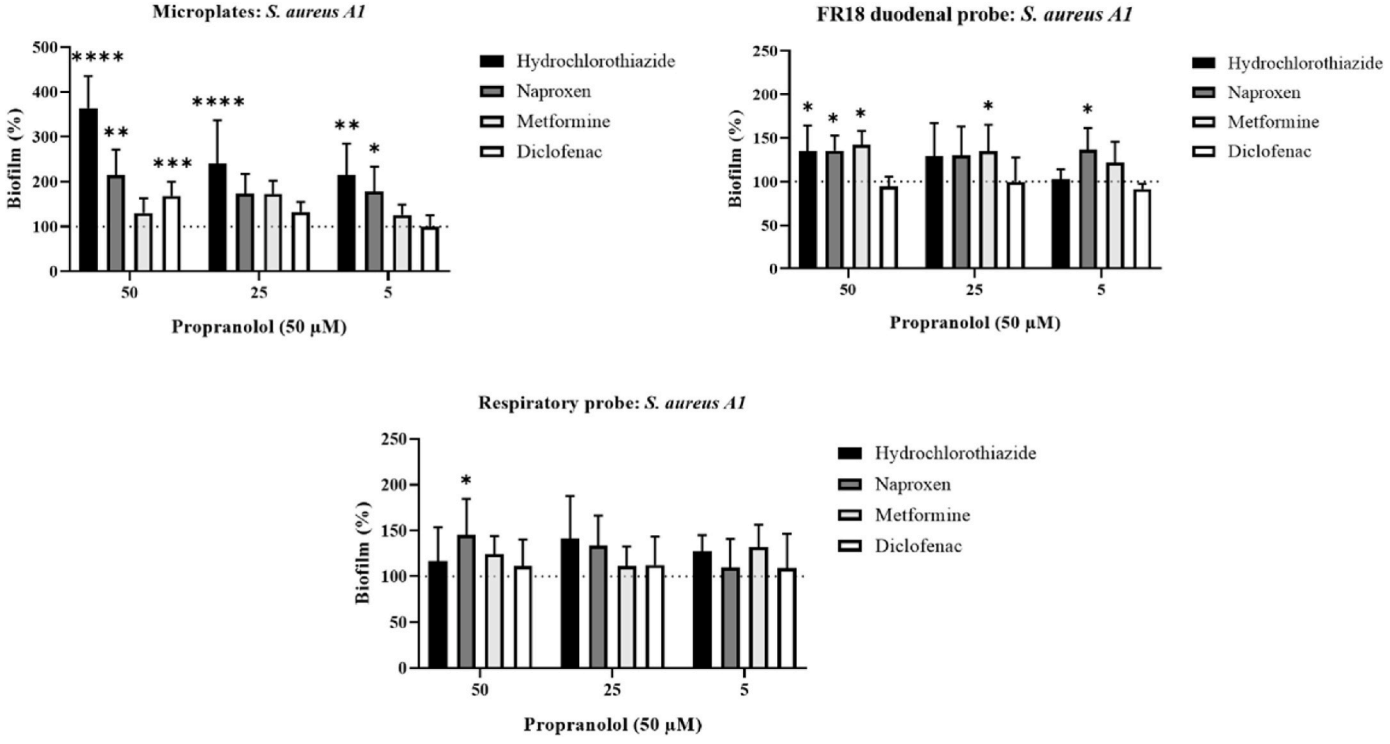

Fig. 7. Biofilm formed in 96-well plates, FR18 duodenal probe, and respiratory probe in *S. aureus* A1 treated with propranolol at 50 μM and mixtures with hydrochlorothiazide, naproxen, metformin, and diclofenac at 50, 25 and 5 μM. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

