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Efficiency of apple cider vinegar individually and in combination with selected antibiotics, and the potential impact of circadian rhythm on bacterial response to treatment in methicillin-resistant *Staphylococcus aureus*

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Abstract

This study evaluated the antibacterial properties of apple cider vinegar (ACV) in two commercial forms, liquid and powder, against Methicillin-resistant *Staphylococcus aureus* (MRSA). The analysis employed the disk diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). Undiluted liquid ACV produced a 7 mm inhibition zone, while the powdered form exhibited inhibition zones of 7 mm, 10 mm, and 12 mm at dilutions of 1:5, 1:125, and 1:40, respectively. The MIC and MBC results were consistent for both forms, with the liquid form achieving MIC and MBC values of 6.25%, and the powdered form showing values of 15.63 mg/mL. Despite variations in geographic origin, the ACV samples demonstrated reproducible antibacterial efficacy. Combining ACV with cefoxitin (Fox30) and vancomycin (VA30) antibiotics, significantly enhanced antibacterial activity, particularly with Fox30, where the inhibition zone increased from 15.6 mm to 19.3 mm. However, combining gentamicin (CN10) with ACV at 500 mg/mL reduced the inhibition zone from 23 mm to 16 mm, indicating an antagonistic effect. Testing the effectiveness of Fox30, VA30, CN10, and neat liquid ACV at three different times of the day (spaced 8 hours apart) revealed no significant time-dependent changes. Slight variations in the powdered form's efficacy warrant further investigation.

Keywords: vinegar, MRSA, antibiotics, synergism, antagonism, MIC, MBC

1. Introduction

Staphylococcus aureus is a bacterium that can exist as part of the normal human microbiota (Chapsa et al., 2023) but is also capable of causing serious infections, as highlighted in numerous studies (Liu et al., 2024). These infections include food poisoning (Pal, 2022), osteomyelitis (Zhang et al., 2021), endocarditis (Chaudry et al., 2018), skin infections (Krasnoselsky

et al., 2020), and bacteremia (Abdollahi et al., 2024). The ability of *S. aureus* to cause a wide range of infections makes it a significant public health concern.

Antibiotics are the primary treatment for bacterial infections and are widely recommended by global health organizations, such as the World Health Organization (WHO, 2024). However, the emergence of antibiotic-resistant strains, particularly Methicillin-resistant *Staphylococcus aureus* (MRSA), has complicated infection management. MRSA arises when *S. aureus* becomes resistant to Methicillin and related antibiotics, leading to treatment failures and increased risks of severe outcomes (WHO, 2024).

MRSA prevalence varies significantly across populations and settings. Among healthy individuals, reported colonization rates include 5.8% among 52 Bhutanese refugees in northeast Ohio, USA (Kadariya et al., 2019), 4.5% among 579 participants in Kirkuk, Iraq (Al-Salihi et al., 2023), and 6% among 100 students in Okada, Nigeria (Okwu et al., 2023). In contrast, hospitalized patients show much higher prevalence rates, such as 51% in an Iranian study (Sarrafzadeh et al., 2021) and 35.5% among 665 patients at Rashid Hospital, Dubai, UAE (Alsabbagh et al., 2023). The WHO recently reported a median global antimicrobial resistance rate of 35% for MRSA across 76 countries, highlighting the growing challenge of effective treatment (WHO, 2023).

MRSA has also been identified in animals and the environment. It has been isolated from mastitic dairy cows (Tesfaye et al., 2021), wild pigeons, and houseflies (Wilson et al., 2024). Additionally, MRSA contamination has been detected on hospital surfaces, such as overbed tables and bedside rails (Kurashige et al., 2016), as well as in public spaces like reception areas and toilet seats (Jaradat et al., 2021).

Vancomycin (VA) remains the first-line treatment for MRSA infections and is recommended by the Infectious Diseases Society of America (IDSA) and the British Society for Antimicrobial Chemotherapy (BSAC) for various conditions, including skin and soft-tissue infections, bone and joint infections, bacteremia, pneumonia, and meningitis (Liu et al., 2011; Brown et al., 2021). Gentamicin (CN), while rarely used alone, is recommended in specific cases, such as catheter-associated urinary tract infections when the MRSA isolate is susceptible (Liu et al., 2011).

