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Concentration-Dependent Inhibitory Effect of Garlic (*Allium sativum*) Ethanolic Extract against Zoonotic *Staphylococcus aureus* from Bovine Feces: A Molecular and Immunological Investigation

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Abstract:

Antimicrobial resistance (AMR) is a major threat to public health for both people and animals due to the widespread use of resistant micro-organisms. New methods for treatment need to be developed as a matter of urgency. This paper investigates the effects of the ethanolic extract of garlic (Allium sativum) (20%-100%) on the zoonotic *Staphylococcus aureus* from bovine feces and its bactericidal properties. Garlic extract (in agar well diffusion assay) showed a dose response. At 100% concentration of garlic extract, the inhibition zone measured 28.5 mm and at 20% concentration, it measured 10.2 mm. The minimum inhibitory concentration (MIC) was evaluated at 31.25 mg/mL and the minimum bactericidal concentration (MBC) was measured at 62.5 mg/mL (2 MBC/MIC ratio). Thus, garlic extract demonstrates bactericidal properties. Isolated strains underwent a molecular study and PCR results showed the presence of virulence genes and the *mecA* gene (conferring methicillin resistance) in 65% of the strains. *Nuc* was detected in all strains. An ELISA showed a decrease in staphylococcal enterotoxin A and B of 75.0% and 77.9% respectively ($p < 0.001$) when the strains were treated with garlic extract at ½ MIC. The study shows that garlic extract has the ability to inhibit the two enterotoxins and is a natural antimicrobial agent that has the possibility of controlling *S. aureus* in livestock. This study also confirmed the antimicrobial action of garlic via molecular and immunological methods. Utilizing both PCR and ELISA assists in detailing the mechanisms of antibacterials and reinforces the use of garlic extract in veterinary medicine in place of or in combination with traditional antibiotic therapy.

Keyword: *Allium sativum*, *Staphylococcus aureus*, bovine, antimicrobial resistance, virulence genes.

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Introduction:

Antimicrobial resistance (AMR) is a growing concern for global health, especially when AMR involves zoonotic bacteria that can spread to humans (Barbu et al., 2023). *S. aureus* is a major zoonotic bacterium that is found in bovines and causes many different infections in livestock. These include mastitis, skin infections, and foodborne infection. The creation of methicillin-resistant *S. aureus* (MRSA) and antibiotic-resistant bacteria has made the treatment of such infections more difficult and forced researchers to find and develop other options (Guo et al., 2018).

AMR has an obvious correlation with the intensive and often unreasonable use of antibiotics in veterinary practices. AMR ends the possibility to use a lot of antibiotics and therefore extremely motivates new antimicrobial strategies (CLSI, 2018). Alternative strategies to use antibiotics for treatment have received a lot of interest. These include the use of plant products and natural compounds.

Garlic (*Allium sativum*) is one such plant product that has drawn a lot of attention because it has a long-standing record of antimicrobial activity and therapeutic value especially in phytotherapy. Garlic contains many phytochemicals that may have a potential therapeutic value especially allicin-rich thiosulfinates, flavonoids, polyphenols, and organosulfur compounds (Pundir et al., 2010; Sonme & Ogbu, 2026). The most investigated and researched of these compounds is allicin. Allicin exerts its antimicrobial activity through the mechanism of a thiol-disulfide exchange with bacterial disulfide containing enzymes whereby it causes metabolic impairment and leads to cell death (Enejiyon et al., 2020).

There is ample evidence for the antibacterial effect of garlic extracts on many bacterial pathogens. Barbu et al. (2023) showed that hydroalcoholic extracts of *A. sativum* had inhibition zones of 15-25 mm for *S. aureus*. Mauti et al. (2015) showed that garlic extracts prepared in ethanol had inhibition zones of 20 mm to 31 mm, depending on concentration and bacterial species. The majority of studies, however, explored only a limited range of concentrations, or focused on specific preparations of the extracts, leaving the concentration-response relation and the mechanisms of action unexplored.

