

# Methicillin Resistance of Food-Borne Biofilm-Forming Staphylococci

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**Abstract:** The aim of this study was to evaluate the biofilm-forming ability and the resistance of planktonic cells and biofilm to methicillin (MIC and MBC, and log CFU/cm<sup>2</sup> reduction, respectively). Thirty-four isolates were used, including coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS), recovered from ready-to-eat (RTE) foods. Biofilm-forming ability was based on enumeration of viable cells from biofilms formed on three types of surfaces, namely stainless steel, polystyrene, and glass. Thirteen of the thirty-four isolates (38.2%) were methicillin-resistant (MIC higher than 4 µg/mL). *Staphylococcus aureus* (CPS) and *Staphylococcus saprophyticus* (CNS) were the species that showed the highest percentage of resistance (50% and 71.4%, respectively). *Staphylococcus warneri* was the only species susceptible to methicillin. In 20 of the 34 isolates, the MBC value was twice the MIC value. The CPS (*S. aureus*) and the CNS (the other species) were not significantly different ( $p > 0.05$ ) on biofilm-forming ability on the three surfaces used. However, when comparing the averages obtained for the biofilm-forming ability of the isolates, the values obtained (log CFU/cm<sup>2</sup>) in polystyrene were significantly higher ( $p < 0.05$ ) than those obtained with the isolates on stainless steel. On the other hand, data from biofilm-forming ability of the isolates on glass and stainless steel are positively and significantly correlated with each other ( $r = 0.54$ ;  $p = 0.02$ ). It was not possible to determine the concentration of methicillin that promotes biofilm removal since log reductions were less than 3 log CFU/cm<sup>2</sup>. The results of this study indicate that foodborne CPS and CNS can form biofilms on different types of material. As these biofilms are resistant to high concentrations of methicillin, their occurrence in food environments and their spread to medical settings can result in staphylococcal food poisoning or, in the worst-case scenario, septicemia, respectively. Good hygiene and good manufacturing practices (GHP and GMP) are therefore mandatory to prevent contamination with *Staphylococcus* spp.

**Keywords:** foodborne *Staphylococcus* spp.; biofilms; methicillin-resistant *S. aureus* (MRSA); minimum inhibitory concentration—MIC; minimum bactericidal concentration—MBC



**Citation:** Salamandane, A.; Correia, J.; Muetanene, B.A.; dos Santos, M.; Malfeito-Ferreira, M.; Brito, L. Methicillin Resistance of Food-Borne Biofilm-Forming Staphylococci. *Appl. Sci.* **2023**, *13*, 7725. <https://doi.org/10.3390/app13137725>

Academic Editor: Anthony William Coleman

Received: 4 June 2023

Revised: 26 June 2023

Accepted: 28 June 2023

Published: 29 June 2023



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## 1. Introduction

Biofilms are complex and highly structured aggregations of sessile bacteria, formed on biotic or abiotic surfaces, embedded in a self-produced extracellular matrix of exopolysaccharides, proteins, and DNA [1–3]. The quorum sensing of microorganisms in the biofilm is oriented to mobilize resources for the survival of microorganisms, such as reducing their metabolism, activating protectors against the host's immune defenses, in addition to protecting against antimicrobial agents, often resulting in persistent and difficult-to-treat infections [1,2]. Biofilm formation involves several steps such as adhesion, accumulation,

maturation, and biofilm dispersion, determined by several genes, mainly from the *ica*ABCD operon encoding a Polysaccharide Intercellular Adhesion (PIA), the main component of biofilms [4,5]. The ability of the bacterial strain to form a biofilm can be assessed by indirect methods, for instance based on the application of crystal violet, and by direct methods based on the viable cell count (CFUs) [6]. The crystal violet method involves growing biofilms in microtiter plates, staining them with crystal violet, and then solubilizing the bound dye to measure its absorbance [5,6]. This method has been widely used for simple high-throughput screening of cultures [7]. However, it is based on the crystal violet binding to viable and non-viable cells and extracellular polymers of the biofilm matrix, not allowing the exact quantification of viable cells in the biofilm [7]. On the other hand, direct methods for measuring biofilm formation rely on removing cells from the surface and plating the resulting cell suspension to determine the number of CFUs [8]. Stainless steel is often used as a surface for the growth of biofilms but, in general, bacteria can adhere and form biofilms on different types of materials, such as polystyrene, Teflon, marble, granite, glass, and polypropylene [6,8,9]. *Staphylococcus* spp. include important human and animal biofilm-forming pathogens that can cause severe infections [1,10]. These bacteria can adhere to implanted medical devices or damaged tissues, causing persistent infections through the formation of biofilms [2,5]. Multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA), which are isolated from various environments, are highly prone to forming biofilms and causing clinically complicated infections, increasing the likelihood of developing persistent, chronic, and recurrent infections [10].

The aim of this study was to evaluate the biofilm-forming ability of *Staphylococcus* spp. recovered from ready-to-eat (RTE) street food. Isolates included coagulase-positive *Staphylococcus* and coagulase-negative *Staphylococcus*. Three different surfaces were used for biofilm formation (glass, stainless steel, and polystyrene). Since in *Staphylococcus* methicillin resistance is closely associated with multidrug resistance, methicillin resistance of planktonic cells and biofilms were also investigated.

## 2. Materials and Methods

This study used 34 isolates of *Staphylococcus* spp. previously recovered from RTE street food sold in Maputo [11]. This set of isolates included 10 *S. aureus* (coagulase-positive staphylococci (CPS)) and 24 coagulase-negative staphylococci (CNS): seven isolates of *S. warneri* and *S. saprophyticus*, respectively, and five isolates of *S. pasteurii* and *S. xylosus*, respectively (Table 1). The procedures for obtaining, identifying, and characterizing these isolates by biochemical and molecular tests were previously described in Salamandane et al. [12]. Briefly, *Staphylococcus* spp. presumptive colonies were obtained after incubation for 48 h at 37 °C onto Chromogenic Baird Parker Agar—CBPA (Biokar Diagnostics, Beauvais, France) according to ISO 6888 [11,13]. Biochemical tests were carried out to characterize the presumptive *Staphylococcus* colonies from CBPA plates, namely: Gram staining, catalase, and oxidase tests [11]. Subsequently, coagulase tests were performed in Coagulase Rabbit Plasma (Biokar Diagnostics, Beauvais, France) and in Baird Parker RPF Agar (Biokar Diagnostics, Beauvais, France), according to ISO 6888-2/A1-2003 [14]. Molecular identification was performed targeting the 16S rRNA gene (1500 bp) with Bac27F forward primer (5-AGAGTTTGGATCMTGGCTCAG-3) and Univ1492R universal reverse primer (5-CGGTTACCTTGTTACGACTT-3). The amplified products were sequenced (STAB VIDA, Caparica, Portugal) and the resulting sequences were submitted to Blastn using the megablast algorithm against Reference RNA sequences database (RefSeq RNA) from the National Center of Biotechnology Information (NCBI) for isolate identification.

