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Biofilm Properties and Their Association to Antibiotic Resistance of *Staphylococcus aureus* from Animal Isolates

Sifat Biofilm dan Hubungannya dengan Resistensi Antibiotik pada Isolat Staphylococcus Aureus Asal Hewan

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ABSTRAK

Staphylococcus aureus (S. aureus) adalah bakteri Gram positif yang memiliki banyak faktor virulensi, salah satunya kemampuan untuk menghasilkan biofilm. Biofilm merupakan salah satu kemampuan penting untuk pertahanan dan resistensi bakteri terhadap antibiotik. Penelitian ini bertujuan untuk mengetahui karakteristik fenotipik dan genotipik isolat S. aureus asal hewan terkait kemampuannya dalam menghasilkan biofilm secara in vitro. Delapan sampel isolat S. aureus yang berasal dari kambing mastitis dan daging ayam segar dievaluasi secara fenotipik terkait kemampuannya dalam menghasilkan biofilm pada 96 plate culture, sedangkan secara genotipik ditentukan dengan mendeteksi gen icaA, icaC dan icaD. Data Minimum Inhibitory Concentration (MIC) dari penelitian sebelumnya digunakan untuk mengklarifikasi korelasinya dengan produksi biofilm dari isolat yang digunakan. Hasil penelitian menunjukkan bahwa 75% (6/8) isolat S. aureus memiliki kemampuan untuk menghasilkan biofilm, sedangkan 50% (4/8) menunjukkan peningkatan produksi biofilm setelah penambahan glukosa. Pengujian PCR menunjukkan bahwa mayoritas isolat positif gen icaA, icaC dan icaD, sedangkan salah satu isolat negatif untuk icaA. Analisis statistik menunjukkan tidak ada korelasi antara optical density produksi biofilm dan MIC antibiotik. Penelitian lebih lanjut diperlukan untuk memperjelas hubungan biofilm dan resistensi antibiotik.

KEYWORDS:
Antibiotic resistance
Biofilm
Glucose
Staphylococcus aureus

ABSTRACT

Staphylococcus aureus (S. aureus) is a Gram positive bacterium that has many virulence factor including the ability to produce biofilm. Biofilm formation is one of the important ability in the persistance and resistant to antibiotic treatment. This study aimed to determine the phenotypic and genotypic characteristics of *S. aureus* isolates from animal origin in their capacity to produce biofilm in vitro. Eight S. aureus isolates collection from goat mastitis and raw chicken meat origin were phenotypically evaluated the ability to produce biofilm in 96 well culture plate, while genotypic were determinated by detecting the *icaA*, *icaC* and *icaD* genes. We employed minimum of inhibitory concentration (MIC) data from previous research to clarify their correlation to biofilm production in respected isolates. The results showed that 75% (6/8) of *S. aureus* isolates had ability to

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produced biofilm, whereas 50% (4/8) showed the elevation of biofilm production after glucose was added. PCR determination showed that majority isolates were positive for *icaA*, *icaC* and *icaD* genes, while one of the isolates was negative for the *icaA*. The statistical analysis tests indicated no correlation between the optical density of biofilm production and MIC of antibiotics. Further research is needed to clarify the association of biofilm and antibiotic resistance.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacteria that recognized to cause of several diseases in animals and humans (Aziz *et al.*, 2022, Miyake *et al.*, 2022, Sato'o *et al.*, 2024). As a pathogen, this bacteria known for its ability to form biofilms, which are structured communities of bacteria encased in a self-produced extracellular polysaccharide matrix (Idrees *et al.*, 2021). This biofilm production is a important virulence factor that contributes to the bacterial persistence and resistance to variety of treatment (Moormeier *et al.*, 2017). The formation of biofilms in *S. aureus* is mainly mediated by the production of Polysaccharide intercellular adhesin (PIA), which is encoded by the *icaADBC* operon. This operon includes genes *icaA*, *icaB*, *icaC*, and *icaD*, which are essential for the synthesis of PIA (Yu *et al.*, 2017, Peng *et al.*, 2022). The PIA is a significant part of the extracellular polymeric substances (EPS) matrix, which provides structural integrity and protection to the biofilm. Moreover, it facilitates the adhesion of bacterial cells to surfaces and to each other, promoting the initial stages of biofilm formation and the development of a robust biofilm structure (Peng *et al.*, 2022) (Wu *et al.*, 2024).