Despite its clinical efficacy, VA is associated with several side effects, including acute kidney injury (Barreto et al., 2019), nephrotoxicity (Zasowski et al., 2018), red man syndrome (Levy et al., 1990), bilateral vestibular hypofunction (Zak et al., 2010), and pancytopenia (Afolabi, 2017). Given the potential for resistance to VA and its associated side effects, researchers have increasingly turned to natural antimicrobial agents as alternative treatments. Many plant-based compounds have demonstrated effectiveness against MRSA, including *Nigella sativa* (Abdullah et al., 2022; EI-Majeed et al., 2023), *Caesalpinia sappan*, *Glycyrrhiza uralensis Fisch*, *Sanguisorba officinalis L.*, *Uncaria gambir Roxb* (Jung et al., 2022), *Piper nigrum*, *Curcuma longa* L. (Khan et al., 2024), *Vernonia polyanthes* (Gitirana et al., 2023), and *Cinnamomum burmannii* (Fadlilah et al., 2021).

This study evaluates the antibacterial potential of apple cider vinegar (ACV) against MRSA, both as a standalone agent and in combination with antibiotics like cefoxitin (Fox30), VA30 and CN30. By exploring the synergistic and antagonistic interactions between ACV and these antibiotics, this research aims to contribute to the development of alternative approaches to managing MRSA infections.

2. Materials and Methods

Bacterial strains

Methicillin-sensitive *Staphylococcus aureus* (MSSA, ATCC 29213) was used as a reference strain to confirm the identification of **MRSA** isolate obtained from the College of Medicine

at Mutah University, located in southern Jordan. The bacterial isolate was initially confirmed through Gram staining, followed by susceptibility testing using the FOX30 disk diffusion method (Hudzicki, 2012).

Apple cider vinegar

AVC in liquid form was sourced from a local market (Bab Mecca, Jordan), while the solid (capsule) form was acquired from (*Laboratorios Bio-Dis España, S.L.*Spain).

Chemicals and media preparation

McFarland Standard 0.5:

The McFarland 0.5 standard was prepared by mixing 0.05 mL of 1% barium chloride solution with 9.95 mL of 1.0% sulfuric acid.

Normal Saline:

Normal saline was prepared by dissolving 0.85 g of sodium chloride (NaCl) in distilled water and adjusting the volume to 100 mL.

Gram Stain

Gram stain solutions (crystal violet, Gram's iodine, and safranin) were purchased from (AZ chem, china). 96% ethanol was prepared by adding enough distilled water to 96 mL of ethanol to reach a final volume of 100 mL.

For the bacterial smear, a loopful of bacterial culture was taken from a Mueller-Hinton Agar plate and spread onto a clean slide. The smear was then air-dried before being passed through a benzene burner for fixation.

The Gram stain process was carried out by adding each solution separately: (crystal violet, Grams Iodine, 96% ethanol and safranin). 1min for each; whereas 96% ethanol (the decolorizer) was added for 20 sec.

Muller-Hinton Agar (MHA) and Muller-Hinton Broth (MHB) media:

A. MHA was prepared by suspending 38.0 g of the powder (Liofilchem, Italy) in 1 liter of distilled water. The medium was then sterilized by autoclaving according to the manufacturer's instructions.

B. MHB was prepared by dissolving 21.0 g of the powder (Liofilchem, Italy) in 1 liter of distilled water. Like the agar, this medium was also sterilized in an autoclave according to the company's instructions.

Kirby-Bauer disk diffusion test

An 18-hour *S. aureus* broth culture was adjusted to the McFarland 0.5 turbidity standard by measuring the absorbance at 600 nm using a UV-1601 spectrophotometer (shimadzu,Japan). A sterile swab (moistened with sterile saline) was then used to inoculate the surface of MHA plates. Antibiotic disks of FOX30, VA30, and CN10 (Bio maxima, Poland), as well as AVC, were applied to the plates using sterile forceps. AVC solutions were deposited onto sterile blank disks at the desired concentrations.

The inoculated plates were incubated upside down in an H19000 incubator (Thermolab, India) at 37°C for 24 hours. The diameter of the inhibition zones was measured in millimetres. All tests were performed in triplicates at a minimum.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration tests (MBC).

A. MIC

AVC solutions were tested for their MIC using the broth microdilution method. Bacteria were grown in MHB for 18 hours, after which the bacterial suspension was adjusted to match a 0.5 McFarland standard turbidity by measuring the turbidity at 600 nm. The 0.5 McFarland suspension was then further diluted to a concentration of 10^6 CFU/mL (achieved by adding 9.9 mL of broth to 0.1 mL of the 0.5 McFarland suspension).

Fifty microliters (µL) of the diluted bacterial suspension was added to each well of a 96-well culture plate (Corning Incorporated Costar®, United States), which had been inoculated with 50 µL of the desired extract concentrations dissolved in MHB to reach the 100 µL final well

volume. The plates were then incubated at 37°C for 18-20 hours. The MIC was determined as the lowest concentration of extract that inhibited visible microbial growth.

