



Apple Cider Vinegar as a Natural Antibiofilm and Antibacterial Agent: An *In Vitro* Analysis against *Staphylococcus aureus*

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Abstract

Biofilms are complex, highly structured communities of microorganisms that are encased in a self-produced extracellular polymeric substance (EPS). Due to the high resistance of biofilms to antibiotics, there is a need to investigate alternative therapeutic strategies. This study investigated the efficacy of apple cider vinegar (ACV) in inhibiting *Staphylococcus aureus* biofilms and its antibacterial activity. A total of 21 *S. aureus* isolates were recovered from 30 mastitic cow milk samples. Out of these 21 isolates, 7 representative isolates (reflecting strong, moderate, and weak biofilm producers) were selected for this study. Identification was done through Gram staining and coagulase tests. Biofilm production was performed using the tissue culture plate method in tryptone soy broth and analyzed using an ELISA auto reader at 450nm and 620nm. The results confirmed that 57.14% (4/7) of the isolates were strong, 14.28% (1/7) were moderate, and 28.57% (2/7) were weak biofilm isolates. Antibiofilm activity revealed that 10% ACV completely inhibited the growth of *S. aureus* biofilm; however, 5% ACV was considered the minimum inhibitory concentration (MIC). Based on these findings, apple cider vinegar may be a potential alternative to modern-day antibiotics in combating antimicrobial resistance.

1. Introduction

Biofilms are intricate microbial communities encased in a self-produced extracellular polymeric substance (EPS). These communities adhere to both living and non-living surfaces and exhibit unique characteristics compared to free-floating microorganisms, such as altered gene expression and the formation of an extracellular matrix. Biofilms are notably

resistant to environmental stressors and antibiotics, making biofilm-associated infections significantly more resistant than those caused by planktonic bacteria. These infections are commonly found in hospital settings and can lead to zoonotic human infections, highlighting the critical need to understand biofilm dynamics (Chakraborty *et al.*, 2018; Fazeli-Nasab *et al.*, 2022). Biofilms are implicated in a variety of infections

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across different host species. For example, *Streptococcus agalactiae* biofilms cause mastitis in animals, while *Mycobacterium avium* subspecies *paratuberculosis* biofilms lead to Johne's disease. In dogs, biofilms formed by *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa*, along with yeast pathogens, are primary causes of otitis externa (Chan *et al.*, 2019). In cattle, *Helicobacter pylori* and *Salmonella enterica* biofilms are major contributors to enteritis (Hathroubi *et al.*, 2018). Additionally, *Escherichia coli* and *Enterococcus spp.* Biofilms are commonly associated with urinary tract infections (Al-Saadi *et al.*, 2016; Bortolami *et al.*, 2019).

The researcher has described the effects of numerous antimicrobial products, as well as their effectiveness against various biofilms via their diverse mechanisms. Antimicrobial peptides, for instance, disrupt essential gene expression, contributing to biofilm formation. Enzymes that degrade biofilm play a crucial role in modifying extracellular matrix components, including polysaccharides, proteins, and extracellular DNA. Additionally, quorum-sensing inhibitors hinder cell-to-cell or cell-to-surface bonds, thereby disrupting biofilm formation (Algburi *et al.*, 2017). Antibiotic therapy with certain modifications is also a worthwhile option against biofilm formation, such as a combination of AMPs with antibiotics like ciprofloxacin, which can disintegrate biofilms of antibiotic-resistant *S. aureus* by affecting the cell membranes of biofilm-embedded cells and causing depolarization. AMPs like melamine and Mel4 have been explored to inhibit *S. aureus* biofilm growth at dose rates below their minimum inhibitory concentrations (MICs), whose mechanism of action is moreover similar to that of *S. aureus* biofilms compared to *S. aureus* cells in suspension, developing membrane depolarization and intracellular content release (Moghaddam *et al.*, 2022).

The extracellular polymeric substances (EPSs) matrix protects microbial cells from external stressful stimuli and upgrades horizontal gene transfer. Therefore, the microorganisms present in a biofilm appear resistant to the host's immune response and antibiotics (Moghaddam *et al.*, 2022). However, biofilm tolerance towards antibiotics may develop through various mechanisms, like neutralization, in which anionic EPSs in the biofilm bind to cationic antibiotics like aminoglycosides, thus limiting their efficacy. In addition, the antibiotic resistance-related genes are increased in biofilm production (Tian *et al.*, 2023). Medicinal herbs such as guava leaf extract, *Ricinus communis*, and *Carica papaya*

leaf extract have been shown to reduce chronic generalized gingivitis by suppressing biofilm formation and inflammation (Moghaddam *et al.*, 2022). Furthermore, *Bacopa monnieri*, *Acalypha indica*, and *Calotropis gigantea* extracts, either alone or in combination, have been found to suppress biofilm formation against *Pseudomonas aeruginosa* and *S. aureus*. However, the combination of these extracts inhibits biofilm growth significantly more than the separate extracts, indicating a potentially favorable herb interaction of phytoconstituents (Tanimu *et al.*, 2022).

