

Research Article

Diclofenac May Induce PIA-Independent Biofilm Formation in *Staphylococcus aureus* Strains

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Received 14 August 2020; Revised 24 December 2020; Accepted 15 January 2021; Published 27 January 2021

Academic Editor: Karl Drlica

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Staphylococcus aureus is a pathogen commonly resistant to antibiotics. Biofilm formation is one of the important factors related to its virulence. Non-antibiotics drugs, such as nonsteroidal anti-inflammatory agents (NSAIDs), have been studied as an alternative for treating infections by multiresistant pathogens and biofilm-associated infections. In this study, the effects of NSAID sodium diclofenac on growth inhibition and biofilm formation of *S. aureus* were evaluated. The minimum inhibitory concentration (MIC) of diclofenac for fifty isolates ranged from 200 to 400 µg/mL. Diclofenac sub-MICs induced biofilm in 32.3% of biofilm-negative strains in tryptic soy broth. All biofilms induced by the drug showed a PIA- (polysaccharide intercellular adhesion-) independent composition, and the scanning electron microscopy showed that the induced biofilm presented a very discrete matrix. The combination of diclofenac with rifampicin sub-MICs induced strong production of PIA-dependent biofilm in three of four strains, while combination of NSAID with NaCl induced the formation of partially polysaccharide biofilm in two strains and PIA-independent biofilm in another strain. The combination of NSAID with glucose resulted in PIA-independent biofilms in all four strains tested. The results showed that diclofenac can commonly induce biofilm production by a PIA-independent pathway. However, when this NSAID is combined with other types of inducing agents, the composition of the biofilm produced may vary.

1. Introduction

Several drugs have a greater or lesser degree of antibacterial activity, although this effect is not their primary therapeutic goal. Compounds with these properties are included in medicine classes such as antihistamines, statins, antipsychotics, local anesthetics, nonsteroidal anti-inflammatory drugs (NSAIDs) [1], anticonvulsants, and sympatholytic agents [2] and have been called non-antibiotics [3].

Among non-antibiotics, many studies have focused on NSAIDs. The NSAIDs are compounds that, in addition to

anti-inflammatory action, have analgesic and antipyretic properties. These drugs are among the most widely used in the world. Their main mechanism of action involves the inhibition of cyclooxygenase, which leads to a decrease in the synthesis of prostaglandins [4].

The most prescribed NSAIDs in clinical practice are drugs such as diclofenac, ibuprofen, indomethacin, and acetylsalicylic acid. Their antimicrobial activity includes bacterial isolates of different species, as well as fungi [5]. Diclofenac (2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid) is a NSAID with an antimicrobial activity in vitro

against various bacterial pathogens [6–9]. Studies have shown that the broad-spectrum antibacterial effect of this drug can also be obtained in vivo [10–12].

The antibacterial mechanism of action of diclofenac seems to reside mainly in the inhibition of DNA synthesis [13]. Besides that, the increase in the uptake of ethidium bromide by *Staphylococcus aureus* cells exposed to diclofenac sodium has provided evidence that NSAIDs may also act by compromising cellular membrane integrity [14]. This drug has also induced extensive alterations in the transcriptome of methicillin-resistant *S. aureus* (MRSA) by changing the expression of hundreds of genes, including those associated with antimicrobial resistance [15].

In addition to investigating their potential for antibacterial activity, researchers have analyzed the effects of diclofenac on biofilms. Biofilms are complex multicellular communities of microorganisms that proliferate in an amorphous polymeric matrix. They play an important role in microbial pathogenicity, protecting against host immune defense mechanisms and conferring survival capacity to antimicrobial agents [16].

In *S. aureus*, an important nosocomial and community pathogen, biofilm formation is mediated by *icaADBC*-dependent and -independent pathways [17]. The genes of *ica* operon encode enzymes involved in the production of the polysaccharide intercellular adhesin (PIA), which mediates the intercellular adherence of bacteria and the accumulation of multilayered biofilm. However, specific proteins can substitute PIA in cell-cell adhesion in the PIA-independent biofilm, and sometimes, extracellular DNA (eDNA) can be the main component of the matrix [18, 19].

Many studies have indicated that diclofenac has anti-biofilm properties on different bacterial pathogens [9, 14, 20]. However, it has not been investigated whether, by contrast, subinhibitory concentrations of this NSAID can induce biofilm production. Therefore, the aim of this study is to investigate the antimicrobial effect of diclofenac against *S. aureus* strains and their action on biofilm induction, alone or combined with other potential inducing agents.

2. Materials and Methods

2.1. Bacterial Isolates. Twenty-five clinical isolates of *S. aureus* (strains A) were obtained from different infected patients admitted to a hospital in Rio de Janeiro (HSE/RJ), and twenty-five nonclinical strains were obtained from the nasal swabs of healthy individuals (strains B). Only one bacterial isolate was considered for each individual. The isolates were submitted to Gram staining, catalase, and tube coagulase tests. The identification and susceptibility to antimicrobial agents were performed using the MicroScan WalkAway-96 System (Dade Behring Inc.). In addition, the isolates were tested for resistance to oxacillin, confirmed by the cefoxitin disk screen test. Species confirmation was made by PCR assay [21]. Bacterial stocks were kept at -80°C in tryptone soy broth (TSB, HiMedia) containing glycerol (50% v/v). The Ethics Committee on Research with Humans of the HSE/RJ approved this study under reference number 000.41.

2.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Diclofenac. The MICs of the diclofenac were determined by the broth dilution method according to the Clinical and Laboratory Standards Institute [22] using 96-well flat-bottomed polystyrene microtiter plates (Kasvi). Briefly, a diclofenac (Sigma) working solution was prepared in methanol, sterilized by filtration, and diluted in TSB. Drug concentrations ranging from 1.600 to $1.5\ \mu\text{g}/\text{mL}$ were obtained by two-fold serial dilutions in TSB, and one hundred microliters of each drug dilution were added to the wells.