B. MBC

For the MBC, a loopful was taken from the well that showed the MIC and subcultured onto an agar plate. The first well that did not exhibit any microbial growth was considered the MBC, as it represented the concentration required to kill the bacteria. MBC testing was conducted based on the MIC broth dilution method recommended by- Clinical and Laboratory Standards Institute (CLSI) (2018), with a modification in the subculture volume to improve specificity for our experimental setup.

3. Results

Phenotypic Identification

A.Gram stain

Phenotypic identification of MSSA (ATCC 29213) and MRSA, obtained from the College of Medicine at Mutah University, using Gram staining confirmed that both strains are Gram-positive cocci arranged in grape-like clusters.

B. Susceptibility to Fox30, VA30, and CN10 using Kirby-Bauer disk diffusion test

Susceptibility to Fox30 is presented in Table 1. The inhibition zone was 15 mm for the MRSA strain and 26 mm for the reference strain MSSA (ATCC 29213). As shown in the same table, susceptibility to VA30 was 18.3 mm for the MRSA strain and 18.6 mm for the reference strain. Additionally, the zone diameters for MRSA and the reference strain were 23 mm and 22 mm, respectively, in response to CN10. The inhibition zones for MRSA and the reference strains against the three antibiotics are depicted in Figure 1.

Table 1. Antibiotic susceptibility of MSSA (ATCC 29213) and MRSA to Fox30, VA30, and CN10.

Bacteria	inhibition zone in mm (mean of three tests)		
	Fox30 (µg)	VA30 (µg)	CN10 (µg)
MSSA(ATCC29213)	26 mm	18.6 mm	22 mm
MRSA	15 mm	18.3 mm	23 mm

Figure 1. Comparison of susceptibility of MRSA and MSSA (ATCC 29213) to FOX30, CN10, and VA30

Evaluation of antibacterial activity of ACV: primary screening, MIC, and MBC against MRSA.

The antibacterial activity of ACV against MRSA was evaluated using the disk diffusion method (Table 2). The results demonstrated that neat liquid vinegar produced an inhibition zone of 7 mm. For ACV powder, the inhibition zones were 7 mm, 10 mm, and 12 mm at concentrations of 100 mg, 250 mg, and 500 mg, respectively. However, diluted solutions of liquid ACV (1:5, 1:25, and 1:40) did not exhibit any antibacterial activity against MRSA.

Table 2. Susceptibility of MRSA to different dilutions and concentrations of liquid (neat) and solid (500 mg/mL) ACV.

ACV	Liquid Vinegar(v/v)				Powder Vinegar(mg/ml)		
	Neat	1:5	1:25	1:40	100 mg	250 mg	500 mg
Inhibition zone in mm (mean of three tests)	7 mm	0 mm	0 mm	0 mm	7 mm	10 mm	12 mm

The MIC and MBC results of ACV confirm the findings from the disk diffusion test regarding its efficiency as an antibacterial agent and clarify the effective concentrations. As shown in Table 3 below, the MIC and MBC for liquid vinegar, used at its stock concentration, were both 6.25% for both tests. Similarly, for solid vinegar (500 mg/mL) dissolved in sterile distilled water, the MIC and MBC were both 15.63 mg for both tests.

Table 3. MIC, and MBC of ACV against MRSA

Extracts	MIC	MBC
ACV (liquid) (Neat).	6.25 %	6.25 %
ACV (powder) (500mg).	15.63 mg	15.63 mg

Synergistic effects of ACV combined with Fox30, VA30, and CN10 against MRSA.

The potential synergistic effects of Fox30, VA30, and CN10 in combination with ACV in both liquid (neat) (ACV N) and solid (500 mg/mL) (ACV S500) forms were tested using the combined disk diffusion method. The mean inhibition zone diameters from triplicate tests are shown in Figures 2, 3, and 4. Some results were reconfirmed using the double disk method, as shown in Figure 5. Statistical analysis was performed using the t-test.

A. ACV with Fox30

In the case of Fox30, the inhibition zones were 18 mm and 19.3 mm for combinations with liquid ACV N and solid ACV S500, respectively, compared to 15.6 mm for Fox30 alone (Figure 2). Statistical analysis revealed $p = 0.002$ for ACV N and $p = 0.001$ for ACV S500 when combined with Fox30. Furthermore, a comparison of Fox30 + ACV N versus Fox30 + ACV S500 yielded $p = 0.016$.