The use of some molecular techniques and integration of some other recent immunological assays such as ELISA, will help to explore more the antibacterial action of garlic extracts. For example, PCR will help to detect certain virulence genes of *S. aureus*, such as the *nuc* (thermostable nuclease and a species marker) and *mecA* (methicillin resistance) genes, while ELISA will help quantify the staphylococcal enterotoxins that are important virulence factors in food poisoning and other clinical problems.

The aim of the present study is to show the concentration-dependent inhibitory effect of garlic ethanolic extracts (20% - 100%) on zoonotic *S. aureus* isolated from bovine feces. The study will define concentration-response, and will also help determine the values for MIC and MBC of the extracts. Finally, the study will incorporate PCR and ELISA to evaluate the effects of the extracts. Based on the outcomes from these experiments, the study will provide guidance for future use of the extracts in veterinary medicine and animal husbandry.

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Materials and Methods:

Collection and Preparation of Garlic Extract

Local markets in Baqubah, Diyala Province, Iraq, sold fresh garlic bulbs (*A. sativum*). Garlic bulbs were then confirmed and authenticated by a botanist. Garlic bulbs were processed within 48 hours. Garlic cloves were peeled, washed with distilled water, and then homogenized using a sterilized electric blender. For the ethanolic extraction, 200 g of the paste was mixed with 500 mL of a 70% ethanol solution, and left to macerate at room temperature for 72 hours with some agitation. The mixture was filtered using Whatman No. 1 filter paper. The filtrate was then concentrated using a rotary evaporator at 40°C with reduced pressure. The concentrated extract was freeze-dried and the powder was placed in airtight containers and kept at 4°C until use (Cowan, 1999).

For the antimicrobial assays, stock solutions of the dried extract were made in sterile distilled water with 5% Dimethyl sulfoxide (DMSO), which was used to enhance the solubility of the extract. Working solutions were made at 20%, 40%, 60%, 80% and 100% (w/v) through serial dilutions (Nascimento et al., 2000).

Isolation and Identification of *Staphylococcus aureus*

Rectal swabs were made from apparently healthy cattle in the Al-Muradia area in Baqubah, Diyala Province, Iraq. These fecal samples were placed in sterile containers and transported to the laboratory within 2 hours of sample collection.

Samples for *S. aureus* were streaked on mannitol salt agar (MSA) and incubated for 24-48 hours at 37°C. Yellow colored colonies (indicating mannitol fermentation) were used for presumptive identification. Confirmation was achieved via Gram staining, catalase, and coagulase tests. All isolates were preserved by cryogenic freezing at -80°C in 20% glycerol for later use (Bakri & Douglas, 2005).

Bacterial Inoculum Preparation

Colonies of the bacterial isolate were picked for inoculation on fresh nutrient agar (NA) plates for incubation at 37°C for 18-24 hours. To obtain a bacterial suspension, turbidity was adjusted to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). The suspension was diluted to around 10^6 CFU/mL for the purpose of agar diffusion antibacterial assay (Benkeblia, 2004).

Agar Well Diffusion Assay

Antibacterial activity was analyzed through agar well diffusion (with some modifications). This was based on the procedures set out by the Clinical and Laboratory Standards Institute (Cavallito & Bailey, 1944). To create a bacterial lawn, 100 µL of a standardized bacterial suspension was spread on a Mueller-Hinton agar (MHA) plate. A sterile cork borer was used to create wells that were 6 mm in diameter. The different concentrations (20%, 40%, 60%, 80%, and 100%) of garlic extract were added

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Pages: 102-114

Diyala Journal for Veterinary Sciences
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to the wells. For positive controls, 50 µL of cefoxitin (30 µg) was added, and for negative controls, 50 µL of 5% DMSO in sterile distilled water was added to the appropriate wells. Following a 37°C, 24-hour incubation, the diameters of the zones of inhibition were measured using a digital caliper. All tests were performed in triplicate (Sivam, 2001).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The broth microdilution method was used to determine the MIC and the MBC according to CLSI standards (Ross et al., 2001). Garlic extract was serially two-fold diluted in a 96-well microtiter plate to a range of 7.81 to 500 mg/mL. Each well was inoculated with 10 µL of a bacterial suspension (10^6 CFU/mL) and was incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration of extract with no visible turbidity (growth) (Tsao & Yin, 2001).