Biofilm formation may enhances the resistance of *S. aureus* to antibiotics (Pokhrel *et al.*, 2024). The biofilm matrix limits the penetration of antibiotics, and the close proximity of cells within the biofilm facilitates horizontal gene transfer, further promoting resistance (Francis *et al.*, 2024). We previously reported phenotypic and genotypic of antibiotic resistance characters of *S. aureus* from animal isolates against β-lactam and tetracycline. It's demonstrated several isolates possess resistance genes characters including *bla*Z^+, *tet*K^+, and *tet*M^+ which showed a linier phenotypics test results of the Kirby Bauer and Minimum Inhibitory Concentration (MIC) tests (Aziz *et al.*, 2023).

This research aims to determine biofilm production phenotypically and genotypically from animal isolates, and to see its relationship with the antibiotic

resistance which has been determined previously. Determining the assosiation of biofilm formation and antibiotic resistance profiles of *S. aureus* is essential for effective infection control, treatment, and the development of new therapeutic strategies (Bhattacharya *et al.,* 2015; Sharan *et al.,* 2024). This dual characterization helps in understanding the pathogenic potential of the bacteria and in devising appropriate measures to combat infections caused by these resilient pathogens.

2. Materials and Methods

2.1. Bacterial Isolate and DNA extraction

This study used 8 *S. aureus* isolates collection which isolated from mastitis milk of Ettawa crossbreed goats and fresh chicken meat from previous research (Maulina, 2022; Putri, 2023; Setyorini, 2023). Isolates in -80ºC glycerol stock were grown on Trypticase Soya Agar media (TSA) (Oxoid, England). Reconfirmation of *S. aureus* was carried out by extracting DNA from pure cultures in TSA agar using *FavorPrep™ Blood/Cultured Cells Genomic DNA Extraction Mini Kit* (FavorPrep, Taiwan). Five to ten colonies were collected using a sterile tube and placed in a 1.5 ml Eppendorf tube containing 200 µl Lysis buffer (20 mg/ml lysozyme; 20 mM Tris-HCl; 2 mM EDTA; 1% Triton X-100, pH 8.0). A total of 5 µl of Lysostaphin (5 mg/ml) (Sigma, USA) was added, then incubated at 37ºC for 30 minutes. The *S. aureus* cell wall was completely lysed using 200 µl of FABG buffer and then incubated at room temperature for 30 minutes. A total of 200 µl of absolute ethanol (Merck, Germany) was added to the mixture and vortexed. The DNA in the mixture was bound to the FABG column matrix by centrifuging at 14,000 x g for 1 minute. The FABG column was then washed using 400 µl W1 Buffer and 600 µl Wash Buffer by centrifugation at 14,000 x g for 1 min respectively. The column was centrifuged again for 3 minutes at 14,000 x g to dry the column matrix. Finally, DNA was eluted in 50 µl Elution Buffer (preheated 70ºC) and stored at -20ºC until use.

2.2. Biofilm Determination

Biofilm determination assay was performed as described previously by Yu *et al.* (2017). *S. aureus* strains from TSA agar were inoculated in Tryptone Soya Broth (TSB, HiMedia, India) at 37°C. The overnight culture was diluted 1:1000 in fresh TSB or TSB supplemented with 1% glucose (HiMedia, India), then 100 µL of each prepared

suspension above was transferred into sterile 96-well cell culture plates (Biologix, China) in triplicates. After incubated at 37°C for 24 hours, the planktonic cells were washed twice with 300 µL sterile phosphate-buffered saline (PBS, pH 7.4, Sigma, USA) and dried at room temperature for 24 hours. The biofilm formation were then stained with 1% crystal violet (Sigma, USA) for 15 minutes and washed twice by submerging the plate in water. The dye bound to the biofilm matrixs was dissolved with 125 µL of 30% acetic acid (Merck, Germany). The optical density (OD) was measured at 595 nm with EZ Read 400 Microplate Reader (Biochrom, United Kingdom), while the non-inoculated medium was used as blank. Subsequently, blank corrected OD values of *S. aureus* tested were used to determine biofilm production. Isolates were considered biofilm producers when their OD values were greater than ODcut (ODavg of blank + 3 × standard deviation [SD] of ODs of blank). They were classified as no (OD ≤ ODcut), weak (ODcut < OD ≤ 2 × ODcut), moderate (2 × ODcut < OD ≤ 4 × ODcut), or strong (OD > 4 × ODcut) biofilm producers as previously described by (Dai *et al.*, 2019).