The bacterial suspension was prepared in a saline solution, and turbidity was adjusted to 0.5 McFarland standard, equivalent to 1.5×10^8 colony forming units (cfu)/mL. The bacterial inoculum consisted of five microliters of the diluted culture to obtain about 5×10^5 cfu/well. TSB without the drug and TSB only with the bacterial inoculum were used as controls. The plates were incubated at 35°C for 24 h and visually read to establish the presence or absence of turbidity. The MIC was defined as the minimum concentration of the drug that inhibited visible bacterial growth. Additionally, optical density ($\text{OD}_{620\text{nm}}$) was measured using a plate reader (Thermo Plate RT6000). The MBC was determined by the subculture, in Tryptic soy agar (TSA, HiMedia), of $10\ \mu\text{L}$ of medium taken from wells that did not show visible growth. The plates were incubated at 37°C for 24 h, and MBC was defined as the lowest drug concentration that resulted in the absence of bacterial growth or in the presence of only one colony.

2.3. Effect of Subinhibitory Concentrations (Sub-MICs) of Sodium Diclofenac on Biofilm Formation. The effect of sub-MICs of diclofenac on biofilm formation was studied using the microtiter plate (MTP) assay [23], with some modifications. Twenty clinical strains and twenty carrier strains were tested. Briefly, two-fold serial dilutions of diclofenac solution were prepared in TSB and added (1:100) to overnight cultures of isolates and reference strains (final concentrations equivalent to 1/2 MIC to 1/16 MIC). Strains with biofilm production induced by diclofenac concentration equivalent to 1/16 MIC were tested for lower drug concentrations. Controls were prepared in TSB without the drug. *S. aureus* ATCC 43300 and *S. epidermidis* ATCC 12228, positive and negative for biofilm production, respectively, were used as reference strains.

After homogenization, the bacterial suspensions were seeded in 96-well flat-bottomed polystyrene microtiter plates ($200\ \mu\text{L}/\text{well}$) and incubated for 24 h at 35°C . Then, the cultures were removed, the wells were washed three times with distilled water, and the attached bacteria were fixed with $200\ \mu\text{L}$ of methanol for 15 minutes. The plates were emptied, air-dried, and stained for 15 min with $200\ \mu\text{L}$ of 2% Hucker's crystal violet solution. After removal of the dye, the plates were washed under running distilled water and air dried. The dye bound to the adherent cells was extracted with $200\ \mu\text{L}$ of 95% ethanol for 30 min, and the optical density ($\text{OD}_{570\text{nm}}$) of biofilm extracts was measured.

All experiments were performed in triplicate and repeated at least three times. In each plate, four wells were used as blanks containing only TSB medium. The cutoff OD value (OD_c) used to differentiate biofilm producer and non-producer isolates was defined as three standard deviations (SD) above the mean OD of the negative control: OD_c = OD average of negative control + (3 × SD of negative control) [24]. Isolates with OD ≤ OD_c were considered as non-producers. The result of the OD average of biofilm extract was used to classify the isolates as weak producer (OD_c < OD ≤ 2xOD_c), moderate producer (2xOD_c < OD ≤ 4xOD_c), and strong producer of the biofilm (4xOD_c < OD) [25].

2.4. Biofilm Detachment Assay. The PIA-independent or PIA-dependent nature of the biofilm matrix induced by diclofenac was evaluated using a degradation test with sodium metaperiodate [26]. The methodology was based on the MTP assay, as described by Izano et al. [27], with some modifications. The isolates were grown overnight in TSB at 35°C and diluted 1 : 100 in TSB without and with the drug (1/2 MIC to 1/8 MIC). Then, the microtiter plates were seeded with 200 μL per well of bacterial suspensions and incubated at 35°C for 24 hours.

Subsequently, the cultures were removed, the wells were washed with distilled water, and 200 μL of 50 mM sodium metaperiodate solution (Sigma) in distilled water was added to triplicate wells. An equal volume of distilled water without the degrading agent was added as a control. The plates were incubated for two hours at 35°C, and then, the wells were washed twice with distilled water. The next steps followed those described in the MTP assay for biofilm.

The PIA-independent or PIA-dependent composition of the matrix was determined based on biofilm degradation levels by metaperiodate and comparing the OD values in the presence of the degrading agent to the untreated control. In this study, biofilm with a PIA-dependent composition was considered that which suffered dissolution by the sodium metaperiodate greater than 50%. A similar procedure to that described for metaperiodate was used for proteinase K (Sigma) (1 mg/mL in 0.1 M PBS-pH 7.0); trypsin (Inlab) (1 mg/mL em Tris 20 mM, pH 7.5) [28], and deoxyribonuclease I type II (Sigma) (100 μg/ml in 150 mM NaCl and 1 mM CaCl₂) [27].

2.5. Composition of the Biofilm Matrix in Strains of *S. aureus* Grown in Medium with Diclofenac Combined with Other Potential Biofilm Inducers. Four positive strains in which biofilm formation was induced by diclofenac were tested for other substances with the potential to induce biofilm production. Previously, the presence of the *ica* operon genes A–D was detected by multiplex polymerase chain reaction (PCR), according to the procedure reported by de Lima e Silva et al. [29].

The substances chosen for the tests were rifampicin, at sub-MICs concentration, glucose (1%) and NaCl (3%). The sub-MICs were determined after previous tests at concentrations equivalent to 1/2 and 1/4 of the MIC (0.004 μg/mL

and 0.002 μg/mL, respectively). Rifampicin sub-MICs were determined after previous tests at concentrations equivalent to 1/2 and 1/4 of the MIC (0.004 μg/mL and 0.002 μg/mL, respectively). Antimicrobial susceptibility tests previously performed showed that these strains were rifampicin sensitive.