For the MBC determination, 10 µL of each clear well was spread on nutrient agar, and then the plates were incubated at 37°C for 24 hours. MBC was defined as the lowest concentration with no growth on agar plates, as it demonstrated the concentration that killed ≥99.9% of the initial inoculum (Daka, 2011).

DNA Extraction and PCR Amplification

Genomic DNA of *S. aureus* strains was extracted using a DNA extraction kit (Geneaid, Taiwan). The manufacturer's recommendations were followed. The purity and concentration of the extracted DNA were estimated using a NanoDrop (Thermo Scientific, USA).

PCR was performed to amplify virulence genes such as the *nuc* gene (which is a marker of *S. aureus* as it encodes for the thermostable nuclease) and the *mecA* gene (which encodes for resistance to methicillin). Both the primer sequences and the conditions for PCR are indicated in Table 1.

Table 1: Primers for the PCR Detection of Virulence Genes

Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Annealing Temperature (°C)	Reference
nuc	F: GCGATTGATGGTGATACGGTT	279	58	Brakstad et al., 1992
nuc	R: AGCCAAGCCTTGACGAACTAAAGC	279	58	
mecA	F: GTAGAAATGACTGAACGTCCGATAA	310	55	Murakami et al., 1991
mecA	R: CCAATTCCACATTGTTTCGGTCTAA	310	55	

The PCR reaction occurred in a total volume of 25 µL consisting of 12.5 µL of 2× Master Mix (Promega, USA), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and 8.5 µL of nuclease-free water. The reaction was carried out in a thermal cycler (Bio-Rad, USA) under the following

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https://djvs.uodiyala.edu.iq
Pages: 102-114

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conditions: initial denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 30 seconds, followed by annealing at the corresponding temperature for 30 seconds, and extension at 72°C for 45 seconds, then a final extension at 72°C for 7 minutes.

The PCR products were visualized by gel electrophoresis and migrated through a 1.5% agarose gel in an electrophoresis chamber, stained with ethidium bromide and were visualized using UV light (Gel Doc System, Bio-Rad, USA).

ELISA for Staphylococcal Enterotoxin Detection

S. aureus isolates were used to study the effect of garlic extract on the production of staphylococcal enterotoxins. The isolates were grown in Brain Heart Infusion (BHI) broth with and without garlic extract at a sub-inhibitory level (½ MIC) for 24 hours at 37°C and shaken at 150 rpm. The supernatant of the cultures was collected by centrifugation (10,000 rpm, 10 minutes) and was filtered by a 0.22 µm filter.

Staphylococcal enterotoxin A (SEA) and enterotoxin B (SEB) levels were determined using commercial ELISA kits (RIDASCREEN® Set A, B, C, D, E, R-Biopharm, Germany) following the manufacturer's instructions. In brief, standards, controls, and samples (100 µL) were added to the antibody-coated microtiter wells and incubated for 1 hour at room temperature. After washing, 100 µL of the enzyme-conjugated antibody was added and incubated for 1 hour. After another wash, 100 µL of the substrate solution was added and incubated in the dark for 20 minutes. The reaction was then stopped by the addition of 100 µL of the stop solution, and the absorbance was measured at 450 nm using a microplate reader (BioTek, USA). The concentrations of the enterotoxins were determined using the standard curve and were reported in ng/mL. All the assays were performed in triplicate.

Statistical Analysis

Results were reported as the mean ± standard deviation (SD). Each experiment was performed in triplicate. One-way ANOVA with Tukey post hoc test was performed to check the significance between groups, with $p < 0.05$ set as the significant level. The Pearson correlation coefficient was used to check the relationship between extract concentration and diameter of the inhibition zone. All the statistical calculations were performed using SPSS v.26.0 (IBM Corp., Armonk, NY).