2.3. Molecular identification of S. aureus and biofilm-encoding genes

S. aureus isolates from glycerol stocks were reconfirmed with species-specific 23S rRNA target gene according to Straub *et al*. (1999). On the other hand, genotypic determination of biofilm was carried out by detecting the *icaA, icaC* and *icaD* genes as in previous studies (Dai *et al.*, 2019). A total of 25 µl of PCR mixture for target gene amplification consisted of 5 µl of mastermix (5X PCR Master Dye Mix, ExcelTaq, SMOBIO, Taiwan), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 16 µl of DDH_2O, and 2 µl of DNA template. The primers used are listed in **Table 1**, and all were purchased from IDT (Integrated DNA Technologies, Inc., Singapore). The mixture in a 0.2 ml PCR tube (Biologix, China) was then homogenized and centrifuged for a few seconds, then the tube was inserted into the SelectCycler II Thermal Cycler (Select BioProducts, USA). The target gene was amplified using the PCR program as in **Table 1**.

PCR products were separated using 1.5% agarose gel (HiMedia, India) in Tris-borate-EDTA (TBE) buffer [89 mM Tris (pH 7.6) (Tris Base, Sigma, USA), 89 mM boric acid (Sigma, USA), and 2 mM EDTA (HiMedia, India)] and FluoroVue DNA staining (SMOBIO, Taiwan). The gel containing the PCR products and 100 bp DNA ladder

(AccuBand, SMOBIO, Taiwan) were then electrophoresed for 20 minutes (voltage 135 volts) using a submarine electrophoresis system (Mupid-exU, Japan). The presence of PCR products was visualized on an LED Transilluminator (BIO-HELIX, Taiwan). The DNA bands that appear are then compared with a DNA ladder to determine the size of the PCR product.

Table 1. Target genes and primer sequences used for the amplification process.

Gene	Primer sequences	Ta (°C)	Size (bp)	Reference
23S rRNA *S. aureus*	F: ACG GAG TTA CAA AGG ACG AC R: AGC TCA GCC TTA ACG AGT AC	64	1250	Straub *et al*. (1999)
icaA	F: TATACCTTTCTTCGATGTCG R: CTTTCGTTATAACAGGCAAG	52	561	Dai *et al*. (2019)
icaC	F: CTTGGGTATTTGCACGCATT R: GCAATATCATGCCGACACCT	55	209	Dai *et al*. (2019)
icaD	F: ATGGTCAAGCCCAGACAGAG R: CGTGTTTTCAACATTTAATGCAA	55	198	Dai *et al*. (2019)

2.4. Correlation analysis of biofilm production and antibiotic resistance

This study employs correlational analysis to explore the relationships between biofilm OD of 8 isolates and MIC data from previous research (Aziz *et al.*, 2023). We used data of TSB (TSB only), TSB+Glc1% (TSB supplemented 1% glucose), MIC_Oksi (MIC of Tetracycline), and MIC_AMP (MIC of Ampicillin), utilizing R version 4.3.1. As an initial step, the distributional properties of each variable were assessed. The Shapiro-Wilk test was employed to evaluate whether the data followed a normal distribution. Subsequently, the Bartlett test was applied to assess the homogeneity of variance among pairs of variables. Based on these preliminary assessments, an appropriate correlation method was selected. For correlation analysis, if the data were determined to be normally distributed and homoscedastic, the Pearson correlation coefficient was utilized to measure the linear relationship between variables. Conversely, if the data deviated from normality or exhibited heteroscedasticity, the Spearman rank correlation coefficient, a non-parametric alternative, was applied. Additionally, scatterplots were generated using the "ggplot2" library to visually inspect the relationships between variables, offering a graphical representation of the correlation patterns. The "ggcorrplot" library was employed for visualizing the correlation matrix. Data were imported from an Excel file

using the "readxl" library, and the "dplyr" library was utilized for data analysis. Furthermore, for the comparison of means between TSB and TSB+Glc1%, either the Mann-Whitney U test or the independent t-test was conducted, depending on the distribution of the data. P value equal or less than 0.05 are considered significant.