The strains grew in TSB containing each of these agents, alone and in combination with diclofenac (50 or 100 μg/mL). In addition, the strains grew in TSB without the agents studied and TSB only with diclofenac. The tests for researching the production and composition of the biofilm were carried out by MTP assay, as described above.

2.6. Quantitative Real-Time PCR (qRT-PCR). One clinical strain of *S. aureus* (SA139) was selected to evaluate the relative expression levels of the *sarA*, *sigB*, *icaA*, and *icaR* genes. Previously, the presence of the *icaADBC* operon was investigated and detected in this strain, according to the technique described by Ninin et al. [30]. The gene sequences were obtained from the GenBank website (<http://www.ncbi.nlm.nih.gov/genbank/>). The primers of the genes used in this study were designed by the Primer Express software (Applied Biosystems®).

The culture was grown in TSB medium until the exponential phase before the addition of the diclofenac solution (50 and 80 μg/ml, final concentration) or an equal volume of sterile methanol (control) and incubated by 20 min. Total RNA extraction was performed based on the phenol-chloroform method at 70°C [31]. Then, the extracted RNA was resuspended in 15 μL DEPC water, and the tube was vortexed for three minutes. RNA purity was evaluated spectrophotometrically on the NanoDrop2000 system (Thermo Fisher Scientific), and the RNA integrity was verified by loading 5 μL of the total RNA into 1.4% agarose gel containing 1.0 × TBE buffer. In addition, RNA concentration was measured using the Qubit® RNA BR Assay Kit (Thermo Fisher Scientific) with the reading of reaction using the Qubit® 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific).

Reverse transcription of the extracted RNA was performed using a LifePro™ gradient thermal cycler (Bioer Technology Co.) and the kit TaqMan® Reverse Transcription Reagents (Applied Biosystems), according to the manufacturer's protocols. The qRT-PCR was prepared in 96-well RT-PCR plates containing the GoTaq® qPCR Master Mix (Promega), according to the manufacturer's instruction. cDNA was diluted (1 : 4), and then, 10 μL of the GoTaq® qPCR Master Mix, 4 μL of the oligonucleotide primer mix (0.8 μM), and 2 μL of DNase-free water were added.

The reaction was performed in triplicate, and negative controls were prepared for each primer oligonucleotide using 5 μL of Milli-Q H₂O with DEPC instead of the first cDNA strand. In all experiments, the *gyrB* gene was used as an endogenous control of reactions. Analysis of data generated in the qRT-PCR reaction was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corporation) and the method of relative quantification of gene expression normalized by endogenous control levels ($2^{-\Delta\Delta CT}$).

2.7. Scanning Electron Microscopy (SEM). Three diclofenac-induced biofilm-producing strains were selected to be cultured on coverslips in TSB without diclofenac (control) and with diclofenac (50 µg/mL). Briefly, a 1:100 dilution of overnight culture was inoculated into 24-well polystyrene microtiter plates (2 mL/well). Subsequently, a sterile Thermanox plastic coverslip (13 mm) (Nalgene-USA) was placed on the bottom of each well. After 24 hours of incubation at 35°C, the coverslips were removed from wells with sterile forceps, rinsed several times with PBS, and then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for one hour at 4°C. After additional washing, the samples were dehydrated with a series of increasing concentrations of ethanol, 30%, 50%, 70%, 80%, and 95% for 30 min each, and pure ethanol for one hour. Finally, the specimens were coated with gold (Baltec SCD 050) and examined using SEM equipment (Zeiss EVO 40) operated at 20 kV.

3. Results

3.1. Antibacterial Activity and Effects of Diclofenac Sub-MICs on Biofilm Formation. The antibacterial activity of diclofenac was tested against 50 strains of *S. aureus*. Among the 25 clinical strains, seven were characterized as MRSA, while among carriers strains, only one strain was identified as community-associated MRSA (CA-MRSA).

The diclofenac MIC ranged from 200 to 400 µg/mL. For the concentration of 800 µg/mL, the action of the drug was bacteriostatic, while the MBC was 1600 µg/mL. Forty isolates were tested for biofilm production. Among 34 negative strains for biofilm production in TSB medium without the drug, supplementation with diclofenac promoted an induction for biofilm production in 11 of them (six clinical strains and five carrier isolates). All these 11 isolates were induced in the sub-MIC of 50 µg/mL, and 9 also were induced in 100 µg/mL. These concentrations were equivalent to 1/2 MIC or 1/4 MIC or 1/8 MIC depending on the strain.

Figure 1 shows the levels of production of biofilm induced by different concentrations of diclofenac in three clinical and three carrier strains. For the A150 strain, diclofenac induced the production of biofilm at concentrations as low as 3.1 and 1.5 µg/mL (Figure 2(a)). These concentrations were equivalent to 1/64 MIC and 1/128 MIC, respectively. Six strains were characterized as biofilm producers in TSB without the drug, and one strain (A116), with the addition of diclofenac to TSB, enhanced the biofilm formation even at a concentration of only 3.1 µg/mL of the drug. At this concentration, the increase was about 32% (Figure 2(b)).

3.2. Diclofenac-Induced Biofilm Type. The type of biofilm induced by diclofenac was characterized based on the degree of degradation after treatment with metaperiodate, trypsin, proteinase K, and DNase. Metaperiodate is an oxidizing agent that cleaves bonds of glucosamine polymers such as PIA, and it is then effective in dispersing polysaccharide biofilms, unlike nonpolysaccharide biofilms, which are resistant to such treatment [32]. Trypsin and proteinase K are

enzymes that degrade biofilms with a matrix of protein composition, while DNase disperses biofilms consisting mainly of extracellular DNA [27, 28].

In both groups of *S. aureus* strains isolated from carriers and those of clinical origin, all biofilms induced by sub-MICs of NSAID were nonpolysaccharides (PIA-independent) in composition: protein (6), protein-DNA (3), and DNA (1). In one isolate, the induced biofilm was not dispersed by any of the tested agents. In the A116 strain, which produced biofilm in TSB without diclofenac and increased production when grown in the presence of the drug, the metaperiodate dispersed the biofilm produced. Thus, the biofilm of this strain has a polysaccharide composition (PIA-dependent) in both growing conditions.