Recent years have witnessed a surge in interest in researching and developing substitutes to resist microbial battle. Apple cider vinegar (ACV), a derivative of cider, has gained attention for its prospective anti-microbial properties. Usually found in shades from light to deep amber, unpasteurized or organic ACV, characterized by the presence of the "mother of vinegar," has a long history of medicinal use spanning thousands of years, attributed to its broad spectrum of health benefits. Although its antibacterial properties are well acknowledged, the ability of ACV to disrupt biofilms requires further investigation. Nonetheless, ACV shows promise as an alternative to commercial antimicrobial agents, suggesting a need for further research and potential integration into clinical practice (Saqib, 2017). The antibacterial effect of ACV has been evaluated against different bacteria; however, its anti-biofilm activity has not yet been investigated. Therefore, the current study was designed to evaluate the anti-biofilm activity of ACV against *S. aureus* biofilm *in vitro*.

2. Materials and Methods

2.1. Study Approval

The study was approved by the Institutional Biosafety Committee of the University of Agriculture, Faisalabad, Punjab, Pakistan (No. 488/QEC, dated August 22, 2024). Sampling was conducted in accordance with standard protocols and with the prior consent of the dairy herd's authority.

2.2. Sample selection and preparation

Mastitic cow milk samples (n = 30) were collected from dairy herds of various sizes in Punjab, Pakistan, specifically in District Faisalabad. The cows were sampled during episodes of mastitis, rather than on a predetermined timetable. The National Mastitis Council's (NMC, 1999) recommendations were followed when collecting milk samples from cows. After collecting 50 mL sterile tubes, the samples were sent to the lab for further processing on ice.

2.3. Isolation and Identification of *S. aureus*

A total of 21 *Staphylococcus aureus* isolates were recovered from 30 mastitic cow milk samples. Out of these 21 isolates, 7 representative isolates (reflecting strong, moderate, and weak biofilm producers) were selected for this study. In the preliminary phase of this study, a specific strain of *S. aureus*, recognized for its robust biofilm-forming capabilities, was meticulously chosen. This careful selection ensured that the strain possessed the essential characteristics necessary for the investigation's objectives. The selected *S. aureus* strain underwent a series of rigorous identification procedures, comprising both morphological and biochemical analyses. These analyses were crucial in confirming the strain's purity and suitability for the study, ensuring that it met the predetermined criteria for inclusion in the experimental design.

2.4. ACV availability and pH measurement

The ACV used in this study was purchased commercially and kept chilled at 4°C until needed. It was purchased from the local market in Faisalabad, Pakistan. Before commencing experimentation, the pH levels of the ACV samples were meticulously measured using a digital pH meter. This precise measurement ensured that the pH values of the ACV samples were consistent and accurately recorded, providing a standardized basis for subsequent analyses and interpretations.

2.5. Antimicrobial activity of ACV against *S. aureus* isolates

The minimum inhibitory concentration (MIC) of *S. aureus* isolates against ACV was determined using the agar well diffusion method; however, the MICs of penicillin-G and Vancomycin were assessed by the microbroth dilution method on 96-well microtiter plates. As a sensitive quality control, *S. aureus* ATCC (American Type Culture Collection, Rockville, Maryland, USA) 25923 was used (CLSI, 2020). Mueller-Hinton agar plates were prepared and inoculated with *S. aureus* cultures. Wells were created in the agar plates using a sterile pipette tip. ACV was dissolved in water, and serial dilutions were prepared. A specific volume (50 µL) of each dilution was added to the respective wells. The plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition around each well were measured (Figure 3).

2.6. Phenotypic detection of *S. aureus* biofilm

Flat-bottom microtiter plates were used in the microtiter plate approach to quantitatively identify biofilm production

(Adame-Gómez *et al.*, 2020). In brief, tryptone soy broth was used as a liquid medium to produce biofilm using the tissue culture plate method. A loop full of the test organism obtained from an overnight culture on nutrient agar was inoculated into the prepared growth medium. The inoculated broth was then incubated at 37°C for 24 hours to allow the bacteria to proliferate and initiate biofilm formation. Following incubation, the bacterial culture was diluted 1:100 with fresh medium to ensure optimal conditions for biofilm formation. Subsequently, 96-well flat-bottom tissue culture plates were filled with 0.2 mL of the diluted bacterial cultures individually. Sterile broth was added to serve as the blank control, while control organisms were included for comparative analysis. The culture plates were then incubated at 37°C for an additional 24 hours to allow biofilm formation. During this incubation period, the bacteria adhered to the surfaces of the wells and began to produce extracellular polymeric substances, forming mature biofilms. The fixed biofilms were stained with 0.1% crystal violet, allowing for the visualization and quantification of biofilm formation. The excess stain was washed away with deionized water, and the plates were carefully dried to prepare them for subsequent analysis.