3. Results and Duscussion

Reconfirmation of species by PCR technique showed that 8 isolates grown from glycerol stock in this study were *S. aureus* species (**Figure 1 A**), indicated by a 1250 bp band which corresponds to the 23S rRNA gene target. We also determined the genotypic properties of biofilm by assesing *ica* operon. In contrast of isolate D, the present study showed majority isolates possess *ica*A, *ica*C and *ica*D genes (**Table 2**). **Figure 1B** indicated the PCR products of *ica*A, *ica*C and *ica*D genes were confirmed as expected molecular size, similar with reference cited (**Figure 1B** and **Table 1**).

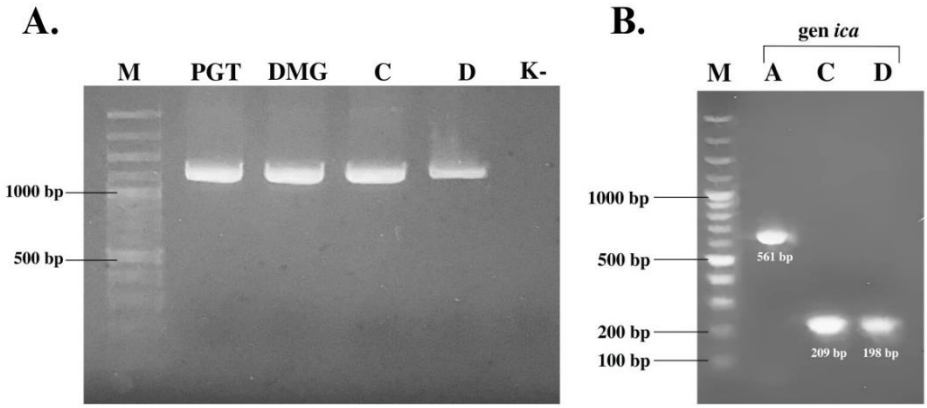

Figure 1. Visualization of PCR products on 23S rRNA and *ica* operon genes. Representative isolates for species spesific by PCR method (A). Biofilm encoded genes of DMG isolate (B).

The microtiter test assay showed that 75% (6/8) isolates in present study had the ability to produce biofilms when the bacterium cultured in TSB medium only (**Figure 2**). By calculating optical density values, those isolates were categorized as weak (+) to moderate (++) biofilm producers. In contrast, the other two isolates do not produce biofilm. Moreover, it's demonstrated that the addition of glucose may affect the ability to produce biofilm. Four out of 8 (50%) isolates showed the increased OD value of biofilm dye when TSB broth supplemented with 1% glucose. In this study, we also noted the mastitis isolates produced biofilm higher than raw meat isolates. Biofilm production assay

in vitro is presented in **Figure 2**. The test was carried out using 96 well culture plate containing TSB or TSB supplemented with 1% glucose. Chrystal violet dye bound to PIA matrix were measured for their optical density by spectrophotometer. The isolates code and biofilm interpretation are indicated. Error bars showed standar deviation from triplicate measurement.