3.3. Composition of Biofilm Induced by Diclofenac When Combined with Other Inducing Agents. Biofilms produced by four strains (A112, A139, A150, and B103) were evaluated after the growth in diclofenac-supplemented media combined with rifampicin sub-MICs, NaCl (3%), and glucose (1%). These strains showed sensitivity to rifampicin in the antimicrobial susceptibility test and harbored the *icaA* and *icaD* genes.

Rifampicin sub-MICs did not induce biofilm production in the isolate A139, while it observed high induction of PIA-dependent biofilm (polysaccharide) in the other three isolates. The mean (OD_{570 nm}) of biofilm extract for 1/2 MIC of rifampicin ranged from 1.263 to 1.659 and from 0.368 to 0.515 for diclofenac.

The polysaccharide composition of the biofilm induced by rifampicin was maintained in the culture with rifampicin + diclofenac, while the biofilm of the control isolate grown only with diclofenac kept its original PIA-independent composition (Table 1). For rifampicin in 1/4 MIC + diclofenac, the results were similar as those of rifampicin in 1/2 MIC + diclofenac, except for the B103 strain, for which the combination of drugs resulted in the production of a partially polysaccharide biofilm (degradation by metaperiodate only about 45%). The biofilm induced by both rifampicin and the combination rifampicin + diclofenac was classified as strong, in contrast to the weak biofilm induced only by diclofenac.

NaCl did not induce biofilm in only one of the four isolates (SA112). For this strain, the biofilm originally induced by diclofenac was inhibited by NaCl + diclofenac. In two strains (A150 and B103), NaCl induced PIA-dependent biofilm formation. However, in the growth with NaCl + diclofenac, the induced biofilm revealed a partially polysaccharide composition, with a biofilm dispersion of about 30% and 36%, respectively, after treatment with metaperiodate (Table 2). Contrary to what we observed for B103 and A150 isolates, NaCl alone and combined with diclofenac induced only PIA-independent biofilm production in the A139 strain.

As for the effects of glucose, there was induction to produce PIA-independent biofilm in all four isolates studied. This result was maintained when the strains were grown in the medium containing glucose + diclofenac (Table 3).

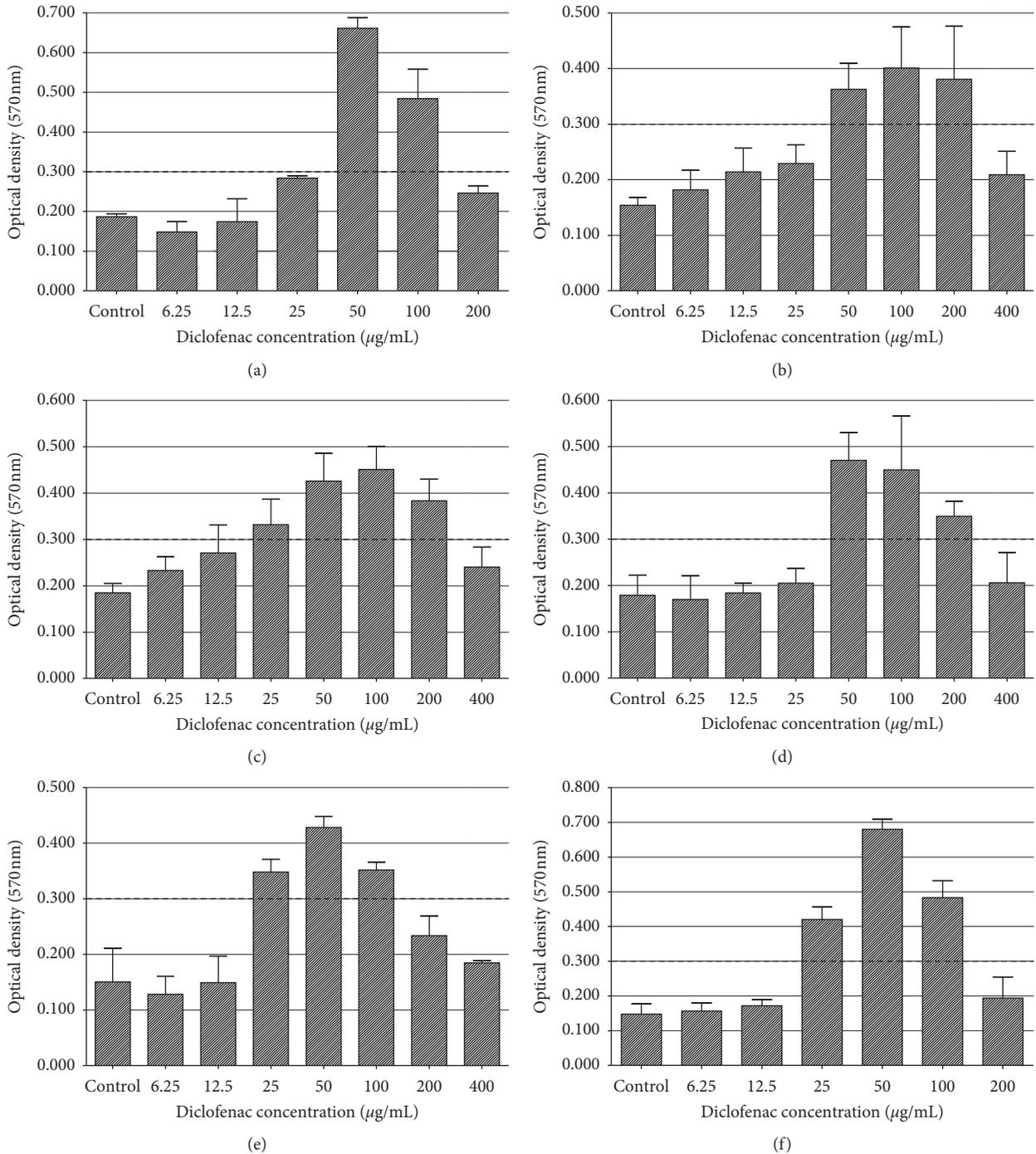


FIGURE 1: Biofilm production by six (a–f) isolates of *S. aureus* in tryptone soy broth (TSB) added of subinhibitory concentrations of diclofenac. The horizontal dashed line indicates the cutoff value that separates production from nonproduction of the biofilm. Assays were repeated three times with similar results, and error bars represent mean with range. (a) A108. (b) A112. (c) A139. (d) B103. (e) B105. (f) B121.