The presence of seven virulence genes, namely encoding toxins (*hly* and *sak*) and staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, and *see*) were previously investigated by multiplex PCR [12]. Fourteen of the thirty-four (41.2%) isolates were also multidrug-resistant. These characteristics are presented in Table 1.

**Table 1.** Antibiotic resistance profile and virulence genes in the isolates used in this study [12].

Isolate Code	Species	Antibiotic Resistance Profile	Virulence Genes
<b><u>11</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>FOX, AMP, MET, VANC, PEN, ERY</u></b>	<i>hly</i>
15	<i>S. aureus</i>	AMP, PEN, ERY	-
<b><u>32</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>FOX, AMP, MET, PEN, SXT</u></b>	<i>hly</i>
<b><u>47</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>VANC, PEN, ERY</u></b>	-
48	<i>S. aureus</i>	PEN, ERY	<i>hly, sea</i>
49	<i>S. aureus</i>	PEN	-
<b><u>50</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>FOX, AMP, MET, VANC, PEN</u></b>	<i>hly</i>
52	<i>S. aureus</i>	VANC	-
<b><u>53</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>FOX, AMP, MET, VANC, PEN, TET, ERY</u></b>	<i>hly, sed</i>
<b><u>68</u></b>	<b><u><i>S. aureus</i></u></b>	<b><u>FOX, MET, VANC, PEN, TET, ERY</u></b>	<i>hly, sec</i>
24	<i>S. pasteurii</i>	PEN	<i>sec</i>
37	<i>S. pasteurii</i>	-	<i>hly</i>
40	<i>S. pasteurii</i>	PEN, TET	-
41	<i>S. pasteurii</i>	ERY	<i>hly</i>
<b><u>43</u></b>	<b><u><i>S. pasteurii</i></u></b>	<b><u>FOX, MET, PEN, ERY</u></b>	<i>hly</i>
6	<i>S. saprophyticus</i>	PEN	-
<b><u>13</u></b>	<b><u><i>S. saprophyticus</i></u></b>	<b><u>FOX, MET, PEN, VANC, TET, ERY</u></b>	<i>sec</i>
<b><u>19</u></b>	<b><u><i>S. saprophyticus</i></u></b>	<b><u>FOX, MET, VANC, PEN, ERY</u></b>	<i>sec, sed, sak</i>
<b><u>26</u></b>	<b><u><i>S. saprophyticus</i></u></b>	<b><u>FOX, MET, PEN, ERY</u></b>	<i>sec</i>
31	<i>S. saprophyticus</i>	PEN, ERY	<i>seb, hly</i>
<b><u>35</u></b>	<b><u><i>S. saprophyticus</i></u></b>	<b><u>FOX, MET, PEN, ERY</u></b>	<i>sec</i>
<b><u>59</u></b>	<b><u><i>S. saprophyticus</i></u></b>	<b><u>FOX, MET, PEN, SXT</u></b>	<i>sec</i>
1	<i>S. warneri</i>	PEN, ERY	<i>sak</i>
18	<i>S. warneri</i>	AMP, PEN, ERY	<i>sec, sak</i>
9	<i>S. warneri</i>	ERY	<i>sec, sak</i>
10	<i>S. warneri</i>	ERY	<i>sak</i>
20	<i>S. warneri</i>	PEN	<i>sec</i>
22	<i>S. warneri</i>	PEN	<i>sec</i>
34	<i>S. warneri</i>	AMP, PEN, GEN, ERY	<i>sec</i>
21	<i>S. xylosum</i>	PEN	<i>sec, sed</i>
23	<i>S. xylosum</i>	AMP, PEN, ERY	-
<b><u>28</u></b>	<b><u><i>S. xylosum</i></u></b>	<b><u>FOX, MET, VAN, PEN</u></b>	-
30	<i>S. xylosum</i>	PEN	-
<b><u>65</u></b>	<b><u><i>S. xylosum</i></u></b>	<b><u>VANC, PEN, ERY</u></b>	<i>seb, sec, sed</i>

The nine antibiotics tested were: Cefoxitin (FOX), Ampicillin (AMP), Methicillin (MET), Vancomycin (VAN), Penicillin G (PEN), Tetracycline (TET), Gentamicin (GEN), Trimethoprim/sulfamethoxazole (SXT), and Erythromycin (ERY). Isolates signed in bold and underlined are multidrug-resistant isolates. Multidrug resistance was considered as non-susceptibility to at least one agent in three or more antimicrobial categories and/or resistance to methicillin [15].

### 2.1. Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The MIC was determined according to the standard broth dilution method (CLSI M07-A8) as indicated by the Clinical and Laboratory Standards Institute (CLSI) [15], evaluating the visible growth of microorganisms in Tryptone–Casein–Soy Broth (TSB) (Biokar Diagnostics, Beauvais, France) at 37 °C for 18 ± 2 h. Briefly, serial two-fold dilutions of methicillin at concentrations ranging from 64 µg/mL to 1 µg/mL were used in 96-well polystyrene microtiter plates (CellStar®, Kremsmünster, Austria). Then, 100 µL of inoculum was then added to obtain a final bacterial concentration of 10<sup>8</sup> CFU mL<sup>-1</sup> (0.5 McFarland's standard) in each well, in a total volume of 200 µL. Cells that were not subjected to the antibiotic at any time and TSB that was not inoculated at any time were controls for inoculum viability

and sterility of the culture medium. The MIC was defined as the lowest concentration of methicillin that prevents visible growth of a selected isolate after a 24 h incubation period.

MBC was determined after MIC determination. In fact, after 24 h incubation at 37 °C, from all the wells that did not show visible bacterial growth, 100 µL were removed, inoculated onto TSA-YE, Trypto–Casein–Soy agar plates (Biokar Diagnostics, Beauvais, France), and incubated at 37 °C, for 24 h. MBC was defined as the lowest concentration of methicillin capable of killing 99.9% of the bacterial population (3 log reduction) [16].