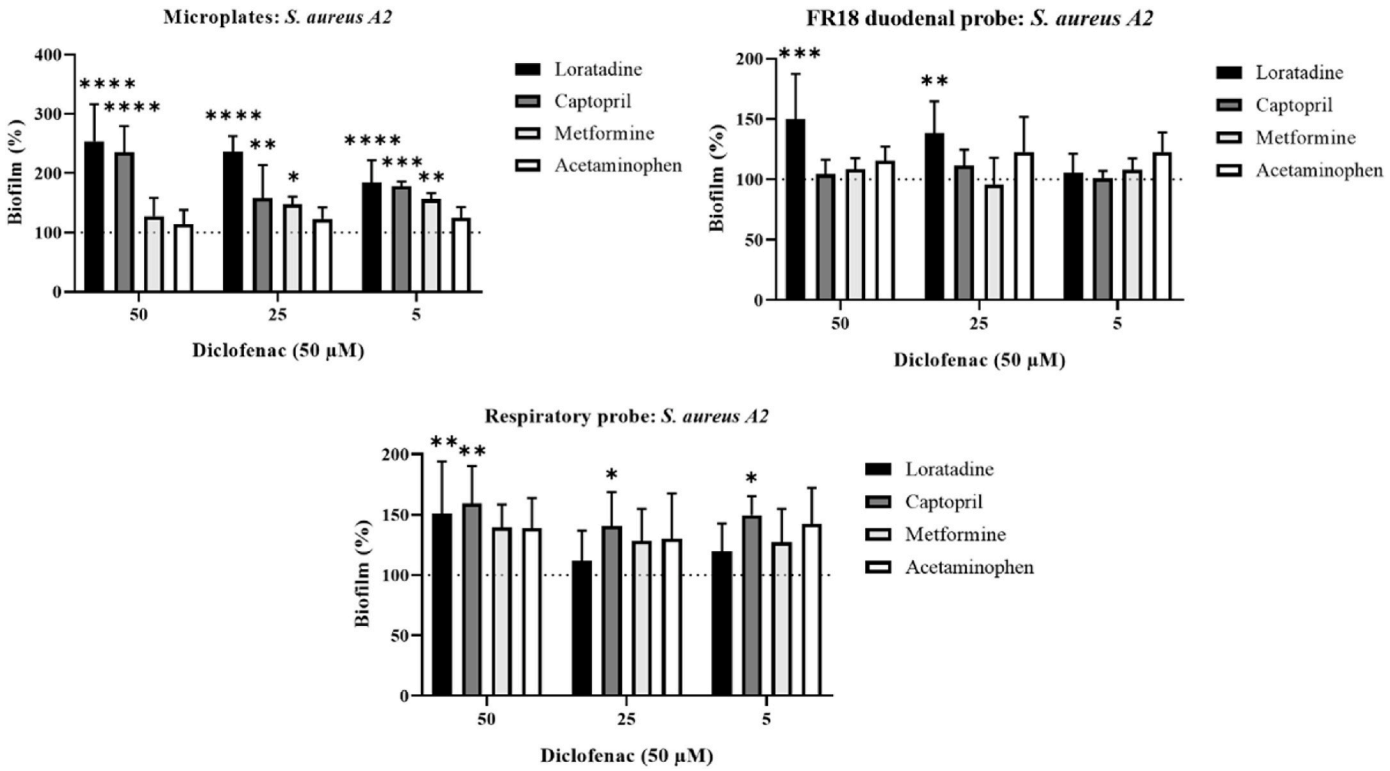

Fig. 8. Biofilm formed in 96-well plates, FR18 duodenal probe, and respiratory probe in *S. aureus* A2M treated with diclofenac at 50 µM and mixtures with loratadine, captopril, metformin, and acetaminophen at 50, 25 and 5 µM. Data represent the mean and standard deviation (±SD), ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

than the effect observed in 96-well plates. On this surface, the mixture of propranolol and metformin increased the biofilm by 42 %, while diclofenac was inactive. However, the biofilm of *S. aureus* A1 in respiratory probes increased by 45 % with the mixture of propranolol and naproxen.