3.4. *Quantitative Real-Time PCR (qRT-PCR)*. The relative expression levels of the *sarA*, *sigB*, *icaA*, and *icaR* genes of the strain A139 were evaluated after treatment with diclofenac (Figure 3). The expression of *icaA* was suppressed in 86% and 54% at concentrations of 50 and 80 µg/mL, respectively, compared to the expressions in the control. In contrast, *icaR*

activity increased about five-fold and three-fold, respectively. Diclofenac also induced an increase in the activity of the two global regulators studied (*sarA* and *sigB*). Concentrations of 50 and 80 µg/mL increased the activity of *sarA* by three times and two times, respectively, in relation to the control, while the increases were 5.6 and about 3 times, respectively, for *sigB*.

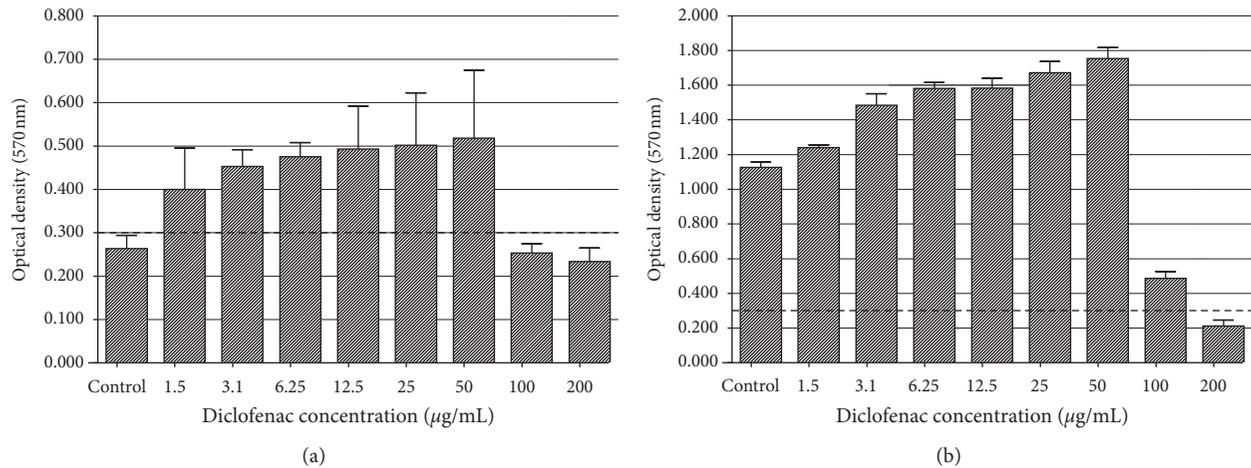


FIGURE 2: Biofilm production by two (a-b) isolates of *S. aureus* in tryptone soy broth (TSB) added of subinhibitory concentrations of diclofenac. The horizontal dashed line indicates the cutoff value that separates production from nonproduction of the biofilm. Assays were repeated three times with similar results, and error bars represent mean with range. (a) A150. (b) A116.

TABLE 1: Degree of degradation of biofilm induced by rifampicin (1/2 MIC) and diclofenac after treatment with sodium metaperiodate in 3 strains of *S. aureus*.

Strain	Control ^a (OD _{570nm})	Rif ^b		Rif + Dic ^d		Dic ^f	
		OD _{570 nm}	SMP ^c (%)	OD _{570 nm}	SMP ^e (%)	OD _{570 nm}	SMP ^g (%)
A112	0.210	1.376	77.9	1.789	70.8	0.368	0.0
A150	0.219	1.263	78.0	1.376	67.9	0.525	0.0
B103	0.212	1.659	75.1	2.186	64.4	0.515	0.0

Rif, rifampicin; Dic, diclofenac (50 µg/mL); SMP, sodium metaperiodate; OD_{570 nm}, optical density at 570 nm. ^aStrain grown on tryptone soy broth (TSB) without supplementation; ^bstrain grown in TSB supplemented with rifampicin; ^cdegradation by metaperiodate (%) of biofilm induced by rifampicin; ^dstrain grown in TSB supplemented with rifampicin + diclofenac; ^edegradation by metaperiodate (%) of biofilm induced by rifampicin + diclofenac; ^fstrain grown in TSB supplemented with diclofenac; ^gdegradation by metaperiodate (%) of biofilm induced by diclofenac.

TABLE 2: Degree of degradation of biofilm induced by NaCl and diclofenac after treatment with sodium metaperiodate in 3 strains of *S. aureus*.

Strain	Control ^a (OD _{570nm})	NaCl ^b		NaCl + Dic ^d		Dic ^f	
		OD _{570 nm}	SMP ^c (%)	OD _{570 nm}	SMP ^e (%)	OD _{570 nm}	SMP ^g (%)
A139	0.182	0.393	0.0	0.580	0.0	0.536	0.0
A150	0.177	0.889	69.4	0.513	29.7	0.386	0.0
B103	0.188	0.915	58.6	0.783	36.7	0.559	0.0

Dic, diclofenac (50 µg/mL); SMP, sodium metaperiodate; OD, optical density. ^aStrain grown on tryptone soy broth (TSB) without supplementation; ^bstrain grown in TSB supplemented with NaCl (3%); ^cdegradation by metaperiodate (%) of biofilm induced by NaCl; ^dstrain grown in TSB supplemented with NaCl + diclofenac; ^edegradation by metaperiodate (%) of biofilm induced by NaCl + diclofenac; ^fstrain grown in TSB supplemented with diclofenac; ^gdegradation by metaperiodate (%) of biofilm induced by diclofenac.