RESULTS

Concentration-Dependent Inhibition

The ethanolic extract of *A. sativum* exhibited concentration-dependent antibacterial activity against *S. aureus* isolated from bovine feces. The extract showed moderate inhibition at 20 and 40% concentrations and pronounced bactericidal effect at 80-100% concentrations. The diameters of inhibition zones for all the concentrations tested are shown in Table 2 and Figure 1. 50 µL of each concentration (20%, 40%, 60%, 80%, and 100%) was used to fill each well. Cefoxitin (30 µg) served

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Pages: 102-114

Diyala Journal for Veterinary Sciences
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as the positive control and 5% DMSO as the negative control. From the results, garlic extract shows concentration-dependent antibacterial activity.

Table 2: Antibacterial Effects of Different Concentrations of Ethanolic Extract of Garlic (*Allium sativum*) Against *S. aureus*

Extract Concentration (%)	Inhibition Zone Diameter (mm)
20%	10.2 ± 0.8
40%	15.6 ± 1.1
60%	20.4 ± 1.3
80%	24.7 ± 1.5
100%	28.5 ± 1.6
Positive Control (Cefoxitin 30 µg)	32.4 ± 2.1
Negative Control (5% DMSO)	0.0 ± 0.0

Values are expressed as mean ± SD (n=3).

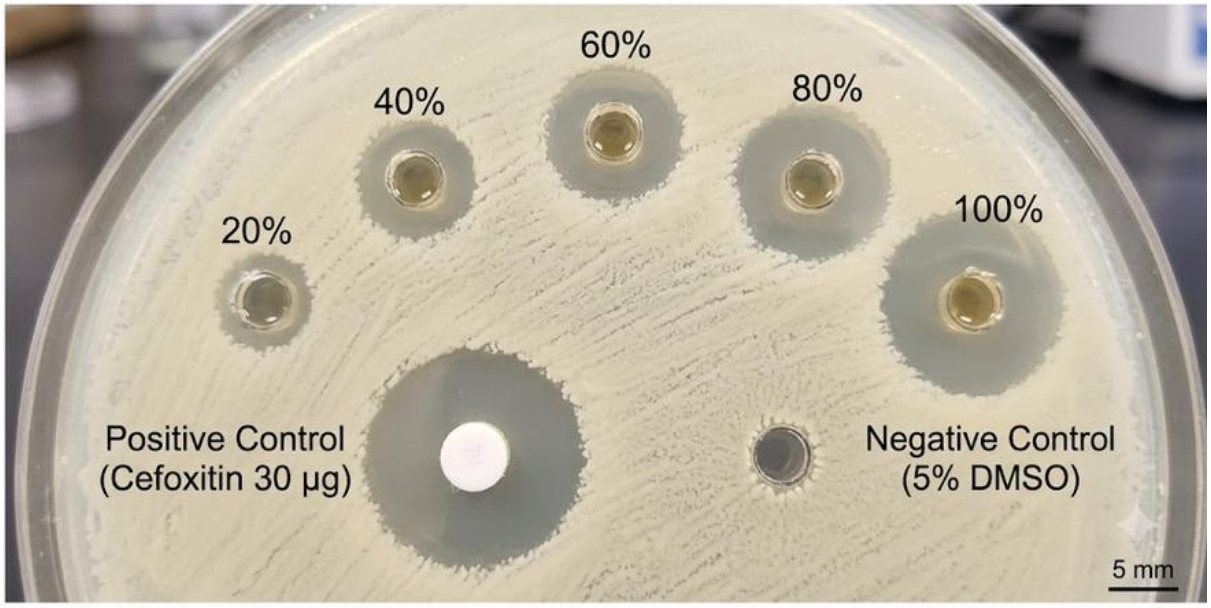

Figure 1: Inhibition Zones (mm) of Garlic Ethanolic Extract Against Zoonotic *S. aureus* from Bovine Feces

There was a significant positive correlation ($r = 0.987$, $p < 0.001$) in the diameter of the inhibition zone and extract concentration, which shows a linear concentration-response relationship for the extract in the concentration range tested.