Regarding *S. aureus* A2M in microplates, significant increases were observed with the mixtures of diclofenac and loratadine (152 %) and captopril (135 %), and to a lesser extent with metformin (56 %) at the lowest concentration (Fig. 8).

However, in the FR18 probe, this effect was only significant with loratadine at the two highest concentrations of 50 and 25 µM (50 % and 38 %, respectively). In the respiratory probe, captopril was the most effective biofilm promoter at all concentrations, with increases of up to 59 %. At the highest concentration, loratadine also showed a high % induction effect of 51 %

3.6. Effect on persister cells in S. aureus A2M

Persister cells were determined by CFU counting from a 24-h biofilm (Fig. 9). Most drugs at 25 µM did not change the free-to-embedded cells ratio in the biofilm, except for diclofenac and cetirizine, for which there was a 100-fold difference in the cell ratio.

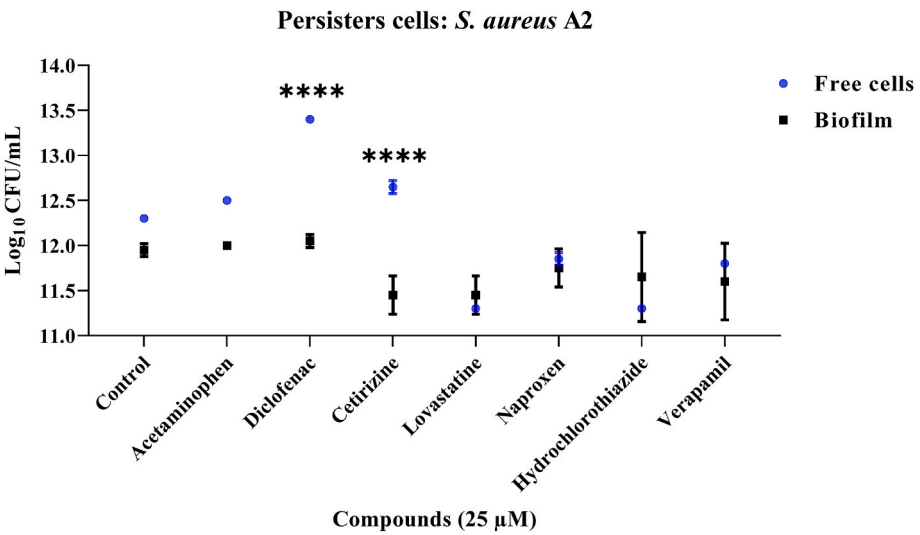

Fig. 9. *S. aureus* A2M persister cells treated with APIs at a concentration of 25 µM. Data represent the mean and standard deviation (±SD), ****p < 0.0001.