B. ACV with VA30

The detailed findings for VA30 revealed inhibition zones of 20.3 mm for both combinations with liquid ACV N and solid ACV S500, compared to 19 mm for VA30 alone (Figure 3). Both combinations demonstrated $p < 0.05$ compared to VA30 alone. In contrast, statistical analysis of VA30 + ACV N versus VA30 + ACV S500 showed no statistically significant difference ($p > 0.05$).

C. ACV with CN10

The results for CN10 demonstrated inhibition zones of 22 mm and 16 mm for combinations with liquid ACV N and solid ACV S500, respectively, compared to 23 mm for CN10 alone (Figure 3). Statistical analysis revealed $p > 0.05$ for the ACV N combination. In contrast, the

combination with ACV S500 showed a significant difference ($p < 0.05$). Furthermore, the comparison between CN10+ ACV N and CN10 + ACV S500 showed weak evidence for a difference ($p = 0.06$).

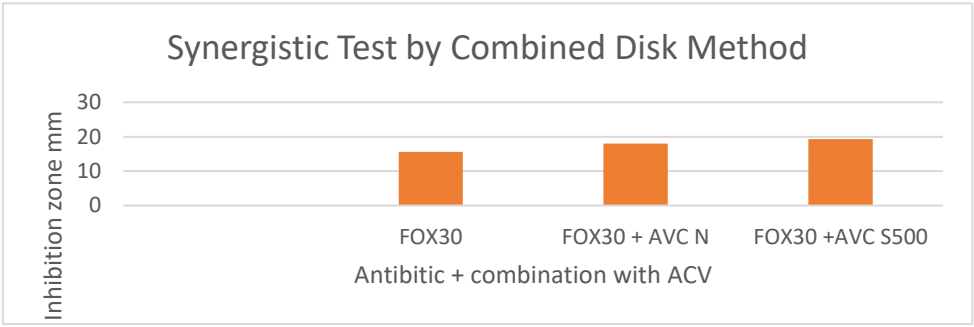

Figure 2. Inhibition zones of Fox30 alone, Fox30 + AVC N, and Fox30 + ACV S500 against MRSA.

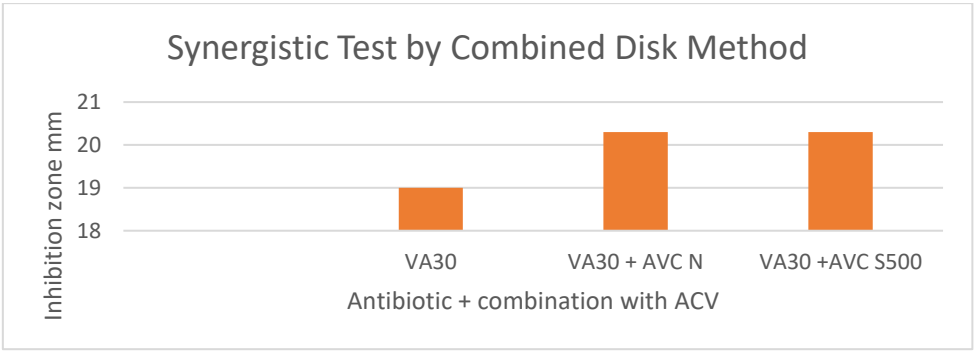

Figure 3. Inhibition zones of VA30 alone, VA30 + AVC N, and VA30 + AVC S500 against MRSA.

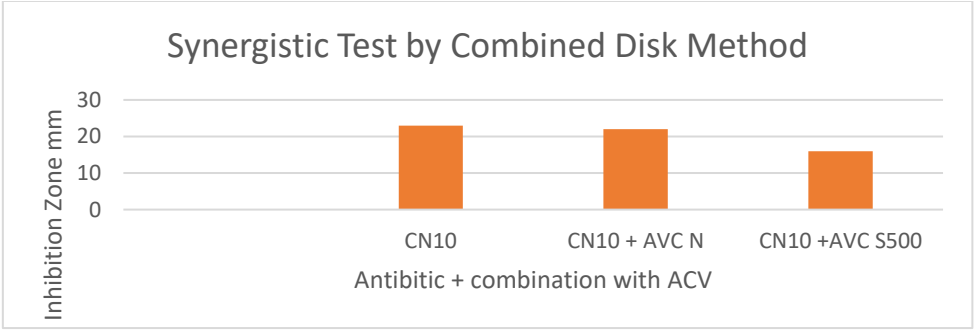

Figure 4. Inhibition zones of CN10 alone, CN10 + AVC N, and CN10 + ACV S500 against MRSA.