## 2.2. Assessment of Biofilm Formation Capacity

The evaluation of the biofilm formation capacity of the isolates was carried out based on the removal of aggregated cells from different surfaces and their respective plating for CFU quantification. Three different surfaces were used, namely: polystyrene, stainless steel, and glass surface. The inocula were always prepared from isolated colonies grown onto TSA-YE for  $22 \pm 2$  h, at 37 °C, which were suspended in TSB to obtain a turbidity equivalent to the McFarlands 0.5 standard (about  $10^8$  CFU mL<sup>-1</sup>).

### 2.2.1. Polystyrene Surface (P24 Microplates)

For this method, 24-well polystyrene microplates (P24 microplates) (Orange Scientific, Braine-l'Alleud, Belgium) were used. Wells were inoculated with 1 mL of the inoculum prepared as described above and the microplates were sealed with Parafilm® (Amcor, Zurich, Switzerland) and incubated for 24 h at 37 °C. Non-inoculated wells (negative controls) were filled with 1 mL of sterilized TSB. After this period, the broth with the culture was removed and each well were subjected to three series of washes with 1.5 mL of Ringer's solution (Biokar Diagnostics, Beauvais, France) (RS). The microplates were inverted onto absorbent paper to remove the remaining washing liquid.

Thirty glass beads of 4 mm of diameter and 1 mL of RS per well were added. The microplate was vortexed on a microplate vortex (Tittertek DSG, Flowlabs, Berlin, Germany) for 2 min at maximum speed to remove biofilm from the surface of the wells. Decimal dilutions were performed, and TSA-YE plates were inoculated by spreading (100 µL). The CFU count was performed after 24 h and confirmed after 48 h of incubation at 37 °C. After calculating the total adhesion surface area, the biofilm formation capacity of the isolates was expressed in log CFU/cm<sup>2</sup>.

### 2.2.2. Stainless Steel Coupons

The stainless-steel coupons (Metalurgica Quinacorte, Lda, Lousa, Portugal) (1 × 1 cm) were previously treated to remove grease and autoclaved for 15 min at 121 °C. The procedure used for the formation of biofilms was in accordance with Costa et al. (2016) [16]. Briefly, each coupon was immersed in a 24-well polystyrene microplate containing 1.5 mL of inoculum. The microplates were sealed with Parafilm® and incubated at 37 °C for 24 h. Coupons used as negative controls (non-inoculated) were immersed in 1.5 mL of sterilized TSB.

After incubation, each coupon was washed with RS (1 mL for each face) in order to remove planktonic cells. Each coupon thus washed was placed in a new P24 microplate with 20 glass beads of 4 mm of diameter per well. Another 30 glass beads of 4 mm of diameter and 1 mL RS were placed on top of each coupon. The P24 microplate was shaken on a microplate shaker (Tittertek DSG, Flowlabs, Berlin, Germany) for 2 min at maximum speed to disaggregate adhered cells. Subsequently, decimal dilutions were performed and inoculated by spreading onto plates of TSA-YE plates. The CFU count was performed after 24 h and confirmed after 48 h of incubation, at 37 °C. After calculating the total adhesion surface area, the biofilm formation capacity of the isolates was expressed in log CFU/cm<sup>2</sup>.

### 2.2.3. Glass Test Tubes

For this method, glass test tubes (16 × 160 mm) with metal caps were previously autoclaved at 121 °C for 15 min and their inner surface used as the adhesion surface. The tubes were added with 5 mL of inoculum and incubated for 24 h, at 37 °C. After this period,

the broth with the culture was removed and each tube was subjected to three series of washes with 7 mL of sterilized RS. The tubes were then inverted onto absorbent paper to remove any remaining washing liquid. Then, 6 mL of RS was added to each tube, which was placed in an ultrasound bath (JP Selecta, Barcelona, Spain) at room temperature for 10 min to release the biofilm. Decimal dilutions were performed and inoculated by spreading onto TSA-YE plates. The CFU count was performed after 24 h and confirmed after 48 h of incubation, at 37 °C. After calculating the total adhesion surface area, the biofilm formation capacity of the isolates was expressed in log CFU/cm<sup>2</sup>.

### 2.3. Evaluation of Methicillin Resistance of Biofilm Produced on Polystyrene Surface (P24 Microplates)

Biofilms were produced on P24 polystyrene microplates as described in Section 2.2.1. For each isolate, six wells were inoculated and incubated at 37 °C, for 24 h. After incubation, three series of washes were performed for each well with RS solution and then 2 mL of methicillin at concentrations of 1, 2, 4, 8, 16, and 32 µg/mL were added. The sixth well was filled with 2 mL of sterilized distilled water. These microplates were again incubated at 37 °C for 24 h. After this period, the antibiotic solution was removed, and each well was washed with three series of 1.5 mL of RS. Thirty glass beads per well and 1 mL of RS were added. The microplate was vortexed for 2 min at maximum speed to remove biofilm from the surface of the wells. After shaking the plates, decimal dilutions were made, and the resulting suspensions were inoculated for CFU counting as described in Section 2.2.1. To calculate log reductions, the controls exposed to sterilized water were used. The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration of methicillin capable of reductions of more than 3 log (99.9% reduction of the bacterial population) [17].

### 2.4. Interpretation of Results and Data Analysis

Assays performed to determine MIC and MBC were always performed in duplicate. Thus, and after the respective incubation period, the wells of the recovery plate that showed visible turbidity in contrast to the non-inoculated controls were considered as positive results. When at least two of the three replicates showed turbidity, the result was considered positive (growth). When only one or none of the replicates showed turbidity, the result was considered negative (absence of growth).

The values obtained for viable cell counts in biofilms (CFU) were transformed into decimal logarithms. Compliance with data normality was determined using the Shapiro–Wilk test. The homogeneity of variance was determined by the Bartlett test. The ANOVA was performed, and the Scheffé test was used to compare the means differences between Log CFU on the three surfaces used. Pearson correlation analyses were performed to determine the relationships between MIC and MBC, and between MIC and biofilm-forming capacity of the isolates. Comparison of results from the three surfaces was obtained by calculating Spearman rank correlation coefficients ( $r$ ), and their relationships were explored subsequently through principal component analysis (PCA). All statistical analyses were performed at 5% in the R programming language (R, 2020).