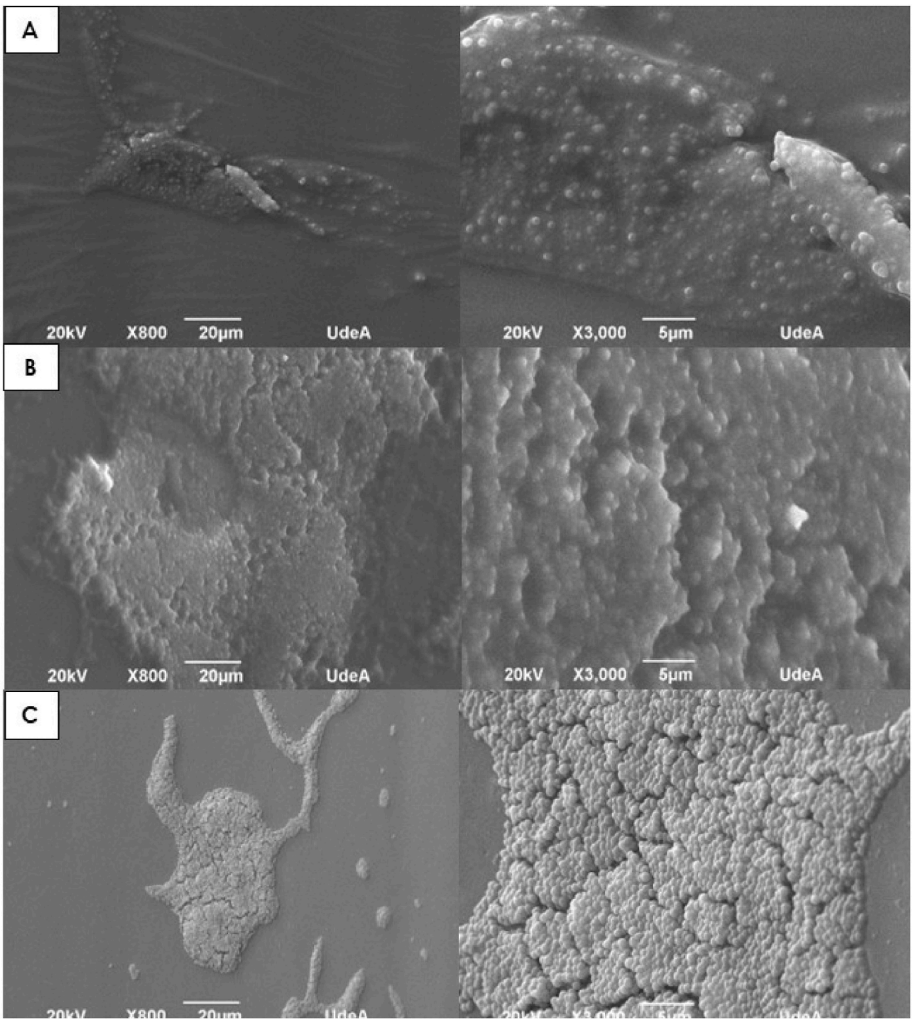

Fig. 10. Effect of APIs on *S. aureus* A2M biofilm in a FR18 probe A. Control experiment. B. With the addition of verapamil at 25 µM in FR18 (induction). C. With lovastatin at 5 µM.

3.7. SEM analysis of S. aureus A2M biofilm formed on medical devices

SEM analysis showed that verapamil increased biofilm production on the FR18 duodenal probe, resulting in a denser structure and greater secretion of the extracellular polymeric matrix (Fig. 10B) compared to the untreated control (Fig. 10A). On the other hand, lovastatin resulted in even more significant biofilm production, with a larger colonized surface and a more compact structure (Fig. 10C).

S. aureus A2M formed an isolated biofilm when treated with cetirizine (Fig. 11B) compared to the untreated control (Fig. 11A). However, a better-structured biofilm was observed when treated with acetaminophen, with a larger colonized surface area and extracellular polymeric matrix (Fig. 11C).

4. Discussion

S. aureus is the primary pathogen responsible for catheter-associated biofilm infections, accounting for approximately 70 % of cases [14]. The World Health Organization (WHO) has designated this microorganism as a top priority due to its role in causing over 20,000 bacteremia-related deaths in the US each year [15,16]. Infections caused by this pathogen are commonly associated with medical devices or implants, such as prostheses, duodenal, and respiratory catheters. These devices facilitate bacterial adhesion, forming an antibiotic-resistant biofilm and making it an ideal surface for infection [17]. Thus, intravascular catheters commonly cause bloodstream infections [17]. The issue of pathogen resistance to antibiotics, mainly by a biofilm, further complicates these infections. Methicillin-resistant *S. aureus* MRSA infections are resistant to various antibiotics, including β-lactams, aminoglycosides, quinolones, and macrolides [18], and a significant concern at the clinical level due to their high morbidity and mortality rates [19].

Biofilm formation is a significant cause of bacterial resistance, rendering most antibiotics ineffective and responsible for nosocomial and recurrent infections, and its production is driven by a complex biochemical process involved in microbial communication called quorum sensing, which is related to bacterial virulence. Several natural and synthetic molecules have been shown to modulate this communication and biofilm production. However, the best known are the antibiotics themselves, such as ampicillin, β-lactam antibiotics, and aminoglycosides, among others, at low concentrations [20]. The same effect on QS could explain the generation of resistance and the very different outcomes when treating a biofilm infection from medical devices with antibiotics [21].