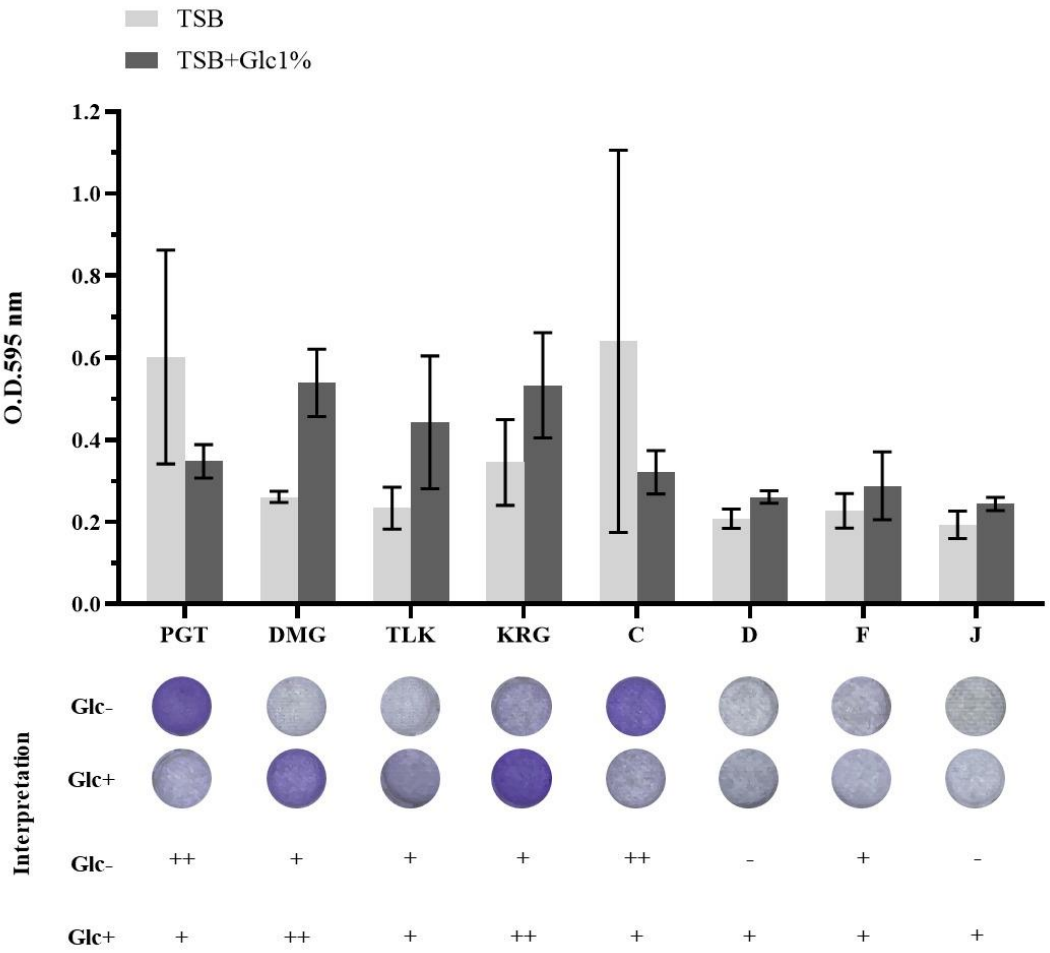

Figure 2. Biofilm production assay in vitro.

In the present study showed the addition of glucose implicated the biofilm production. Mann-Whitney U test was employed to compare TSB and TSB+Glc1%. The test revealed a p-value of 0.41, indicating no statistically significant difference in two datasets. Although statistically not significant, the increase of OD values in overnight cultured TSB medium supplemented with 1% glucose indicated an increase the biofilm production. Vasu *et al.* (2017) study suggested glucose exposure will causes phosphorylated the sugar via glucokinase (*glkA*) to forming glucose-6-phosphate

metabolites, which enters anabolic pathways that promote the production of biofilms in *S. aureus*. Related to the origin of isolate used in this study, this shows that improper handling of animal products with the addition of carbohydrates containing glucose may increase the persistence of *S. aureus* in meat or other food stuff surface. The presence of glucose in the growth medium enhances biofilm formation on different surfaces, such as polystyrene, polypropylene, glass, and stainless steel (Lee *et al.*, 2015). However, the addition of glucose does not always increase biofilm production which presented in TLK and F isolates (**Figure 2**). Michu *et al.* (2011) and Lade *et al.* (2019) studies on *S. aureus* and *S. epidermidis* revealed that although glucose can sometimes promote the production of biofilms, it is not always successful. For example, sodium chloride (NaCl) by itself posed a higher risk of biofilm formation than glucose by itself in milk processing settings. Furthermore, biofilm measurements varied significantly when glucose and NaCl were present simultaneously, indicating that glucose by itself is not a reliable promoter of biofilm formation.

Table 2. The characteristics of biofilm production and antibiotic resistance of 8 isolates in the study.