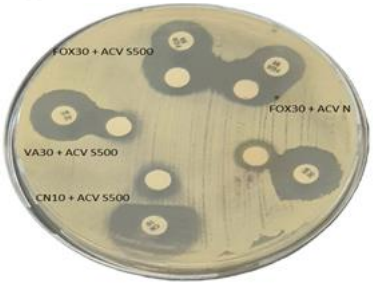

Figure 5. Synergistic test by double disk method of VA30 with ACV S500, CN10 with ACV S500, FOX30 with ACVS 500, and FOX30 with ACV N against MRSA.

Potential Circadian Effect

To investigate the potential circadian effect on the susceptibility of MRSA to Fox30, VA30, CN10, ACV N, and AVC S500 experiments were conducted at three different times of the day (8 AM, 4 PM, and 12 AM). MRSA cultures were prepared 8 hours before the designated treatment times, and the bacterial suspension was adjusted to match a 0.5 McFarland standard turbidity by measuring the turbidity at 600 nm. The results (Table 4) were assessed 24 hours after treatment using the disk diffusion method. Results showed no significant time-dependent variation in the effectiveness of Fox30, VA30, CN10, and ACV N, as the p-values were > 0.05. However, ACV S500 exhibited minor fluctuations in efficacy ($p = 0.045$) between 4 PM and 12 AM.

Table 4. Investigation of the potential effect of treatment time on the efficacy of Fox 30, VA30, CN10, ACV N, and ACV S500.

Treatment time	Antibiotic/ Extraction	Fox30	VA30	CN10	ACV N	ACV S500
8 AM	Inhibition zone mm	15	18	23.33	6.66	11
4 PM	(mean of three tests)	14.66	18.33	24	6.66	9.33
12 AM		14.66	18	23.66	7	10.66

4. Discussion

Phenotypic Identification

Susceptibility to Fox30, VA30, and CN10 using Kirby-Bauer disk diffusion test

The findings provide insights into the resistance and sensitivity patterns of the tested strains. As illustrated in Table 1 and Figure 1, the MRSA strain demonstrated resistance to Fox30, consistent with CLSI guidelines (2018), which classify inhibition zones below the breakpoint as indicative of resistance. In contrast, MSSA showed sensitivity, aligning with its expected susceptibility profile.

For VA30, both MRSA and MSSA exhibited sensitivity. These findings align with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024), which highlights the rarity of VA30-resistant isolates. This consistency underscores the continued efficacy of Fox30 as a reliable treatment option for MRSA and MSSA infections.

Regarding CN10, the sensitivity observed for both strains aligns with EUCAST guidelines, emphasizing its effectiveness against MRSA. The comparable inhibition zones for MRSA and MSSA further confirm this antibiotic's potential role in combating resistant strains.

These findings reinforce the importance of adhering to standardized guidelines in the assessment of antimicrobial susceptibility for accurate diagnosis and effective treatment strategies.

Evaluation of Antibacterial Activity of ACV: Primary Screening, MIC, and MBC Against MRSA

Liquid and solid ACV demonstrated notable antibacterial efficacy against MRSA (Table 2). Yagnik (2014) reported that the minimum dilutions of liquid ACV required for growth inhibition against MRSA in the disk diffusion test were 1:25 for liquid ACV and 200 μg/mL for ACV tablets. In contrast, my study demonstrated that ACV N and 100 mg of ACV powder exhibited inhibitory effects under similar conditions. These findings align with several studies that have highlighted the antibacterial properties of ACV, effectively inhibiting the growth of a wide range of pathogens.

ACV has been shown to act against *Enterococcus faecalis* (*E. faecalis*), a leading cause of root canal failures in dental clinics (Alyamany et al., 2022), as well as clinical strains of *Escherichia coli* (*E. coli*), *S. aureus*, and *Candida albicans*(C. *albicans*) (Yagnik et al., 2018), indicating broad-spectrum efficacy covering both Gram-positive and Gram-negative bacterial species. Furthermore, ACV has exhibited activity against foodborne pathogens such as enterohemorrhagic *E. coli* (EHEC) O157 and *Salmonella serovars* (*S. serovars*) (Entani et al., 1998; El-Demerdash et al., 2021), in addition to MRSA (Yagnik et al., 2014). The broad spectrum of affected organisms suggests that ACV may employ multiple mechanisms to inhibit microbial growth.