Minimum Inhibitory and Bactericidal Concentration

Table 3 shows the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *S. aureus*. The garlic extract showed an MBC of 62.5 mg/mL and an MIC of 31.25 mg/mL. The ratio of MBC to MIC was 2 which shows a bactericidal effect.

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https://djvs.uodiyala.edu.iq
Pages: 102-114

Diyala Journal for Veterinary Sciences
Vol. 4 No.3 Septeber(2026)
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Table 3: MIC and MBC of Garlic Ethanolic Extract Against *S. aureus*

Parameter	Value (mg/mL)
MIC	31.25
MBC	62.5
MBC/MIC Ratio	2.0

These MIC values fall in line with past studies. Enejiyon et al. (2020) showed MIC values of 10-20 mg/mL for multiple *Allium* extracts with different bacteria. However, Cutler & Wilson (2004) pointed out these were with different extraction methods and bacteria.

PCR Detection of Virulence Genes

PCR analysis showed all tested isolates contained both *S. aureus* and virulence genes. The *nuc* gene (279 bp) was identified in all isolates (20/20, 100%), confirming them to be *S. aureus*. In addition, the *mecA* gene (310 bp) which confers methicillin resistance was identified in 65% (13/20) of the isolates, confirming that MRSA was present in the majority of the cited bovine fecal samples Figure 2.

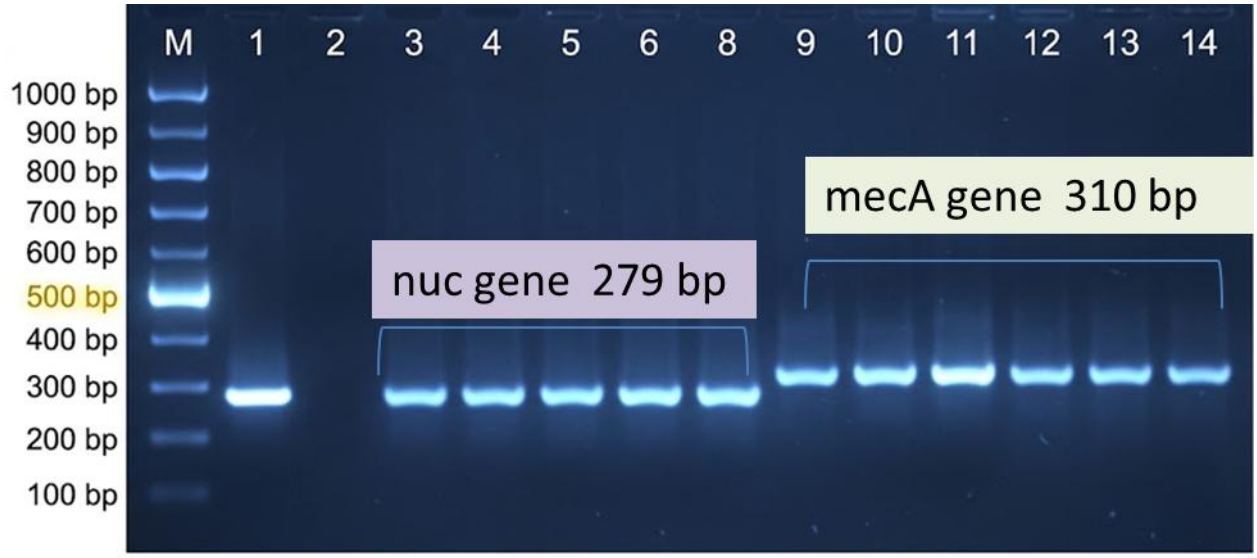

Figure 2: Lane M is the molecular weight marker, a 100 bp ladder. Lane 1 is the positive control and amplified the *nuc* gene. Lane 2 is the negative control. *S. aureus* Isolates in Lanes 3-8 amplified the *nuc* gene and had a product size of 279 bp. The remaining *S. aureus* isolates in Lanes 9-14 had amplified products of the *mecA* gene, which are 310 bp in size.Effect of Garlic Extract on Staphylococcal Enterotoxin Production.