No	Isolate Code	Isolate origin	Biofilm (TSB w/o Glc-)	Genotype			MIC (Aziz *et al*., 2023)	
				icaA	*icaC*	*icaD*	*Ampicillin*	*Tetracycline*
1	C	Goat milk mastitis	++	+	+	+	< 0.125 (S)	< 0.125 (S)
2	D	Goat milk mastitis	-	-	+	+	< 0.125 (S)	< 0.125 (S)
3	F	Goat milk mastitis	+	+	+	+	< 0.125 (S)	< 0.125 (S)
4	J	Goat milk mastitis	-	+	+	+	< 0.125 (S)	0.5 (S)
5	DMG	Raw chicken meat	++	+	+	+	2 (R)	0.25 (S)
6	TLK	Raw chicken meat	+	+	+	+	32 (R)	256 (R)
7	PGT	Raw chicken meat	+	+	+	+	2 (R)	256 (R)
8	KRG	Raw chicken meat	++	+	+	+	32 (R)	32 (R)

Note: S (Susceptible) or R (Resistant)

Next we explored the association between biofilm production and ampicillin or tetracyline resistance pattern. Multiple studies suggested that biofilm-producing *S. aureus* strains exhibit higher levels of antibiotic resistance compared to non-biofilm producers. Biofilm producers were more resistant to erythromycin, clindamycin, and ciprofloxacin compared to non-biofilm producers (Pokharel *et al.*, 2022; Pokhrel *et al.*, 2024). However, in present study, the results of statistical tests in figure 3 showed no correlation among both parameter.

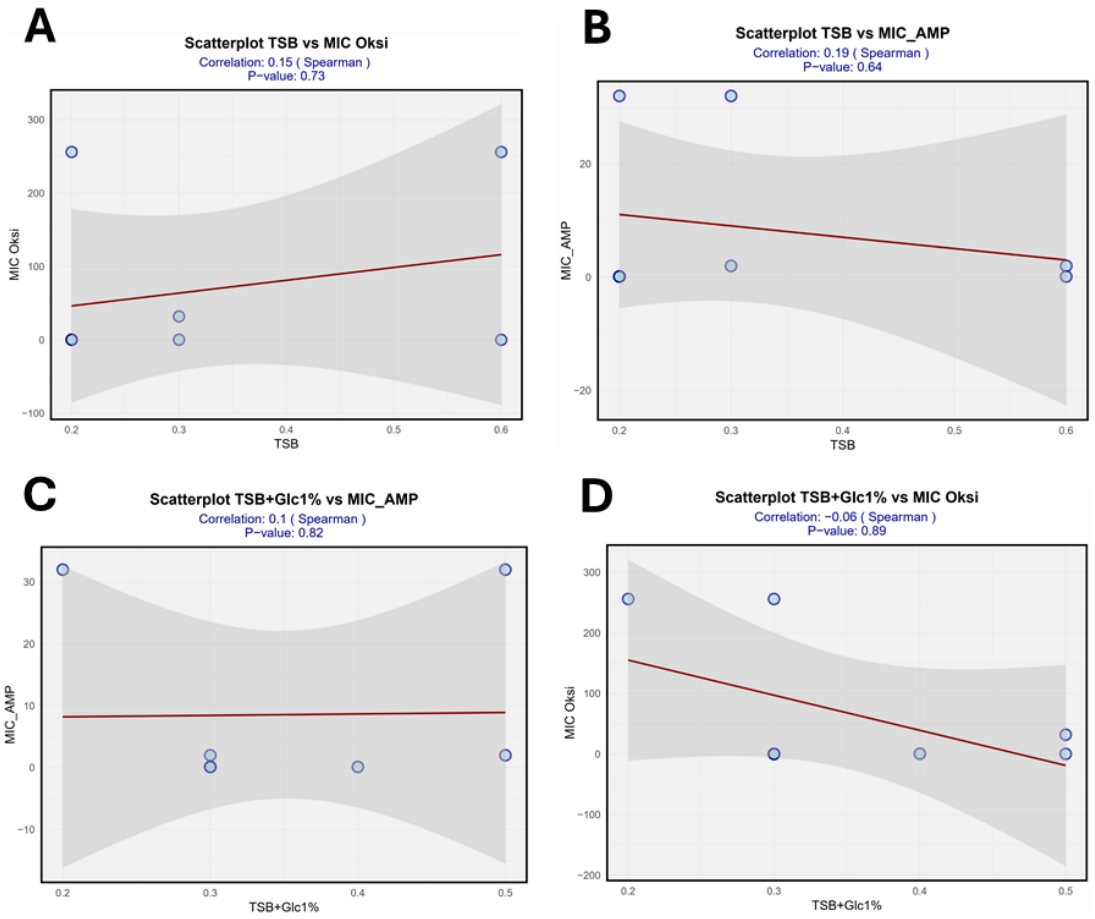

Figure 3. The scatterplots of the corelation between biofilm production and antibiotic resistance pattern. The panel A to D showed parameter tested and Spearman's rank correlation coefficient. P-value of each test was also indicated.