Composition analysis of ACV has identified the presence of various organic acids, including acetic, citric, formic, lactic, malic, and succinic acids (Budack et al., 2014), as well as quinic, tartaric, and propanedioic acids (Xia et al., 2020). Additionally, it contains phenolic compounds such as gallic, catechin, epicatechin, chlorogenic, caffeic, and p-coumaric acid (Budack et al., 2014; Xia et al., 2020).

The antibacterial efficacy of weak organic acids is often attributed to their ability to disrupt microbial cells. Dissociated acids release protons outside the cell, while undissociated acids cross the cell membrane and dissociate internally. This process leads to proton export, which consumes cellular adenosine triphosphate (ATP), causing energy production and regulation to become uncoupled. Consequently, internal pH imbalances disrupt enzymatic activity, protein synthesis, and DNA/RNA synthesis (Mani-Lopez et al., 2012; Ricke, 2023).

In a study examining the effects of organic acids on *E. coli*, acetic acid caused the highest membrane damage, while citric and malic acids specifically targeted the cell wall, resulting in alkaline phosphatase leakage. A combination of these acids exhibited broader antimicrobial effects, primarily due to their multiple destructive mechanisms (Ji et al., 2023).

In *S. aureus*, treatment with ACV led to the absence of critical proteins such as chaperone protein DnaK and FtsZ, while in *E. coli*, proteins like DNA starvation protein, citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase were affected. Similarly, in *C. albicans*, enzymes such as pyruvate kinase, 6-phosphogluconate dehydrogenase, and fructose biphosphate aldolase were absent, highlighting ACV's multi-faceted antimicrobial potential (Ricke, 2023).

These findings underscore the diverse and potent antimicrobial mechanisms of ACV and its components. Furthermore, scanning electron microscope (SEM) images revealed significant morphological changes in MRSA treated with *Portulaca oleracea L.* (*P. oleracea L.*) organic acid extracts, including extensive cell wall damage and high levels of protein and nucleic acid leakage (Liu et al., 2023).

Polyphenols, which are secondary plant metabolites, play a vital role in protecting plants from ultraviolet (UV) radiation and diseases. Among these compounds, catechin is known for its antimicrobial activity (Rhman et al., 2022). Caffeic acid has been shown to reduce pyocyanin production and inhibit biofilm formation in *Pseudomonas aeruginosa* (*P. aeruginosa*)

(Ugurlu et al., 2016). Studies on *Tagetes lucida Cav* extracts have identified phenolic compounds, including gallic acid and caffeic acid, with demonstrated antibacterial activity. These compounds permeabilize the membrane of *S. aureus* by forming pores, as observed microscopically, and also cause bacterial DNA fragmentation (Villa-Silva et al., 2020). Since catechin, gallic acid, and caffeic acid are also present in ACV, it is plausible that they contribute to its antibacterial effects. However, it is worth noting that other components in ACV might also play a role in its activity against bacteria, warranting further investigation into its full range of bioactive compounds.

The observed inhibition zones and the MIC/MBC results (Table 3) further corroborate the potential of ACV as a natural antimicrobial agent, emphasizing its relevance in combating antibiotic-resistant strains like MRSA.

The quality of vinegar is influenced by factors such as apple variety and production methods (Kara et al., 2021). Additionally, cider's specific and typical geographical characteristics are well-recognized (Sousa et al., 2020). Given the global nature of vinegar production, variations in the effective MIC and MBC concentrations of ACV are to be expected. Furthermore, MIC and MBC values can differ significantly across various organisms and species. For instance, the MIC of solid ACV against *Salmonella spp.* ranged from 2 to 16 µg/mL (El-Demerdash et al., 2021), while for *Candida spp.*, the MIC was 2500 µg/mL (Mota et al., 2015). In my study, the MIC against MRSA was 15.63 mg/mL, which is within an acceptable range given the complex mixture of compounds in ACV that may contribute to its inhibitory effect. These findings align with studies on *P. oleracea* organic acid extracts, which demonstrated a MIC of 12.5 mg/mL against *S. aureus* (Liu et al., 2023).
For liquid ACV, MICs of 0.625% have been reported against *Streptococcus mutans* (*S. mutans*) and *E. faecalis*, while against *Lactobacillus casei* (*L. casei*), it was 1.25%. In my study, the MIC was 6.25% against MRSA, confirming that MICs can vary among species. The 6.25% MBC for liquid ACV against MRSA observed in my study is similar to the MBC values reported for *S. mutans* and *E. faecalis* 5% in the study by Chandraseharan et al. (2023).

In contrast to previous studies, my research highlights a unique feature: the identical values for MIC and MBC in both the liquid and solid forms of ACV (Table 3). This consistency is noteworthy, as most studies report differences between MIC and MBC values. These results suggest that ACV in both forms has the potential to act as both a bacteriostatic and bactericidal agent at the same concentration, setting it apart from other antimicrobial agents reported in the literature.