2.7. Measurement of biofilm production and Antibiofilm activity of ACV

The optical density of the stained biofilms was measured using a microtiter plate reader at appropriate wavelengths (450 and 620 nm). This measurement provided quantitative data on the extent of biofilm formation, which could be further analyzed statistically to assess the efficacy of antibiofilm agents or to compare biofilm-forming capabilities among different bacterial strains.

The crystal violet staining experiment was used to assess the antibiofilm properties of various ACV doses, aiming to inhibit the production of *S. aureus* biofilms and prevent bacterial colonization. Sterile polystyrene 96-well flat-bottom tissue culture plates served as the experimental platform. Following the establishment of biofilms on these plates using the previously described protocol, the antibiofilm agents were separately added to the wells. The plates were then incubated under control conditions for a specified duration, allowing the anti-biofilm agents to interact with the pre-existing biofilms. After the incubation period, the biofilms were fixed to preserve their structure and integrity for subsequent analysis. Fixation was achieved by

treating the biofilms with sodium acetate, ensuring that they remain intact during the staining process.

2.8. Staining, optical density, and antibiofilm activity measurement

The fixed biofilms were stained with crystal violet, allowing for the visualization and quantification of the adhering bacteria within the biofilms. Excess dye was removed, and the plates were allowed to dry. The microtiter plate reader was operated at a primary wavelength of 620 nm (with 570 nm used as the reference filter during crystal violet absorbance measurement). The optical density measurements obtained from the microtiter plate reader were used to assess the extent of biofilm inhibition by the antibiofilm agents. Three duplicates of each experiment were run. Biofilm inhibition% = (Control OD_{545 nm} – Test OD_{545 nm}) / (Control OD_{545 nm}) × 100 was the formula used to determine the biofilm inhibition percentage (Raja *et al.*, 2011).

2.9. Statistical analysis

Statistical analyses were performed to compare the optical densities of treated and untreated samples, allowing for the determination of the effectiveness of the antibiofilm agents in inhibiting biofilm formation by the *S. aureus* strain. All statistical comparisons were performed using one-way analysis of variance (ANOVA) with a post hoc Bonferroni correction for pairwise comparisons (GraphPad Prism, 5.0). p-value of <0.05 was considered significant.

3. Results and Discussion

3.1. Isolation and Identification of *S. aureus*

Microbiological examination revealed 21 *S. aureus* isolates among 30 milk samples from mastitis cases. Colonies on blood agar plates exhibited typical *S. aureus* morphology, appearing circular, convex, and smooth (Figure 2a). Gram staining revealed that these isolates of *S. aureus* were Gram-positive cocci (Figure 2b). The bacterial strains were cultured on Mueller-Hinton agar. Wells were created with the assistance of a micropipette tip (Figure 2c). These wells were then filled with 5% ACV and incubated for 24 hours. The plates were observed after 24 h visually to confirm the zone of inhibition around the wells. ACV diffused in the agar from the wells and caused the inhibition of bacterial growth in those specific areas. This inverse relationship between the antimicrobial agent concentration and the zone of inhibition is a common observation in antimicrobial susceptibility testing. Higher concentrations of an antimicrobial agent generally led to larger zones of inhibition, indicating greater

Table 1. Minimum inhibitory concentration (MIC) of ACV

Drug name	Drug Conc. µg/ml	Log Conc.	A (Diameter of zone in mm)	Square of zone diameter (A ²)
ACV	500µg/ml	2.7	30 mm	900
ACV	250 µg/ml	2.4	27 mm	729
ACV	125 µg/ml	2.1	24 mm	576
ACV	62.5 µg/ml	1.8	20 mm	400
ACV	31.25µg/ml	1.5	15 mm	225

ACV= Apple cider vinegar, Conc = concentration

antimicrobial potency. By analyzing the zone diameters and their squares, researchers can determine the minimum inhibitory concentration (MIC) of ACV against the tested microorganism. Based on the data provided, the MIC of ACV is likely to be between 31.25µg/ml and 62.5µg/ml, as the diameter starts to decrease significantly at concentrations below 62.5µg/ml (Table 1 and Figure 2d). This information can be valuable in understanding the antimicrobial potency of ACV and its potential application as an alternative or complementary antimicrobial agent against the target pathogen.