TABLE 3: Degree of degradation of biofilm induced by glucose and diclofenac after treatment with sodium metaperiodate in 4 strains of *S. aureus*.

Strain	Control ^a (OD _{570nm})	Glu ^b		Glu + Dic ^d		Dic ^f	
		OD _{570 nm}	SMP ^c (%)	OD _{570 nm}	SMP ^e (%)	OD _{570 nm}	SMP ^g (%)
A112	0.202	0.722	3.1	0.586	0.0	0.411	0.0
A139	0.185	0.821	0.0	0.722	0.0	0.655	0.0
A150	0.200	0.876	0.0	0.579	0.0	0.387	0.0
B103	0.192	0.642	0.0	0.602	0.0	0.625	0.0

Glu, glucose; Dic, diclofenac (50 µg/mL); SMP, sodium metaperiodate; OD, optical density. ^aStrain grown on tryptone soy broth (TSB) without supplementation; ^bstrain grown in TSB supplemented with glucose (1%); ^cdegradation by metaperiodate (%) of biofilm induced by glucose; ^dstrain grown in TSB supplemented with glucose + diclofenac; ^edegradation by metaperiodate (%) of biofilm induced by glucose + diclofenac; ^fstrain grown in TSB supplemented with diclofenac; ^gdegradation by metaperiodate (%) of biofilm induced by diclofenac.

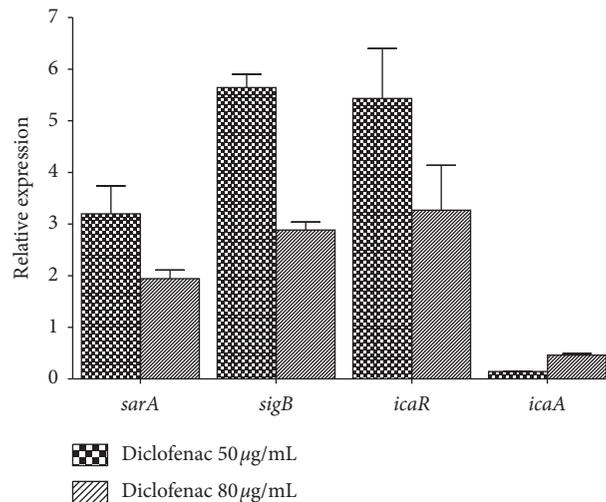


FIGURE 3: Relative expression of *sarA*, *sigB*, *icaA*, and *icaR* genes in a *S. aureus* strain. The SA139 strain was grown to the exponential phase and then treated with diclofenac (50 and 80 µg/mL). Medium without diclofenac was used as the control. The *gyrB* gene was used as an endogenous reaction control. Each bar represents the mean of relative expression with range for biological triplicates.

3.5. Scanning Electron Microscopy (SEM). The B103, A139, and A150 strains were chosen for morphological evaluation (SEM) of diclofenac-induced biofilm (Figure 4). In strains grown on the surface of Thermanox plastic membranes in TSB medium without diclofenac (control), the images revealed very few adhered cells. Conversely, after cultivation in medium with 50 µg/mL of NSAID, there were large amounts of cell aggregates. Magnified images of the cultures showed cell aggregates with a small amount of extracellular matrix material, mainly in the B03 strain. No significant changes were observed in the morphology of bacterial cells grown in the medium with NSAID.

4. Discussion

Bacterial multidrug resistance is a growing phenomenon worldwide. New resistance mechanisms have been described for different bacterial pathogens. The difficulty in effectively treating common infectious diseases with conventional antibiotics available has stimulated the study of the antimicrobial action of drugs known as “non-antibiotics” [1, 3, 5, 33]. This group of drugs includes diclofenac, an NSAID that has been shown to have a broad-spectrum antimicrobial activity in vitro and in vivo [6–9, 13, 34]. On the other hand, it has not been investigated whether sub-MICs of this drug can induce biofilm formation, as in the case for certain antibiotics [29, 35].

In the present study, the MIC of the diclofenac ranged from 200 to 400 µg/mL, with slight differences regarding the origin of isolates (clinical or carrier). These concentrations are well above the therapeutic plasma levels normally attained [36]. However, it should be considered that there are topical formulations of this NSAID that can locally determine the presence of concentrations much higher than those achieved in plasma with the systemic use of the drug [37]. Therefore, the possibility of effects under these

conditions on the microorganism in the interaction with the drug cannot be excluded.

The literature reports the MIC of diclofenac for *S. aureus* with values ranging from 50 to >1000 µg/mL, which may be due to intrinsic physiological characteristics of isolates studied, as well as to the methodological procedures, such as the way for drug solubilization, culture medium, and the technique [8]. The MBC was detected only at 1600 µg/mL, since plating in TSA of aliquots of wells containing ≤800 µg/mL resulted in spots with bacterial growth. This result is in contrast with earlier findings, which pointed to a bactericidal action of diclofenac in concentrations lower than 1600 µg/mL [10, 13, 38, 39].

Positive and negative isolates for biofilm production in TSB medium were tested to evaluate the effects of diclofenac sub-MICs on biofilm production. Diclofenac induced biofilm production in 11 of 34 biofilm-negative isolates grown in TSB, and all induced biofilms showed a non-polysaccharide composition. These results add new data on drugs with the potential to induce production of non-polysaccharide biofilm in *S. aureus*, since this phenomenon has not yet been reported for diclofenac. Although diclofenac at a concentration equal to that achieved for human serum has limited the formation of strong biofilms by clinical isolates of *S. aureus* grown on the surface of polypropylene mesh, Reśliński et al. [40] showed that the strains producing weak biofilm increased from 1.4% (control) to 10.0% (diclofenac). However, the chemical nature of this biofilm was not characterized by the authors.