Synergistic effects of ACV combined with Fox30, VA30, and CN10 against MRSA.

A. ACV with Fox30

The results demonstrate a notable enhancement of Fox30 antibacterial activity when combined with ACV (Figure 2). Statistical analysis in results confirmed these improvements were significant.

B. ACV with VA30

Similar to the results with Fox30, combinations of liquid and solid ACV with AV30 (Figure 3) demonstrated a statistically significant improvement. whereas, statistical analysis of the effects of liquid ACV N and solid ACV S500 on VA30 revealed no significant difference suggesting that both concentrations of ACV provided similar levels of enhancement and significantly improved VA30 antibacterial activity against MRSA. Further investigations, such as MIC or checkerboard assays, are needed to confirm this observation.

Fox30, a β-lactam antibiotic, and VA30, a glycopeptide antibiotic, are both known to inhibit bacterial cell wall synthesis (Patel et al., 2024; Paterson et al., 2014). In relation to the mechanisms of action of ACV, the citric and malic acids present in ACV target similar cellular structures (Ji et al., 2023). This is further supported by scanning electron microscopy (SEM) images, which showed significant morphological changes and leakage of proteins and nucleic

acids in MRSA after treatment with organic acid extracts (Liu et al., 2023). Specifically, citric acid is known to cause membrane damage (Ji et al., 2023). This effect is also observed with gallic and caffeic acids in *S. aureus* treated with ACV, where membrane pores were microscopically detected, alongside DNA fragmentation (Villa-Silva et al., 2020). Additionally, the heat shock protein, typically involved in protein repair, was absent in *S. aureus* treated with ACV (Yagnik et al., 2018).

Given these observations, combining Fox30 and VN30 with ACV could lead to potential synergistic effects due to the multiple destructive mechanisms involved. Furthermore, the dissociation of organic acids, which release protons outside the bacterial cell and alter the extracellular pH (Mani-Lopez et al., 2012; Ricke, 2023), could enhance the antibacterial effects of Fox30. It is known that Fox30 exhibits increased activity against *S. aureus* as the pH decreases (Tobe et al., 1977), and VA30 is activated in slightly acidic environments (Yu et al., 2018). Thus, the weak acidic environment created by diluted ACV may facilitate the enhanced activity of both antibiotics.

C. ACV with CN10.

In the case of combinations with CN10, statistical analysis revealed no significant difference for the ACV N combination, suggesting that there was no notable change in antibacterial activity between CN10 alone and CN10 combined with ACV N. In contrast, the combination with ACV S500 showed a significant difference, suggesting a potential antagonistic effect. This antagonistic effect of ACV was further supported by a p-value of 0.06 when comparing the two forms of ACV combined with CN10, providing weak evidence for a difference. Figures 4 and 5 illustrate the possible antagonistic effect of solid ACV on CN10.

As discussed earlier, composition analysis of ACV has revealed the presence of various organic acids (Budack et al., 2014; Xia et al., 2020). Some of these acids dissociate outside the bacterial cell, releasing protons, which lowers the extracellular pH (Mani-Lopez et al., 2012; Ricke, 2023). This reduction in pH was shown in this study to inhibit the action of CN10. These findings are consistent with Baudoux (2007), who reported that gentamicin's efficacy is significantly hindered as the pH decreases from neutral to acidic levels. Specifically, MIC and MBC values increased by 72-fold when the pH was reduced from 7.4 to 5 during tests against *S. aureus*.

Gentamicin, an aminoglycoside antibiotic, weakens bacterial protein synthesis by binding to ribosomes (Mingeot-Leclercq et al., 1999; Karunarathna & Bandara, 2024). It targets the 30S ribosomal subunit, causing misreading of the genetic code and inhibiting translocation (Yoshizawa et al., 1998). Aminoglycosides are large, highly polar molecules, and their penetration through porin channels in the cell membrane is unlikely. Instead, they electrostatically bind to anionic sites on the cell surface, particularly phospholipids and teichoic acids, during the energy-independent phase of antibiotic uptake (Taber et al., 1987). The subsequent transport across the cytoplasmic membrane requires energy and is referred to as the energy-dependent phase, which is inhibited by low pH (Mingeot-Leclercq et al., 1999).