3.2. Biofilm production by *S. aureus* isolates

Biofilms were produced in 96-well microtiter plates, and biofilm presence was confirmed using an ELISA reader based on optical density. Biofilm was considered strong when the optical density value was greater than 0.29, moderate when the optical density value ranged from 0.15

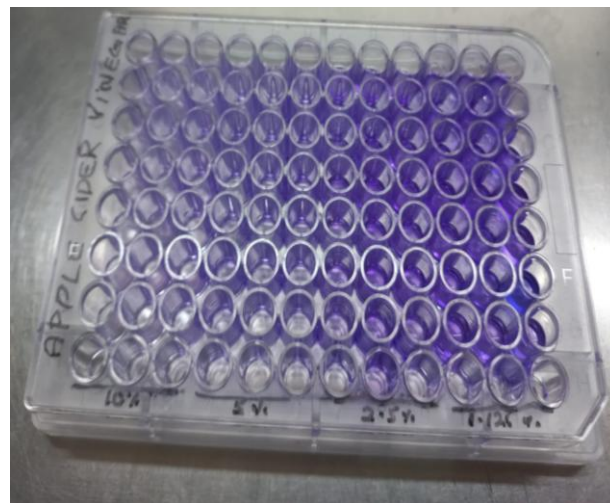


Figure 1. Microtiter plate evaluating the anti-biofilm activity of ACV against *S. aureus* biofilm