## 3. Results

### 3.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Methicillin

Thirty-four *Staphylococcus* isolates were evaluated for methicillin MIC and MBC. MIC values ranged from 1 µg/mL to over 32 µg/mL (Table 2). Of the 34 isolates tested in this study, 13 (38.2%) had MICs higher than 4 µg/mL (resistant to methicillin according to CLSI breakpoints) (Table 2). None of the seven *S. warneri* isolates was methicillin resistant (MICs ranging from 2 µg/mL and 4 µg/mL) (Figure 1 and Table 2). On the contrary, among *S. aureus* and *S. saprophyticus*, the highest percentage of resistance was found (50% and 71.4%, respectively) (Figure 1). The four isolates that showed MIC values higher than 32 µg/mL included two *S. aureus*, one *S. pasteurii* and one *S. saprophyticus* (Table 2).

MBC values followed the same trend as MIC. In 20 of the 34 isolates, the MBC value was twice the MIC value.

**Table 2.** MIC and MBC of methicillin for *Staphylococcus* isolates and MBC/MIC ratio.

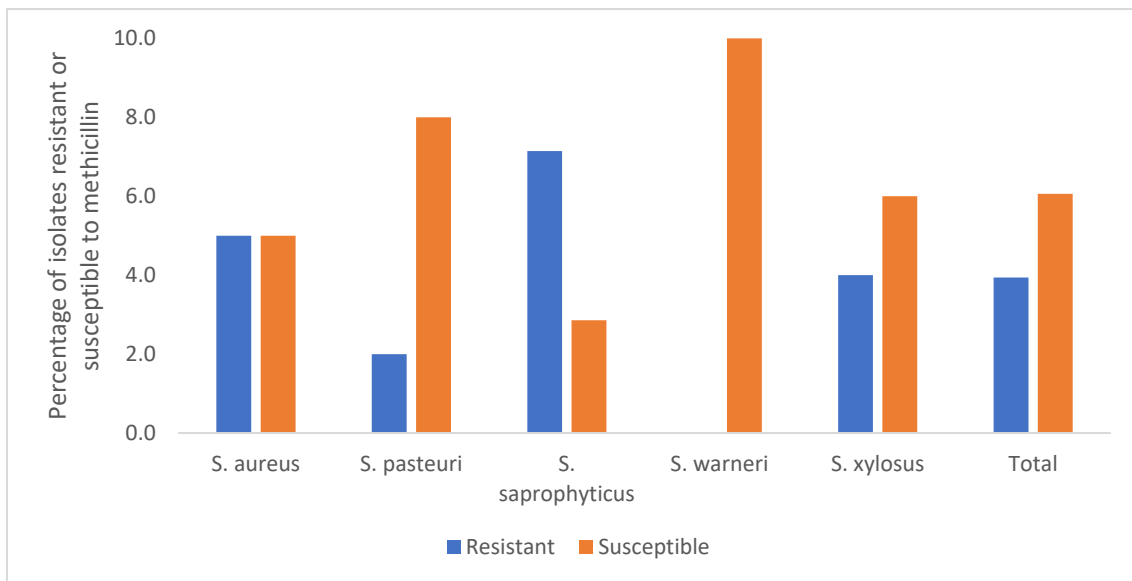
Strain Code	Species	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )	MBC/MIC
11	<i>S. aureus</i>	8	8	1
15	<i>S. aureus</i>	2	4	2
32	<i>S. aureus</i>	16	32	2
47	<i>S. aureus</i>	2	4	2
48	<i>S. aureus</i>	4	4	1
49	<i>S. aureus</i>	4	8	2
50	<i>S. aureus</i>	32	>32	-
52	<i>S. aureus</i>	4	8	2
53	<i>S. aureus</i>	>32	>32	-
68	<i>S. aureus</i>	>32	>32	-
41	<i>S. pasteurii</i>	4	4	1
24	<i>S. pasteurii</i>	4	8	2
37	<i>S. pasteurii</i>	4	4	1
40	<i>S. pasteurii</i>	4	4	1
43	<i>S. pasteurii</i>	>32	>32	-
6	<i>S. saprophyticus</i>	2	4	2
13	<i>S. saprophyticus</i>	8	8	1
19	<i>S. saprophyticus</i>	>32	>32	-
26	<i>S. saprophyticus</i>	8	16	2
31	<i>S. saprophyticus</i>	4	16	4
35	<i>S. saprophyticus</i>	8	16	2
59	<i>S. saprophyticus</i>	32	>32	-
1	<i>S. warneri</i>	4	8	2
18	<i>S. warneri</i>	2	4	2
9	<i>S. warneri</i>	4	8	2
10	<i>S. warneri</i>	2	4	2
20	<i>S. warneri</i>	2	2	1
22	<i>S. warneri</i>	2	4	2
34	<i>S. warneri</i>	2	4	2
21	<i>S. xylosum</i>	4	8	2
23	<i>S. xylosum</i>	1	2	2
28	<i>S. xylosum</i>	32	>32	-
30	<i>S. xylosum</i>	4	4	1
65	<i>S. xylosum</i>	32	>32	-

### 3.2. Evaluation of Biofilm-Forming Ability on Tree Types of Surfaces

In order to evaluate the ability of biofilm formation in three different materials, 18 isolates of *Staphylococcus* spp. (8 CPS and 10 CNS) were used. On stainless-steel, the biofilm-forming ability expressed in Log (CFU/cm<sup>2</sup>) ranged between  $4.58 \pm 0.02$  and  $6.86 \pm 0.02$  in CPS isolates, and between  $4.30 \pm 0.04$  to  $6.97 \pm 0.01$  in CNS isolates (Table 3).

In the glass, the results were between  $6.30 \pm 0.01$  and  $7.66 \pm 0.01$  log CFU/cm<sup>2</sup> in the CPS isolates, and between  $6.10 \pm 0.01$  and  $7.67 \pm 0.03$  log CFU/cm<sup>2</sup> in the CNS isolates (Table 3).