The concentrations that largely determined this effect were equivalent to 1/2 MIC, 1/4 MIC, and 1/8 MIC, according to the strain. In a nonbiofilm producing strain (SA150), induction of biofilm production by diclofenac was noted at a concentration as low as 1.5 µg/mL. Besides that, in a producing strain (SA116), an increase in biofilm production at concentrations from 3.1 µg/mL of the drug was

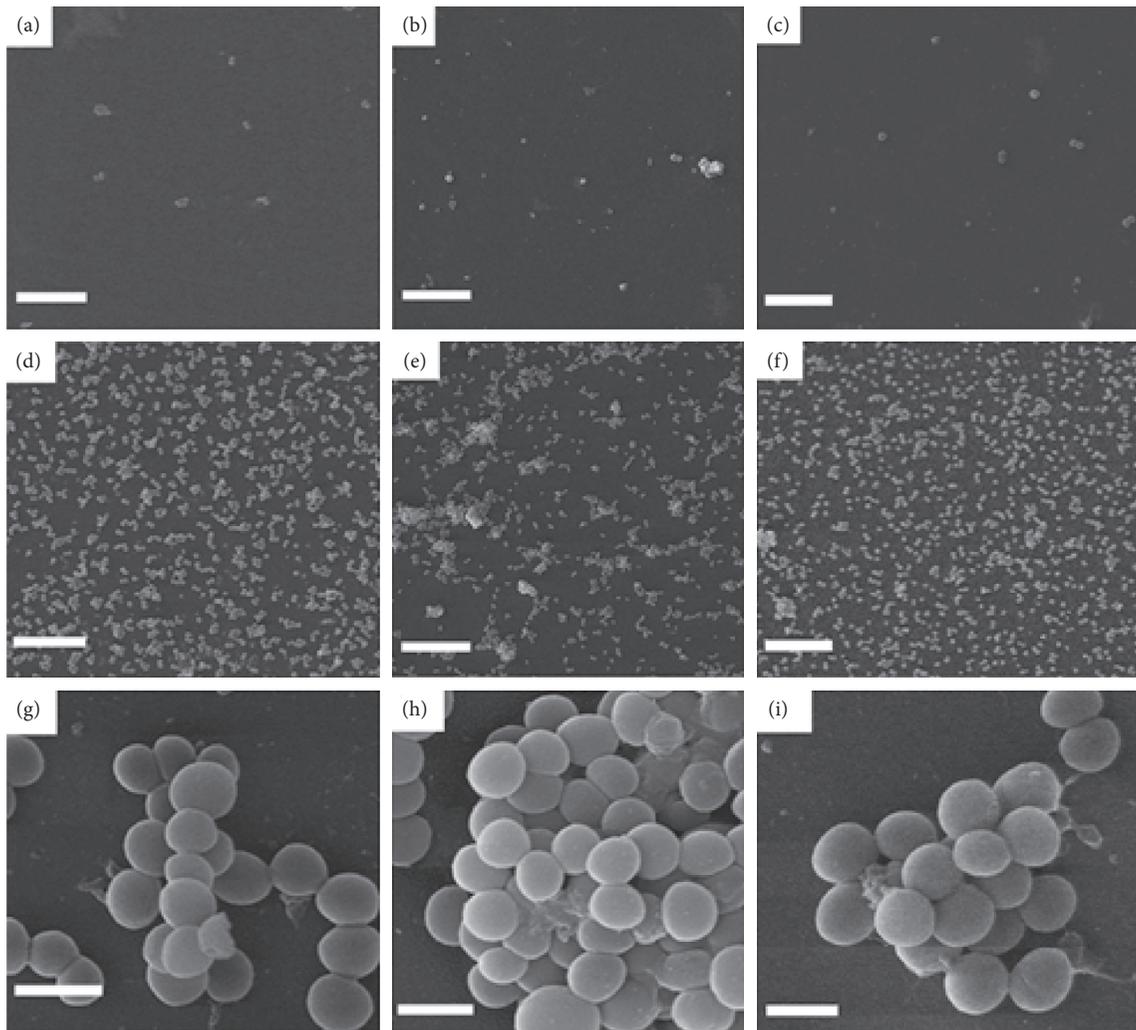


FIGURE 4: Scanning electron microscopy images of three strains of *S. aureus*. Bacteria were grown in twenty-four well tissue culture plates with Thermanox plastic coverslips at 35°C for 24 h. (a, b, and c) SA139, SA150, and B103 strains, respectively, grown in medium without diclofenac (magnification: 4000x); (d, e, and f) SA139, SA150, and B103 strains, respectively, grown in medium with diclofenac (magnification: 4000x); and (g, h, and i) images of the respective strains grown in medium with diclofenac (magnification: 40000x). Scale bars: (A, B, C, D, and F) = 10 μm ; (G, H, and I) = 1 μm .

observed. It is important to note that these concentrations are within the plasma levels that the drug can reach [36].

The nonpolysaccharide composition of the biofilm matrix induced by diclofenac, indirectly determined in this study by the degree of degradation after treatment with sodium metaperiodate solution, suggests that the drug activates PIA-independent pathways to produce biofilm. In fact, complementary tests using trypsin and proteinase K, in addition to DNase, showed that the induced biofilm had a protein composition in most isolates, while in others, the matrix composition was eDNA-protein, eDNA, or not affected by the dispersing agents used. On the other hand, in a strain (SA116) that naturally produces biofilm in TSB without the drug, diclofenac determined an increase in the production of polysaccharide biofilm. It suggests that depending on the strain, the NSAID could stimulate *ica*- (PIA-) dependent pathway in order to increase biofilm production.