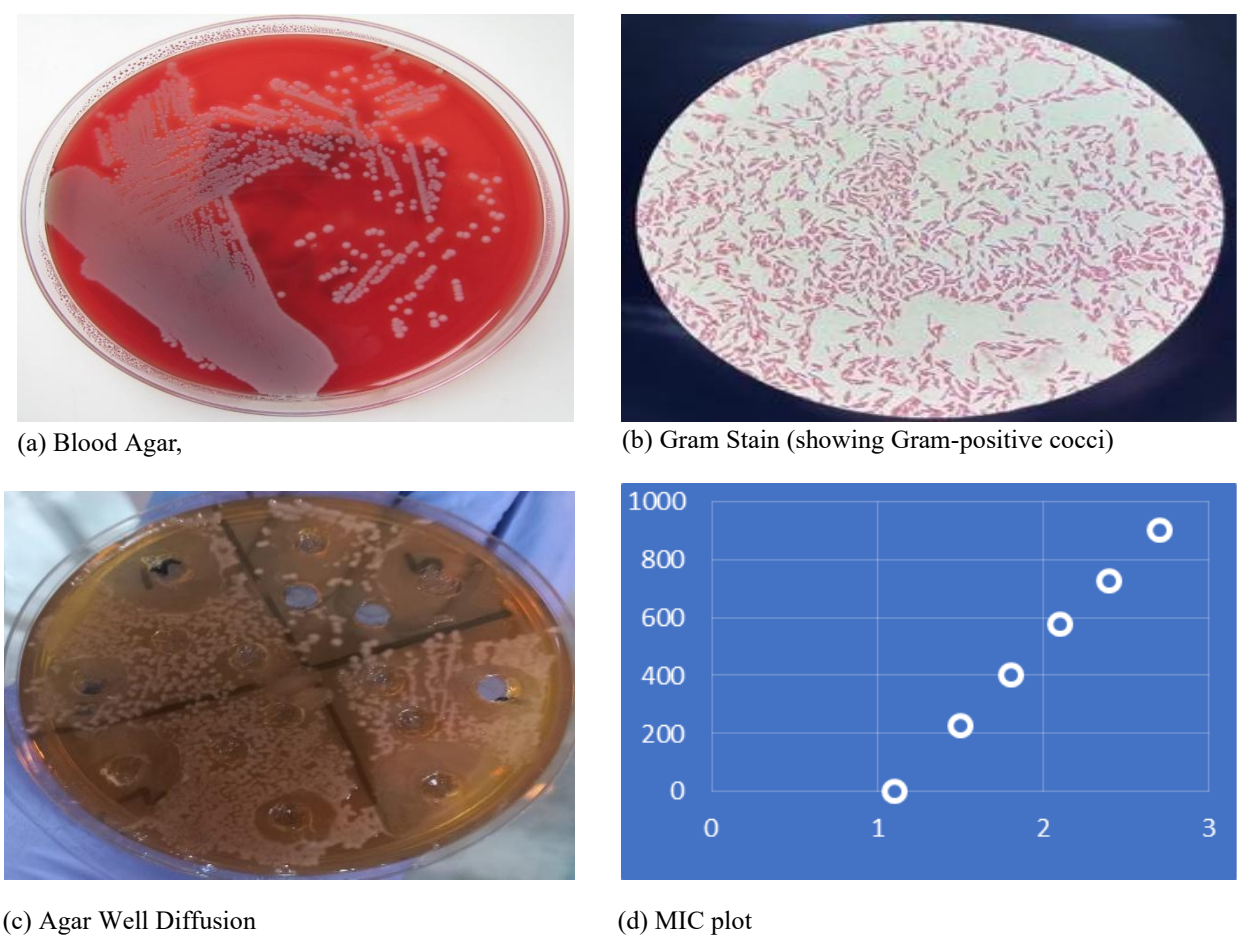


Figure 2. Panel (a) is Blood Agar, panel (b) Gram Stain (showing Gram-positive cocci), panel (c) Agar Well Diffusion, and panel (d) is the MIC plot.

to 0.29, and weak when the optical density value was below 0.15. Optical density values revealed that 57.14% (4/7) of isolates were strong, 14.28% (1/7) were moderate, while 28.57% (2/7) were weak biofilm producers (Figure 3a).

3.3. Anti-biofilm activity of ACV, Penicillin, and Vancomycin

The results regarding the anti-biofilm activity of ACV are indicated in Figure 3b. The data indicated a significant reduction in biofilm formation, with 2.1% of cultures producing strong biofilms, 8.14% producing moderate biofilms, and 89.66% producing weak biofilms. The results regarding the anti-biofilm activity of Penicillin are presented in (Figure 3c). Post-treatment data indicated a significant reduction in biofilm formation, with 4.07% of cultures producing strong biofilms, 10.7% producing

moderate biofilms, and 85.23% producing weak biofilms. The results regarding the anti-biofilm activity of Vancomycin are shown in Figure 3.d. post-treatment data indicated a significant reduction in biofilm formation, with 4.8% of cultures producing strong biofilms, 11.43% producing moderate biofilms, and 84.78% producing weak biofilms.

3.4. Effect of ACV, Penicillin, and Vancomycin with different time intervals

The results regarding the effect of ACV, Penicillin, and Vancomycin with time are indicated in Figures 4, 5, and 6. The biofilm produced was treated with ACV, and the initial results showed a significant decrease in biofilm production. The results for ACV showed that the OD values after 72 hours were significantly lower than the OD values after the