On polystyrene, the biofilm-forming ability varied between  $7.04 \pm 0.13$  and  $8.47 \pm 0.53$  log CFU/cm<sup>2</sup> in CPS isolates, and between  $6.68 \pm 0.11$  and  $8.13 \pm 0.05$  log CFU/cm<sup>2</sup> in CNS isolates (Table 3). The lowest and highest production capacity on polystyrene were verified for the species *S. pasteurii* where counts ranged from  $6.64$  log CFU/cm<sup>2</sup> to  $9.72$  log CFU/cm<sup>2</sup>, followed by *S. xylosum* with counts ranging from  $7.53$  log CFU/cm<sup>2</sup> to  $9.4$  log CFU/cm<sup>2</sup> (Table 3). *S. pasteurii* and *S. saprophyticus* were the species with the highest biofilm productivity, with mean counts above 8 log CFU/cm<sup>2</sup> (Table 3).

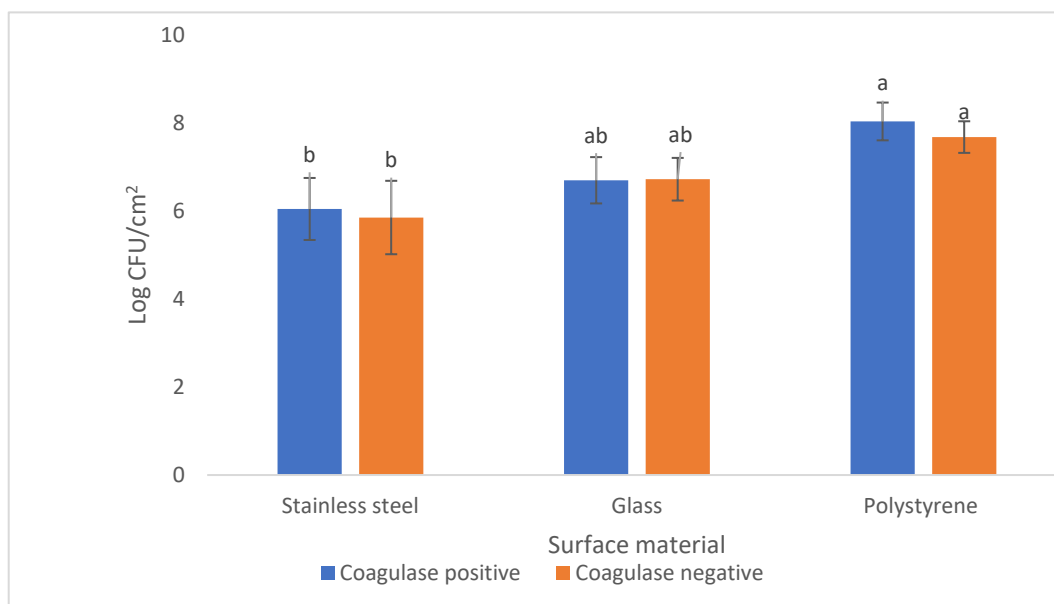


**Figure 1.** Methicillin resistance profile (%) of *Staphylococcus* isolates (susceptible—MIC  $\leq 4$   $\mu\text{g/mL}$ ; resistant—MIC  $> 4$   $\mu\text{g/mL}$ ). At least two biological replicates were performed, with two technical replicates each.

**Table 3.** Biofilm-forming ability of the *Staphylococcus* isolates on three types of surfaces (stainless steel, glass, and polystyrene).

Isolate Code	Species	Biofilm-Forming Ability (log CFU/cm <sup>2</sup> )		
		Stainless Steel	Glass	Polystyrene
11	<i>S. aureus</i>	4.98 $\pm$ 0.01	6.99 $\pm$ 0.04	8.32 $\pm$ 0.02
15	<i>S. aureus</i>	6.86 $\pm$ 0.01	6.30 $\pm$ 0.02	8.47 $\pm$ 0.53
32	<i>S. aureus</i>	5.85 $\pm$ 0.06	6.36 $\pm$ 0.09	7.56 $\pm$ 0.11
42	<i>S. aureus</i>	6.26 $\pm$ 0.07	6.56 $\pm$ 0.07	8.05 $\pm$ 0.19
47	<i>S. aureus</i>	4.58 $\pm$ 0.02	6.30 $\pm$ 0.01	8.00 $\pm$ 0.01
49	<i>S. aureus</i>	6.24 $\pm$ 0.07	7.63 $\pm$ 0.04	7.04 $\pm$ 0.13
53	<i>S. aureus</i>	6.25 $\pm$ 0.01	7.66 $\pm$ 0.01	8.20 $\pm$ 0.47
48	<i>S. aureus</i>	5.53 $\pm$ 0.01	6.83 $\pm$ 0.01	7.83 $\pm$ 0.07
1	<i>S. warneri</i>	4.30 $\pm$ 0.04	6.41 $\pm$ 0.03	7.72 $\pm$ 0.04
18	<i>S. warneri</i>	6.49 $\pm$ 0.09	7.67 $\pm$ 0.03	8.04 $\pm$ 0.02
6	<i>S. saprophyticus</i>	6.32 $\pm$ 0.01	6.72 $\pm$ 0.10	7.66 $\pm$ 0.03
19	<i>S. saprophyticus</i>	5.32 $\pm$ 0.02	6.10 $\pm$ 0.01	7.68 $\pm$ 0.03
35	<i>S. saprophyticus</i>	4.85 $\pm$ 0.06	6.53 $\pm$ 0.01	7.60 $\pm$ 0.09
21	<i>S. xylosum</i>	6.28 $\pm$ 0.01	7.11 $\pm$ 0.01	7.40 $\pm$ 0.16
28	<i>S. xylosum</i>	4.85 $\pm$ 0.06	6.33 $\pm$ 0.02	7.37 $\pm$ 0.05
30	<i>S. xylosum</i>	5.41 $\pm$ 0.03	6.72 $\pm$ 0.03	8.13 $\pm$ 0.05
40	<i>S. pasteurii</i>	6.28 $\pm$ 0.01	7.13 $\pm$ 0.01	6.85 $\pm$ 0.11
43	<i>S. pasteurii</i>	6.97 $\pm$ 0.01	7.46 $\pm$ 0.01	7.97 $\pm$ 0.12

The comparison between means for CPS (*S. aureus*) and for CNS (the other species) were not significantly different ( $p > 0.05$ ) on the three surfaces used. However, when comparing the averages obtained for the biofilm-forming ability of the 18 isolates, the values obtained (log CFU/cm<sup>2</sup>) in polystyrene were significantly higher ( $p < 0.05$ ) than those obtained with the isolates on stainless steel (Figure 2).