Thus, daily exposure to food, beverages, cosmetics, and even drugs poses a significant microbial risk because these substances may also be inducers of quorum sensing and biofilm. It is important to recognize this biofilm-modulating effect of exogenous molecules such as drugs to avoid their consumption or contact, to rationalize drug therapies, and to design new inhibitory molecules based on their structure. Therefore, this study investigated the effect of 12 commonly used APIs on biofilm

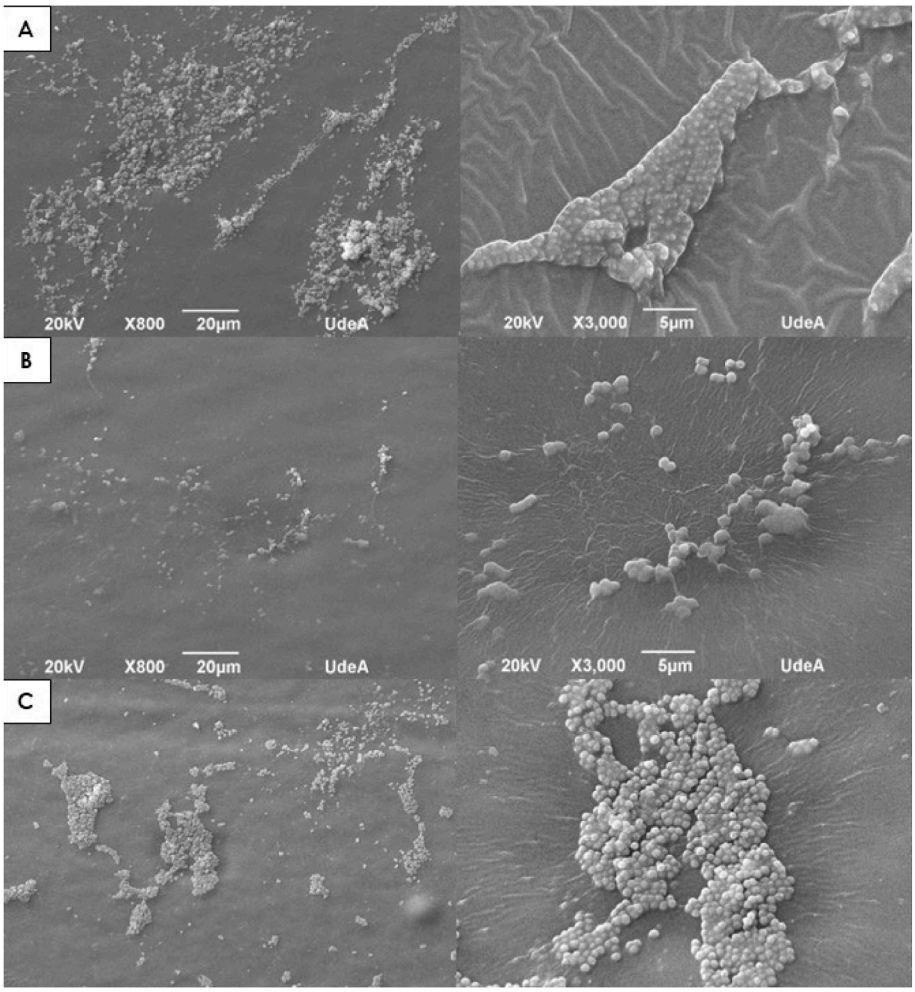

Fig. 11. Effect of drugs on biofilm *S. aureus* A2M in a respiratory probe A. Control experiment. B. With cetirizine (biofilm inhibitor) C. With acetaminophen at 5 μM (biofilm inducer).

formation in two clinical isolates of *S. aureus* on two medical devices frequently associated with recurrent infections. Although most drugs were tested at plasma concentrations, acetaminophen, naproxen, ibuprofen, and metformin were tested at concentrations lower than those in plasma (Supplementary Material #2).