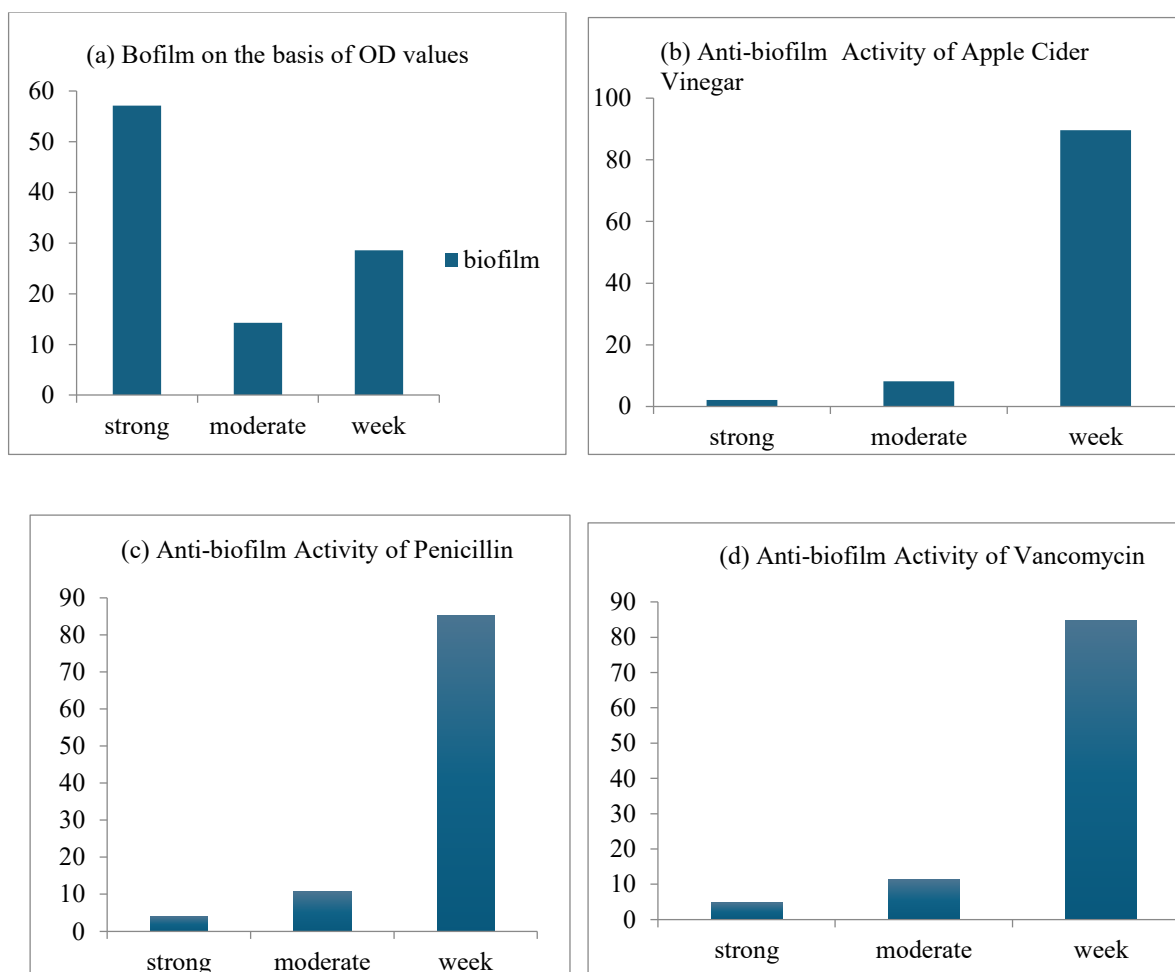


Figure 3. Distribution of the *S. aureus* isolates based on their ability to produce biofilm as strong, moderate, and weak biofilm producers, based on (a) OD values and after treatment with (b) ACV, (c) Penicillin, and (d) Vancomycin

initial treatment with the compound (Figure 4). It is evident from these values that after 72 hours of treatment, the vast majority (89.66%) of isolates were shifted to weak biofilm producers, while only 2.1% remained strong biofilm producers.

The results for penicillin showed that the OD values after 72 hours were significantly lower than the OD values after the initial treatment with the compound (Figure 5). It is evident from these values that 85.23% of isolates were strong biofilm producers, 10.7% were moderate, and the remaining (4.07%) were weak biofilm producers.

The results for vancomycin showed that the OD values after 72 hours were significantly lower than the OD values after the initial treatment with the compound (Figure 6). It is

evident from these values that 84.78% isolates were strong biofilm producers, 11.43% were moderate, and the remaining 4.8% were weak biofilm producers.

3.5. Discussion

This research investigated the antibacterial and anti-biofilm effects of ACV, Penicillin, and vancomycin against *S. aureus in vitro*. Biofilm formation is a common survival strategy for bacteria, enabling them to thrive in various environments, including natural, industrial, and clinical settings (Rather *et al.*, 2021). In this study, as the concentration of the ACV decreased, the diameter of the zone of inhibition also decreased, a typical observation in antimicrobial susceptibility testing. By analyzing the zone diameters and their squares, the researchers determined the MICs of

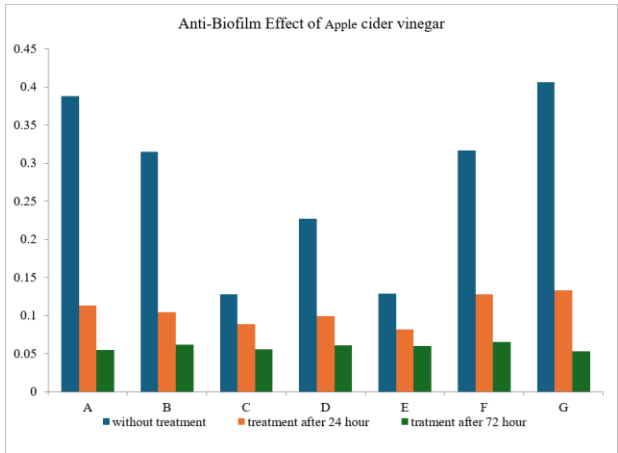


Figure 4. Distribution of the *S. aureus* isolates on their ability to produce biofilm as strong, moderate, and weak biofilm producers based on OD values after treatment with ACV at 24 and 72 hours post-treatment.