Since the dissociated organic acids in ACV lower the pH (Ricke, 2023), it becomes evident that the inhibitory effect of ACV on CN10, as demonstrated in this study, is linked to this mechanism. Once inside the cell, undissociated organic acids from ACV cross the membrane and dissociate, leading to proton export and ATP consumption. This disrupts the coupling of energy production and regulation, depriving CN10 of the energy necessary for its binding to ribosomes (Mani-Lopez et al., 2012; Ricke, 2023).

In summary, the decrease in acidity caused by vinegar inhibits CN10 entry into the cell and its binding to ribosomes. This inhibition, driven by reduced pH, disrupts the energy-dependent processes required for CN10 uptake and activity. As a result, CN10 effectiveness is diminished, as it is unable to efficiently bind to ribosomes and interfere with bacterial protein synthesis. These findings further highlight the significant impact of pH changes on the antimicrobial activity of antibiotics, as demonstrated in this study.

It is noteworthy that the ACV N used in this study was a Jordanian product, while the ACV S500 was sourced from Spain. These geographical origins, along with factors such as apple variety, production methods, and cider-specific characteristics, may have influenced the observed effects (Villa-Silva et al., 2020; Kara et al., 2021). However, it is evident that ACV causes cell envelope damage, which could support the action of Fox30 and VA30. Conversely, changes in extracellular pH may lead to antagonistic effects when ACV is combined with CN10 as a result of ATP consumption.

Potential Circadian

Results that showed no significant time-dependent variation in the effectiveness of Fox30, VA30, CN10, and ACV N, indicating the stability of their efficacy throughout the 24 hours. Whereas ACV S500 exhibited minor fluctuations in efficacy between 4 PM and 12 AM, suggesting a potential circadian.

Life on Earth has evolved in tandem with the sun, with nearly all fundamental biological processes closely linked to its daily cycles of light and darkness. In mammals, the circadian clock is regulated by feedback loops involving activator and repressor transcription factors (TFs) that drive oscillatory transcription and translation, recurring approximately every 24 hours (Kim &Lazer, 2021). Melatonin, a hormone secreted by the pineal gland during the night, serves as a critical time cue for the biological clock. In humans, the sharp increase in sleep propensity typically occurs about two hours after the onset of endogenous melatonin production (Zisape,2018).

Interestingly, melatonin also appears to play a role in microbial behavior. Colonies of *E. aerogenes* were found to proliferate significantly faster in the presence of melatonin, with maximal growth responses observed within the physiological range of gut melatonin levels (paulose et al., 2016). This observation supports the hypothesis that the host's circadian clock regulates the enteric microbiome. MRSA, as both a human commensal and a pathogen (Chapsa et al.,2023; Liu et al., 2024) is inherently influenced by host physiology. It cannot be fully studied in isolation from the human body's effects. Melatonin has demonstrated antimicrobial effects against MRSA, suggesting that this pathogen may also be susceptible to host circadian influences (Tekbas- et al.,2008). In the present in vitro study, the efficacy of Fox30, VA30, CN10, and ACV against MRSA was evaluated under controlled incubation conditions. The results indicated that the stability of these agents' antimicrobial activities was unaffected by the controlled environment, which could be considered an advantage for their reliability as effective treatment options.

Bridging in Vitro Findings to Clinical Applications: The Role of in Vivo Studies

It is essential to acknowledge the limitations of in vitro studies in replicating the complexity of human physiological conditions. Further in vivo studies are necessary to explore how factors such as circadian rhythms, host-microbe interactions, and dynamic environmental changes might influence the efficacy of antibacterial agents against MRSA. Such investigations would provide deeper insights into optimize ng treatment strategies for MRSA infections in real-world scenarios.

This study highlights the promising in vitro effects of apple cider vinegar (ACV) and antibiotics, but in vivo studies are essential to validate these findings in a biological environment. Future research should focus on testing ACV-antibiotic combinations in animal models to evaluate efficacy, safety, and dosage optimization.

Clinically, combining ACV with antibiotics could enhance antimicrobial effectiveness, reduce required doses, and address antibiotic resistance. Preclinical and clinical studies are needed to confirm these benefits and explore their potential for treating resistant infections. By addressing these gaps, future work can translate laboratory findings into practical therapeutic strategies.

5. Conclusions

The identical MIC and MBC values for ACV highlight its unique bactericidal efficacy, comparable to Fox30 and VA30, though it should be avoided with CN10 due to its antagonistic effect. In vitro stability was confirmed for Fox30, VA30, CN10, and ACV, with minor fluctuations observed for ACV S500 in time-dependent treatments. Further in vivo studies are essential for a deeper understanding of ACV's potential and interactions.

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Conflict of interests

The authors affirm that they have no competing interests to disclose.

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