Drug effects (Table 1). The results considered the type of clinical isolate, the medical device or bacterial support surface, the substance tested, and its concentration. Most APIS-induced biofilm formation in ESBL *S. aureus* isolates A1 and ESBL and methicillin-resistant *S. aureus* strains (A2M). Next, acetaminophen, lovastatin, and captopril were the most potent inducers. Verapamil and cetirizine were biofilm inhibitors on *S. aureus* isolate A2M, whereas propranolol was on *S. aureus* isolate A1 respiratory probes. Analysis by SEM microscopy revealed that cetirizine produced more biofilms isolated on the respiratory probe surface in *S. aureus* A2M (Fig. 11B).

The clinic isolates of *S. aureus* exhibited differential behaviors. Thus, the biofilm induced by acetaminophen in *S. aureus* A1 was increased by 57 % and 48 % in microplates and respiratory tubes, respectively, while in *S. aureus* A2M was 44 % and 31 %. Previous reports suggest that the mechanism of action of the drugs and the determination of the clonal lineages of each microorganism can explain the results obtained [22].

Medical devices. Regarding medical devices, the respiratory tube was less susceptible to bacterial biofilm formation, which may be attributed to its more uniform structure than the duodenal tube. Patients who depend on medical devices risk acquiring bacterial infections due to the potential contamination of these devices' external and internal surfaces [23].

Thus, biofilm has become a target, and significant progress has been made in *S. aureus* [24–26]. The combination of antibiotics with QS inhibitors has been proposed to overcome microbial resistance [27] by biofilm formation or resistance-associated events such as β-lactamase activity and efflux pumps. Similarly, miconazole and phenothiazine reduced *P. aeruginosa* biofilm, hemolytic and protease activity, among other effects [28], and daptomycin, a new antibiotic, in addition to its biocidal impact on *S. aureus,* was also able to eradicate up to 96 % of biofilm cells, acting as a potent clearing agent. Microbiana [24].

Drug mixtures. On the other hand, drug combinations containing both an inducer, such as diclofenac and an inhibitor, such as propranolol, are intended to mimic commonly used polyvalent pharmacotherapies and to identify synergistic combinations. Surprisingly, when combined with inducers and other drugs, propranolol has a strong inducing effect, mainly when used with hydrochlorothiazide and naproxen in *S. aureus* A1, and the same pattern was detected with diclofenac, especially with loratadine in *S. aureus* A2M. Even drugs that do not induce biofilm formation can act synergistically, increasing the risk for frequent users. However, drug mixtures have also been studied as biofilm eradicators on Gram-negative and Gram-positive biofilms [29].

Persister cells. Concerning persister cells, diclofenac and cetirizine cause a significant difference between free and embedded cells in the biofilm; this makes the biofilm more unstable due to the release of cells from its surface, making it more susceptible to conventional antibiotic treatments [30,31]. However, this contrasts with the strong inducing

Table 1
Total APIs effects on *S. aureus* A1 and A2M biofilm in 96-well microplates, duodenal and respiratory probes, and persister cells.