Biofilms with a PIA-independent extracellular matrix have been associated with MRSA strains [41, 42]. Among the clinical isolates tested, seven were MRSA, but diclofenac did not induce biofilm production in any of these strains. There are no other studies on the chemical nature of diclofenac-induced biofilms; however, it is important to note that Dotto et al. [43] reported that NSAID salicylic acid, the main biometabolite of acetylsalicylic acid, promoted the formation of biofilm in *S. aureus* in a PIA-dependent way.

It is not yet fully known how the biofilm matrix can vary in response to different inducing agents. A better understanding of the chemical composition of biofilms induced by different conditions could be useful in strategies for treating infections associated with biofilm, since, for example, cells in biofilms with the protein matrix have a different organization of growing compared with those in PIA-dependent biofilm.

Four isolates that were positive for biofilm induction by diclofenac were evaluated for the effects of combining NSAID with other potential biofilm-inducing substances (rifampicin, glucose, and NaCl). The aim of these tests was to assess whether the combination of diclofenac with these substances could induce changes in the composition of the biofilm matrix and which pathway predominates, whether PIA-dependent or PIA-independent.

The results showed that rifampicin stimulated high polysaccharide biofilm production, both alone and in combination with diclofenac, suggesting that the activation of the *ica*-dependent pathway promoted by this antibiotic was strong enough to overlap the *ica*-independent stimulus implemented by diclofenac. It is important to note that this effect can be dose-dependent, since in one of the strains studied, the combination of a lower concentration of rifampicin (1/4 MIC) with diclofenac resulted in the formation of a biofilm that was only partially polysaccharide.

Unlike rifampicin, both glucose alone and the combination of glucose with diclofenac induced the formation of a nonpolysaccharide biofilm. This result is not surprising, since this sugar is considered a common activator of biofilm production through a PIA-independent pathway [41, 44, 45]. However, it is interesting to note that the combination of diclofenac + glucose did not result in an additive stimulatory effect of increasing biofilm production, compared to the effect of these substances in isolation.

As for NaCl, this salt has been considered an activator of the *ica* operon in non-MRSA strains [41], which is in agreement with the results obtained for two of the 4 isolates tested. However, the biofilm produced by these two isolates in the medium supplemented with NaCl + diclofenac was poorly dispersed by metaperiodate (only 29% and 36%), suggesting that, in this case, a greater stimulation of NSAID in the activation of an *ica*-independent pathway has predominated, while the *ica*-dependent pathway stimulated by NaCl was not completely inhibited, resulting in low PIA production. On the other hand, the third isolate (A139) produced a PIA-independent biofilm both in the presence of NaCl and in the medium containing this salt and diclofenac, although this strain previously showed the presence of *ica* operon genes (*icaA* and *icaD*). This result is suggestive of the complexity of the regulation of biofilm production in *S. aureus*, since it can also involve characteristics specific to each strain.

The results obtained with qrt-PCR to some extent corroborate the phenotypic results found for the production of diclofenac-induced PIA-independent biofilm in strain SA139. The relative expression of *icaA* was downregulated by diclofenac treatment, while the activity of *icaR* increased considerably. *icaR* is a negative regulator of *icaADBC* because it binds to the *icaA* promoter region [46]. It was also observed that diclofenac increased the activity of the global regulators *sigB* and *sarA*.

sigB, an alternative sigma factor of RNA polymerase that is activated in stress response, has been shown to regulate numerous genes involved in virulence, including genes involved in biofilm formation [47]. Although the effects of this regulator on *ica* transcription may vary depending on

the study, Lauderdale et al. [48] demonstrated that it was essential for *ica*-independent biofilm formation in *S. aureus*.

SarA, on the other hand, is a global regulatory protein affecting the expression of many genes in *S. aureus*, including a diversity of genes involved in pathogenesis. This regulator seems to affect both *ica*-dependent and *ica*-independent biofilm formations [19, 49]. A mechanism of action of SarA in the production of *ica*-independent biofilm seems to be associated mainly with its capacity to repress bacterial proteases, allowing the formation of a protein-dependent biofilm [50].

Magnified SEM images confirmed the induction of PIA-independent biofilm production in the SA139 strain by diclofenac. The presence of little extracellular material was noted. According to Vergara-Irigaray et al. [51], the morphology of cells visualized by SEM in a PIA-rich biofilm matrix is different from that of proteinaceous biofilm. The authors showed that in the case of PIA-dependent biofilm, cells are embedded in an abundant extracellular matrix mesh that interconnected the bacteria. However, cells growing in a proteinaceous biofilm formed by FnBP showed dense bacterial aggregates with close contact between cells, without an appreciable extracellular amorphous matrix.

5. Conclusion

Diclofenac showed relatively high MIC values (200–400 µg/mL) for the strains tested, and the bactericidal activity occurred only at concentrations greater than 800 µg/mL. In addition, this study is the first to show that diclofenac sub-MICs can induce the production of nonpolysaccharide biofilm in strains of *S. aureus* negative for biofilm production in TSB, suggesting that this drug activates a PIA-independent pathway in *S. aureus*. However, in strains naturally producing PIA-dependent biofilm in TSB without additives other than diclofenac, the drug can stimulate an increased production of this type of biofilm.

Despite the limited number of strains studied here, the results suggest that if diclofenac is combined with other potential biofilm inducers, such as rifampicin sub-MICs or NaCl, the composition of the biofilm matrix may show variations probably depending on the type of inducing stimulus or characteristics of the bacterial strain. Such stimulus may result in the formation of a PIA-dependent or only partially polysaccharide biofilm. In contrast, when diclofenac is combined with glucose, the resulting biofilm maintains the PIA-independent composition observed for diclofenac, perhaps because both substances activate a single pathway to biofilm formation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors are grateful to UNIRIO for student scholarship. The authors also are so much thankful to Dr. Ernesto Hofer for reading the manuscript and for the valuable contributions.

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