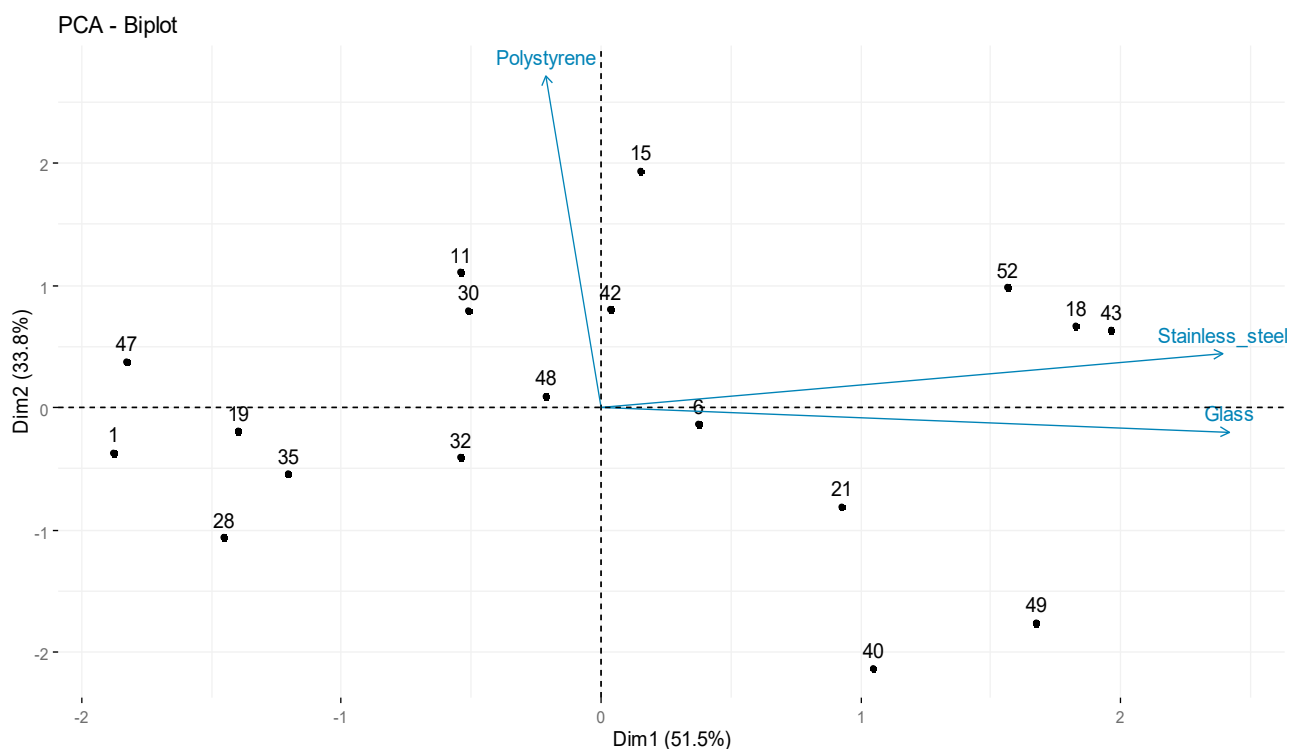
**Figure 2.** Biofilm-forming ability of CPS and CNS isolates assessed on the three tested surfaces. Means followed by the same letter do not differ statistically from each other, according to the Tukey test at a 5% probability level.

The count values obtained on the three surfaces (log CFU/cm<sup>2</sup>) by the 18 isolates were transformed into rank orders and evaluated by principal component analysis (PCA) (Figure 3). The initial three-dimensional space (three variables–three surfaces) was reduced to a plane (Dim1/Dim2) defined by the two principal components that account for 85.3% of the variability of the data (51.5% in the first component and 33.8% in the second component).

When the 18 isolates were projected in this plane (Dim1/Dim2), the biofilm-forming ability was not consistently determined on the three tested surfaces. The capacity for biofilm formation on polystyrene is positively correlated with Dim2, indicating that higher values of Dim2 correspond, in general, to an increase in the biofilm-forming ability of the isolates. Likewise, variations in the opposite direction indicate a decrease in the biofilm forming-ability of the isolates. This can be illustrated by isolates 15 (*S. aureus*) and isolate 30 (*S. xylosum*) as good biofilm producers and by isolates 49 (*S. aureus*) and isolate 40 (*S. pasteurii*) as poor biofilm producers (Figure 3).

Data from the assessment of the biofilm-forming ability of the 18 isolates on glass and stainless steel are positively correlated with each other ( $r = 0.54$ , Table 4) and with Dim1 (Figure 3), indicating that higher Dim1 values correspond, in general, to an increase in the capacity of biofilm formation of the isolates. Likewise, variations in the opposite direction indicate a decrease in the biofilm formation capacity of the isolates. This can be illustrated by isolates 18 (*S. warneri*) and 43 (*S. pasteurii*) as good biofilm producers and by isolates 28 (*S. xylosum*) and 47 (*S. aureus*) as poor biofilm producers (Figure 3).

On the other hand, the enumeration method based on cell counts on stainless steel surfaces is positively correlated with the two components, Dim1 and Dim2, which indicates that higher values of Dim1 added to the contribution of Dim2 correspond to an increase in the ability to form biofilm (isolate 43—*S. pasteurii*) (Figure 3).



**Figure 3.** Principal component analysis of data from enumeration of cells from biofilms of the 18 *Staphylococcus* isolates on the three surfaces used: stainless steel (coupons); glass (test tubes), and polystyrene (24–well polystyrene microplates–24 microplates).