API	*S. aureus* A1			*S. aureus* A2M			
	Microplate	FR18 duodenal probe	Respiratory probe	Microplate	FR18 duodenal probe	Respiratory probe	**Persister cells
Naproxen	*50 μM: 48 %	5 μM: 33 %	WE	50 μM: 61% 25 μM: 83%	WE	WE	WE
Diclofenac	50 μM: 42% 25 μM: 38%	WE	WE	50 μM: 119% 25 μM: 64%	WE	WE	++
Acetaminophen	50 μM: 57% 25 μM: 39% 5 μM: 44%	WE	50 μM: 48%	50 μM: 44% 25 μM: 33% 5 μM: 43%	WE	5 μM: 31%	WE
Ibuprofen	5 μM: 74%	WE	WE	WE	50 μM: 41%	WE	NT
Metformin	WE	WE	WE	WE	WE	WE	NT
Lovastatin	25 μM: 47%	50 μM: 80% 25 μM: 72%	WE	50 μM: 40 % 25 μM: 62%	25 μM: 56% 5 μM: 55%	WE	WE
Loratadine	25 μM: 52% 5 μM: 109%	25 μM: 61% 5 μM: 76%	WE	WE	WE	WE	NT
Propranolol	WE	WE	25 μM: 36%	WE	WE	WE	NT
Hydrochlorothiazide	50 μM: 40%	WE	50 μM: 54 % 25 μM: 65 %	WE	50 μM: 61%	WE	WE
Verapamil	WE	50 μM: 54% 25 μM: 30% 5 μM: 39%	WE	WE	25 μM:85 % 5 μM: 92%	50 μM: 21 % 25 μM: 37%	WE
Captopril	25 μM: 57% 5 μM: 55%	50 μM: 35%	WE	WE	WE	50 μM: 38%	NT
Cetirizine	WE	50 μM: 27%	WE	WE	WE	50 μM: 40 % 25 M: 40 %	++

*% of biofilm effect: inhibitor (blue) or inducer (red).
**Persister cells: Inducing impact of the population of free cells (+).
WE: Without effect; NT: Not evaluated.

effect of diclofenac, as it induces a strong biofilm but is not very stable. In contrast, cetirizine has an exciting profile as an inhibitor that destabilizes biofilm and exposes bacteria to the environment. However, the difference in persister cells may be caused by an instability in the biofilm that supports them, probably acting as an eradicator, and possibly the mechanism of cetirizine and verapamil in this work.

Mechanism of action. Defining a mechanism of action in this work is challenging because the 12 APIs have very different structures and show little similarity with the known autoinducers. Moreover, to have a system close to natural conditions, the assays were performed on two clinical isolates, with two medical devices on which biofilm formation is frequent, and the drugs were tested at plasmatic concentration and, in some cases, sub-plasmatic. Moreover, QS induction and biofilm formation mechanisms are very diverse and complex. For example, some antibiotics such as azithromycin, imipenem, cefepime, and piperacillin/tazobactam at sub-MIC bacteriostatic concentrations significantly reduced the virulence of *Pseudomonas aeruginosa* [32], affecting biofilm production, proteases, hemolysin, and pyocyanin, among others. Furthermore, the same virulence reduction was found in *Proteus mirabilis* with subMIC doses of ciprofloxacin [33]. Flufenamic acid, a potent anti-inflammatory in MRSA infections, inhibited peptidoglycan biosynthesis, β-lactam resistance, and biofilm formation [34]. Similarly, at low concentrations, the antihypertensive felodipine enhanced the efficacy of gentamicin, blocked MRSA resistance protein expression, and eradicated biofilm and persister cells [35].

5. Conclusions

Using some normal-like conditions, this paper studied the effect of 12 APIs on biofilm formation and stability in two clinical isolates of *S. aureus*. Most were potent inducers, although the response depended on several factors, including clinical isolate, compound, concentration, and type of medical device. Propranolol, verapamil, and cetirizine were inhibitory in respiratory probes, and the latter also modified the population of biofilm-embedded cells. This work, therefore, provides information about the probable clinical implications of drugs in older people and patients using medical devices or undergoing surgical procedures. However, using inhibitory drugs as drug repurposing or templates also makes designing new and more potent biofilm inhibitors possible. Nonetheless, further epidemiologic analyses and *in vivo* assays are needed to confirm these findings and to establish a correlation between drug use, the onset of bacterial infections in patients, and the use of medical devices.

In addition, it is essential to recognize that antibiotic activity studies cannot be reduced to determining the minimum inhibitory concentration of their growth and their effects on the biofilm and other processes associated with QS, such as lytic enzymes and siderophores.

CRediT authorship contribution statement

Maria L. Carmona-Orozco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Fernando Echeverri: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2024.106863.

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