In detail, the analysis of the relationships between TSB, TSB+Glc1% , MIC_Oksi, and MIC_AMP revealed non-normal distributions for most variables, as indicated by the Shapiro-Wilk test: TSB (W=0.71, p=0.00), MIC_Oksi (W=0.61, p=0.00), and MIC_AMP (W=0.60, p=0.00), while TSB+Glc1% showed a non-significant deviation from normality (W=0.86, p=0.12). Homogeneity of variances, assessed by Bartlett's test, was rejected across all comparisons, with p-values consistently below 0.00, indicating significant differences in variances. Given the non-normality and heteroscedasticity, Spearman's rank correlation was chosen, a non-parametric method robust to these violations. For TSB vs MIC_Oksi (**Figure 3A**), a weak positive correlation was observed (rho=0.15, p=0.73); for TSB vs MIC_AMP (**Figure 3B**), a slightly stronger weak positive correlation (rho=0.19, p=0.64); for TSB+Glc1% vs MIC_AMP (**Figure 3C**), a very weak positive correlation (rho=0.10, p=0.82); and for TSB+Glc1% vs MIC_Oksi (**Figure 3D**), a very

weak negative correlation (rho=-0.06, p=0.89). The scatterplots for each correlation would visually depict these relationships, showing scattered points with no clear linear trend, reflecting the weak correlations observed. The scatterplots would show the distribution of the data points, and the lack of a clear linear pattern would visually confirm the low rho values.

Several studies also notable a variation of correlation between biofilm formation and antibiotic resistance. Aniba *et al*. (2024) reported a strong correlation was observed between biofilm-forming ability and antibiotic resistance in *S. haemolyticus* from human urinary tractus infection patients, with multidrug-resistant strains showing higher biofilm production. In contrast, Li *et al*. (2021) reported *Acinetobacter baumannii* isolates from hospital in China showed that non-multi drug resistant (MDR) strains tended to form stronger biofilms than MDR strains. Their suggested a negative correlation between biofilm-forming ability and resistance profiles. Moreover, Donadu *et al*. (2022) study revealed there is no correlation between biofilm-forming capacity and antibiotic resistance of *Staphylococcus spp.*

In this study we demonstrated almost all isolates possesing *ica* gen operon. The *ica* operon is crucial for biofilm formation in *S. aureus*, with the *icaA* and *icaD* genes being particularly important for this process (Torlak *et al.,* 2017). However, the presence of the *ica* operon does not always correlate with biofilm production, as some strains with the operon do not form biofilms (Eftekhar and Dadaei 2011). Chan et al. (2024) study suggested the presence of biofilm-associated genes such as *icaA, icaD*, and *agrI* has been linked to both biofilm formation and antibiotic resistance. For instance, the *agrI* gene was significantly associated with resistance to oxacillin, cefoxitin, and fluoroquinolones. Eventhough the *ica* operon is crucial for the synthesis of PIA as a major component of the biofilm matrix, biofilm formation in *S. aureus* is a complex process regulated by multiple genes and regulatory systems such as *sarA*, *Rbf*, *ArlR-MgrA*, *SigB* and *Rsp* (Cheung *et al.,* 2021; Pokharel *et al.,* 2022; Wu *et al.,* 2024). Our study implies the regulation of biofilm formation in isolates resistant to ampicillin and tetracycline antibiotics may differ from other classes of antibiotics. While there is no direct statistical correlation between biofilm production and MIC values, biofilm formation still significantly impacts the treatment, often requiring higher concentrations of desinfectant for eradication and also protect the bacteria from host immune system. The relationship

is complex and influenced by various factors, including the type of bacteria, and the specific antibiotics used.

4. Conclusion

The majority (6/8) *S. aureus* isolates from animal origin had the ability to produce biofilms and 50% (4/8) showed the response to glucose, increased biofilm production capacity. Almost all isolates possesing *ica* gen operon, while one isolates negative for *icaA* gene. There is no statistical correlation between biofilm production to MIC values of ampicillin and tetracycline antibiotics. Further research is needed to clarify the regulatory mechanism of *S.aureus* biofilm production and their association to ampicillin and tetracycline.

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