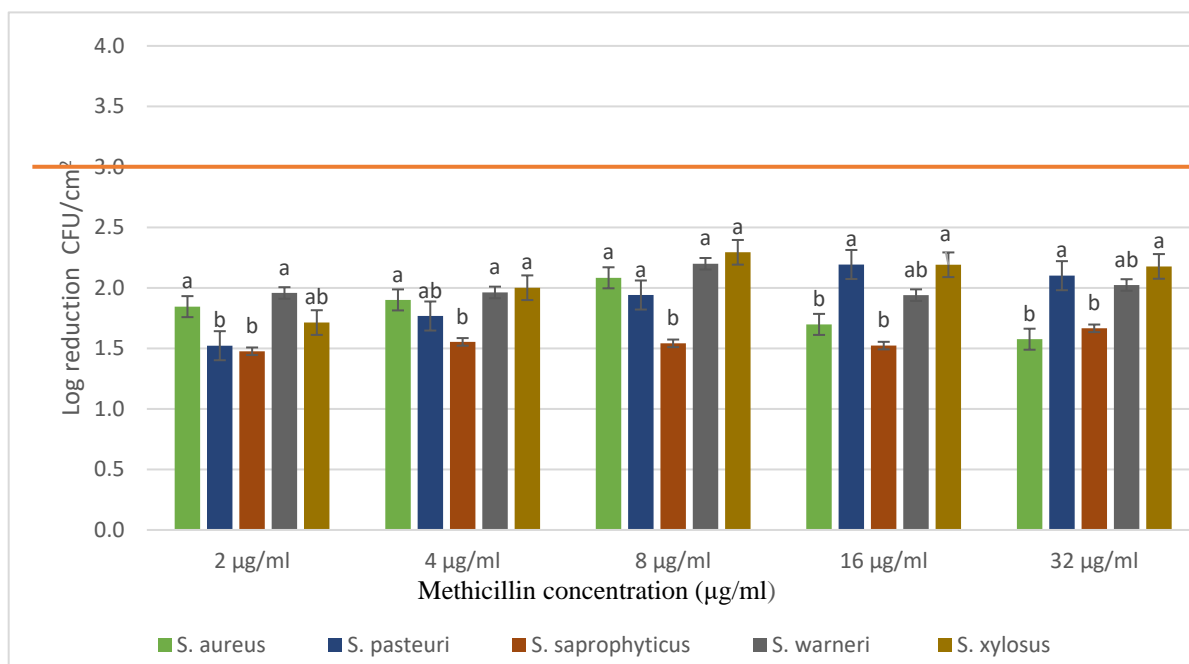
**Table 4.** Pairwise Spearman rank order correlation coefficient ( $r$ ) for biofilm analysis on three different surfaces.

	Glass	Polystyrene
Stainless steel	$r = 0.54$ $p\text{-value} = 0.02$	$r = 0.04$ $p\text{-value} = 0.86$
Polystyrene	$r = -0.09$ $p\text{-value} = 0.72$	

### 3.3. Evaluation of Methicillin Resistance of Biofilms Produced on Polystyrene Surface (P24 Microplates)

Regarding the evaluation of the susceptibility of biofilms to methicillin, high resistance was observed with all methicillin concentrations tested (up to 32  $\mu\text{g}/\text{mL}$ ).

Figure 4 shows the logarithmic reduction of biofilm after exposure to the different concentrations of methicillin used. In general, reductions ranging from 1.5 to 2.3 Log CFU/cm<sup>2</sup> were observed. The species *S. saprophyticus* showed reductions of less than 1.6 Log CFU/cm<sup>2</sup> with all concentrations tested. *S. warneri* isolates showed no difference in reduction with the different methicillin concentrations tested. *S. aureus* showed reductions of less than 1.7 log CFU/cm<sup>2</sup>, with high concentrations of the antibiotic (16 and 32  $\mu\text{g}/\text{mL}$ ). For *S. xylosum*, log reductions varied as a function of antibiotic concentration, from 2  $\mu\text{g}/\text{mL}$  to 8  $\mu\text{g}/\text{mL}$ . With the highest concentrations (16 and 32  $\mu\text{g}/\text{mL}$ ) of methicillin there was no difference in the logarithmic reductions of the isolates. The greatest reduction in viable cells observed were obtained in *S. warneri* and *S. xylosum* species with 8  $\mu\text{g}/\text{mL}$ . On the other hand, the smallest reductions (greater difficulty in eradicating biofilms) were observed in the species *S. saprophyticus* (close to 1.5 Log CFU/cm<sup>2</sup> with all methicillin concentrations tested).



**Figure 4.** Logarithmic reductions (Log CFU/cm<sup>2</sup>) obtained after exposure of biofilms to five concentrations of methicillin, for 24 h at 37 °C. Means followed by the same letter do not differ statistically from each other, according to the Tukey test at a 5% probability level. Yellow horizontal line: Break-point of minimum biofilm eradication concentration (MBEC).

The antibiotic would be considered effective if a 3-log reduction in viable bacterial counts had been achieved. For each methicillin concentration tested, different letters in the columns indicate significant differences ( $p < 0.05$ ). At least two biological replicates were performed, with two technical replicates each.

#### 4. Discussion

In this study, the ability of 34 *Staphylococcus* isolates recovered from RTE street foods to form biofilms was evaluated. This set included coagulase positive *Staphylococcus* (CPS) as well as coagulase negative (CNS). The CNS have emerged as pathogens with risk potential for humans and animals similar to coagulase-positive *Staphylococcus* (CPS) [12,18]. These isolates were selected from a group of 70 *Staphylococci* previously characterized by antibiotic resistance profile and presence of virulence and antibiotic resistance genes [12]. Some of these isolates (namely, *S. aureus* isolate 68, *S. saprophyticus* isolate 19, and *S. xylosus* isolate 65) are not only multidrug-resistant but also present more than one of the seven virulence genes investigated most associated with their pathogenicity.

Three different surfaces were used, namely stainless steel, polystyrene, and glass surface. The methodology used was based on the removal of aggregated cells and enumeration of CFUs after plating (log CFU/cm<sup>2</sup>). Of the three tested surfaces, polystyrene was the surface on which both CPS and CNS showed the highest biofilm production. However, for each surface tested, no statistical differences were found between CPS and CNS isolates. There was a high and significant correlation between stainless steel and glass counts. This indicates that these two surfaces have similar properties related to biofilm adhesion. On the other hand, there was a low correlation between stainless steel and polystyrene, and between glass and polystyrene. This indicates differences in biofilm adhesion between polystyrene and the other two materials, glass, and stainless steel.