penicillin and ACV, providing valuable insights into the antimicrobial potencies of these agents against the tested bacterial strain. The antimicrobial efficacy of ACV was assessed by Saqib (2017), who compared its efficacy with ciprofloxacin. Their finding demonstrates that *S. aureus* was susceptible to 0.31 ± 0.0548 $\mu\text{g/ml}$ of ACV, which supports our finding, as the MIC of ACV was 0.275 $\mu\text{g/ml}$. The study's findings are also supported by Kalaba et al. (2019), who reported an 11.33 mm zone of inhibition produced by ACV against *S. aureus*. Similar findings are recorded in the current study, where ACV produced a 15mm zone of inhibition against *S. aureus*. The bactericidal activity of ACV is attributed to acetic acid, which alters the osmoregulation of *S. aureus* (Shweta et al., 2021). Initially, *S. aureus* cultures showed 57.14% strong, 14.28% moderate, and 28.57% weak biofilm production. Following treatment with ACV, penicillin, and vancomycin, the same cultures exhibited a significant shift in biofilm production, with 2.1%, 4.07%, and 4.8% strong biofilm producers, 8.14%, 10.7%, and 11.43% moderate producers, and 89.66%, 85.23%, and 84.78% weak producers, respectively. This demonstrates the bacteria's high tendency to create resilient biofilms, aligning with existing literature that identifies *S. aureus* as a significant biofilm former in various infections. Their synergistic interactions enhance biofilm stability and resistance to treatment (Baldas et al., 2018). Additionally, this significant reduction in the biofilm-forming ability of cultures suggests that ACV, penicillin, and vancomycin

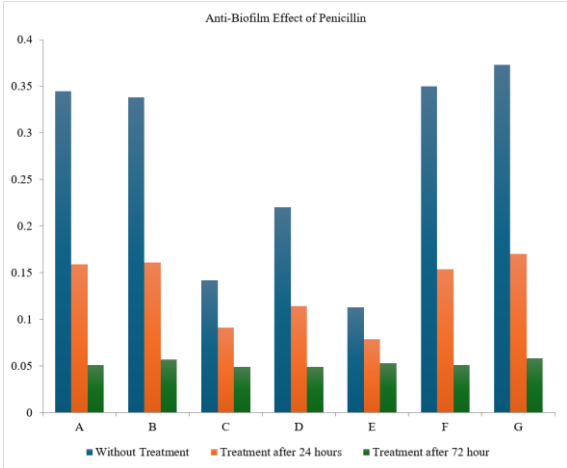


Figure 5. Distribution of the *S. aureus* isolates on their ability to produce biofilm as strong, moderate and weak biofilm producers based on OD values after treatment with penicillin at 24 and 72 hours post-treatment.

effectively disrupt the biofilm structure and reduce the bacterial community's ability to form strong and moderate biofilms. The effect of ACV, penicillin, and vancomycin on the biofilm produced by *S. aureus* was also determined after 72 hours. The OD values indicated that ACV, penicillin, and vancomycin inhibited biofilm formation. The observed antibiofilm activity can be attributed to the multifaceted effects of ACV, penicillin, and vancomycin. *S. aureus* is thought to be the cause of chronic or persistent infections because of its capacity to produce biofilms, which aid in the bacterium's survival under harsh conditions within the host (Christensen et al., 1985; Bernardi and Pizzolitto, 2007). Microtiter plate assays were performed for biofilm detection of *S. aureus*, and out of 946 wells, 98.5% were found to be biofilm producers. Further assessment revealed that 55.67% of these strains were strong biofilm producers. These findings are in line with current research, which confirms that 57.14% of strains were strong biofilm producers. Kivanç (2018) studied the biofilm-producing ability of *S. aureus* in 83 isolates from diabetic patients. They elaborate that 30% of the isolates were weak biofilm producers, 24% were moderate biofilm producers, and 30% were strong biofilm producers. They evaluated the antibiotic susceptibility of these strains, which confirmed that 80% of weak biofilm-producing strains were susceptible to penicillin. However, moderate and vigorous biofilm-producing strains were found to be 14% and 4% susceptible to penicillin, respectively. Biofilm disruption

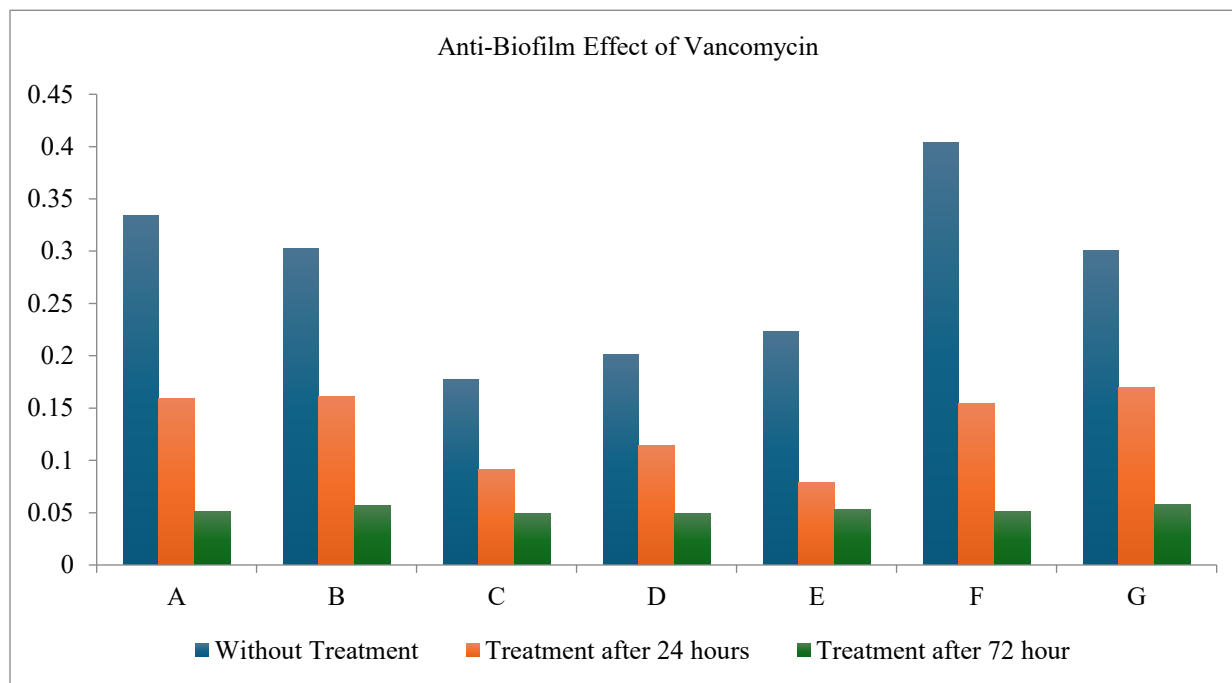


Figure 6. Distribution of the *S. aureus* isolates on their ability to produce biofilm as strong, moderate, and weak biofilm producers based on OD values after treatment with vancomycin at 24 and 72 hours post-treatment

is caused by changing membrane integrity (Savu *et al.*, 2022). These findings support our study, as 85.23% activity was noted against weak biofilm producers, 10.7% against moderate biofilm producers, and 4.07% against strong biofilm-producing strains. The present study is also supported by Nosheen *et al.* (2017), who demonstrated antibacterial activity against *S. aureus*, which inhibits cell wall synthesis (Yao *et al.*, 2011).

Da Costa Júnior *et al.* (2018) studied vancomycin against various species of *Staphylococcus*, including *S. aureus*, and observed that vancomycin has antibiofilm activity against weak strains of *S. aureus*; however, resistance was noted among strong biofilm-producing strains. This study aligns with current research, as 87.78% reductions in weak biofilm-producing strains of *S. aureus* were observed following the action of vancomycin. Within a few years after the beginning of the antibiotic era (Kirby, 1944), *S. aureus* developed antibiotic resistance, which today encompasses all known antibiotic classes (D'Costa *et al.*, 2006). It is concerning that *S. aureus* is becoming increasingly resistant to vancomycin in both wealthy and developing nations. Vancomycin only has a bactericidal effect on Gram-positive bacteria when it prevents NAM-NAG-polypeptide from being incorporated

into the expanding peptidoglycan chain (Chakraborty *et al.*, 2012).

Overall, the findings of this study highlight the significant potential of ACV as an effective antimicrobial agent against *S. aureus*, including drug-resistant and biofilm-forming strains of this bacterium. The superior antimicrobial activity of ACV compared to conventional antibiotics suggests that it could be a promising alternative or complementary approach for managing *S. aureus* infections, particularly in cases where antibiotic resistance and biofilm formation pose significant challenges. These results warrant further investigation into the clinical applications and potential therapeutic uses of ACV in the management of *S. aureus* infections.

Conclusion

The findings of this study illustrate the potent antibacterial and anti-biofilm properties of ACV against *S. aureus* biofilms. The marked reduction in biofilm production, observed in both strong and moderate cases, after treatment underscores the potential of these agents as effective biofilm disruptors. The results suggest that ACV can significantly hinder the biofilm-forming abilities of these bacteria, which holds significant implications for both clinical and industrial applications. Further research is recommended to investigate

the detailed mechanisms of ACV action and its potential synergistic effects with other antimicrobial agents.

Declarations

Ethical Approval

Not applicable

Conflict of Interest

The authors declare no conflict of interest.

Funding

Not applicable

Author Contributions

Muhammad Zakir: Conceptualization, methodology, research trial, and data collection, writing review and editing; Misbah Ijaz: Conceptualization, methodology, supervision, formal analysis, investigation, and writing review and editing; Muhammad Shuaib: Software, formal analysis, data curation and visualization and writing-original draft preparation; Asad Manzoor, Talat Bilal Yasoob, Muhammad Tahir Mohy-ud-Din, Faiza Hassan, Asif Nawaz, Shoaib Sultan, Shahzad Hussain: Data curation, validation, writing-review & editing. All authors have read and approved the final manuscript.

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