Polystyrene is more hydrophobic and has low surface energy, while stainless steel is moderately hydrophilic and has a slight negative charge [7]. Therefore, polystyrene surfaces, which are naturally hydrophobic, promote greater adhesion of microbial cells when compared to other hydrophilic surfaces, such as stainless steel and glass [19]. In

a study using *S. epidermidis*, Koseki et al. (2014) [20] showed a decrease in biofilm formation on the cobalt-chromium molybdenum (Co-Cr-Mo) alloy, which was attributed to the increase in hydrophilicity in this alloy [20,21]. However, other studies have shown opposite results. For instance, the yeast *C. albicans* has been shown to have less metabolic activity on polycarbonate and stainless steel (hydrophilic surfaces) compared to Teflon (hydrophobic surface) [22]. Similarly, some studies claim that hydrophilicity has only trace impact on biofilm formation, as shown by the fact that *S. epidermidis* biofilm formation was not significantly altered by differences in surface wettability [23]. Another determining factor that influences cell adhesion and biofilm production are the electrostatic interactions established between the surface and the cells due to the formation of an electrostatic double layer on each part [24]. The formation of this double layer depends on the ionic strength and the pH value of the surrounding medium. For example, *S. aureus* has been described as adhering more to positively charged polymeric surfaces than to negatively charged ones [24,25]. This observation corroborates our results, in which a greater production of biofilm was observed on polystyrene surfaces than on glass and stainless steel.

As already mentioned, the isolates used in this study were previously characterized in terms of their antibiotic resistance profile as having a high level of resistance to  $\beta$ -lactam and to non- $\beta$ -lactam antibiotics (such as vancomycin, erythromycin, tetracycline, and trimethoprim/sulfamethoxazole) [12]. In the present work, the  $\beta$ -lactam antibiotic, methicillin, had MICs ranging from 1  $\mu\text{g}/\text{mL}$  to over 32  $\mu\text{g}/\text{mL}$ , with 13 of the 34 isolates having MICs greater than 4  $\mu\text{g}/\text{mL}$  (38% of methicillin resistant isolates). In 20 of the 34 isolates, the MBC value was twice the MIC value. In fact, most antibiotics show this ratio between MBC and MIC [26].

Regarding the biofilms formed, it was not possible to determine the concentration of methicillin that promotes their removal. Several factors have been suggested to explain the extraordinary resistance of biofilm bacteria to antibiotics. Among them are the low metabolic rates and reduced growth rates exhibited by biofilm bacteria, particularly those in the innermost layers. All together with the activation of antibiotic resistance mechanisms can make biofilm cells between 10 and 1000 times more resistant than their planktonic counterparts [27,28]. The gene that encodes resistance to methicillin resistance *mecA* was previously identified in the *Staphylococcus* isolates used in this study [12]. This gene is located in a mobile genetic element called SCCmec (staphylococcal cassette chromosome mec) and encodes a modified penicillin-binding protein called PBP2a or PBP2' (penicillin-binding protein 2a or 2-prime) [29]. The bacterial community of the biofilm can exhibit altered gene expression, including the *mecA*. This altered expression can lead to an increase in the production of resistance proteins such as PBP2a that deactivate or bind to antibiotics, reducing their effectiveness [30]. Consequently, even in the presence of high methicillin concentrations, the methicillin-resistant *S. aureus* (MRSA) and the methicillin-resistant *Staphylococcus* biofilm community in general can continue to synthesize their cell wall, which allows it to survive and reproduce.

In this study, the maximum biofilm removal (reduction of 2 log CFU/cm<sup>2</sup>) was observed with 8  $\mu\text{g}$  of methicillin/mL. The exception was the species *S. pasteurii* (reduction of 1.5 log CFU/cm<sup>2</sup>), although this was one of the species that showed the highest susceptibility to methicillin (80% of the isolates with MIC = 4  $\mu\text{g}/\text{mL}$ ). These results suggest that the higher concentration of methicillin may limit its own penetration into the biofilm matrix. However, several authors [31–34] have suggested that reduced penetration of antibiotics cannot fully explain the resistance of biofilms to antibiotics. Other mechanisms that may explain the resistance and tolerance of biofilms to antibiotics may be related to persistent cells and adaptive responses [35]. Antibiotics such as fluoroquinolones, rifampin, and ampicillin have good penetration into the biofilm matrix, although they fail to eradicate bacteria from the biofilm (log reductions < 3 log CFU/cm<sup>2</sup>) [36]. Moreover, even in the case of compounds with low diffusion within biofilms, they all end up reaching the bacteria in the biofilm, although with different times of action and efficiencies [37]. Hoiby et al. [38] suggested the use of a combination of several strategies for the management of *S. aureus*

biofilms, including early physical removal of colonized materials or local administration of high doses of antibacterial chemotherapy.

The occurrence of biofilm-forming strains in the food environment is of significant relevance, particularly when considering species that are multidrug-resistant, as was the case in this study. In the food environment, biofilms can develop on various equipment and surfaces, such as food processing machinery, utensils, and storage containers. These biofilms provide a favorable environment for the growth and survival of pathogenic bacteria, including *S. aureus* [35,39]. Furthermore, the presence of virulence genes in multidrug-resistance biofilm-forming strains enhances their pathogenic potential. This combination of multidrug resistance and virulence genes increases the risk of food contamination and poses a significant threat to public health.

## 5. Conclusions

The results of this study show the ability of different species of foodborne *Staphylococcus*, coagulase positive (CPS), and coagulase negative (CNS) to equally produce biofilms on different surfaces. As these biofilms are resistant to high concentrations of methicillin, the spread of these food-borne biofilm-forming staphylococci to clinical environments with the formation of biofilms on medical devices can lead to the occurrence of difficult-to-treat bacteremia, which can culminate in fatal septicemia. Our study highlights that biofilm formation depends not only on the characteristics of the strains used, as observed by the variability of values obtained for different *S. aureus* strains, but also on the physical and chemical properties of the surface materials employed.

**Author Contributions:** Conceptualization, A.S., M.M.-F. and L.B.; methodology, A.S., B.A.M., M.M.-F. and L.B.; validation, A.S., B.A.M., M.M.-F. and L.B.; formal analysis, A.S., B.A.M., J.C. and M.d.S.; investigation, A.S., J.C., M.d.S., M.M.-F. and L.B.; writing—original draft preparation, A.S., J.C. and M.d.S.; writing—review and editing, A.S., M.M.-F. and L.B.; supervision, M.M.-F. and L.B.; funding acquisition, M.M.-F. and L.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by national funds through FCT—Foundation for Science and Technology, I.P., under the scholarship grant with the reference PRT/BD/151521/2021, and institutional support of project UIDB/04129/2020 of LEAF-Linking Landscape, Environment, Agriculture and Food, Research Unit.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The authors confirm that the data supporting the findings of this study are available within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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