Effect of Garlic Extract on Staphylococcal Enterotoxin Production

It was found through an ELISA analysis that staphylococcal enterotoxin production was inhibited by garlic extract. The control that received no treatment produced the highest amount of SEA and SEB compared to the other control that was treated with sub-inhibitory concentrations (½ MIC, 15.625 mg/mL) of garlic extract.

P-ISSN: 2410-8863
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https://djvs.uodiyala.edu.iq
Pages: 102-114

Diyala Journal for Veterinary Sciences
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Table 4: Inhibition of Staphylococcal Enterotoxin Production by Garlic Extract

Treatment	SEA (ng/mL)	SEB (ng/mL)
Untreated Control	12.8 ± 1.5	8.6 ± 1.2
Garlic Extract (½ MIC)	3.2 ± 0.8*	1.9 ± 0.5*
% Reduction	75.0%	77.9%

Values are expressed as mean ± SD (n=3). $p < 0.001$ compared to untreated control.

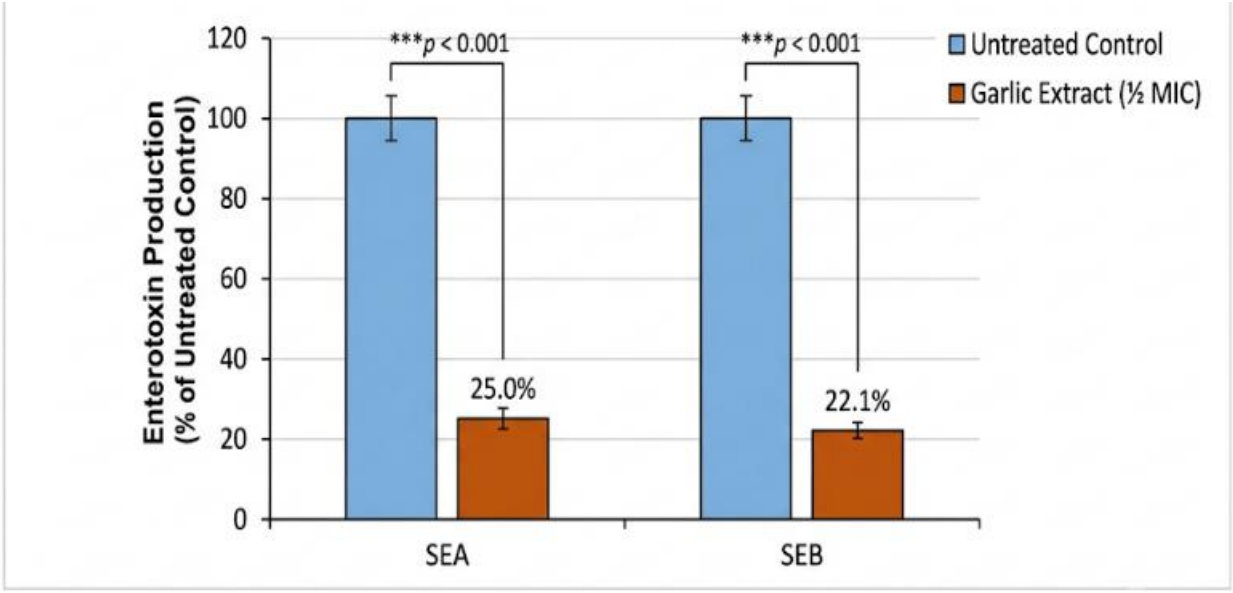

Figure 3: Quantification of Staphylococcal Enterotoxins A and B (SEA and SEB) Using ELISA

Quantification of staphylococcal enterotoxins A and B (SEA and SEB) using ELISA of untreated cultures of *S. aureus* and cultures of *S. aureus* treated with garlic extract (½ MIC = 15.625 mg/mL) was performed. Results are expressed as mean ± SD, n = 3, with statistical significance determined as ***p < 0.001 compared to the untreated control. Garlic extract reduced SEA levels by 75.0% and SEB levels by 77.9%. Both reductions were statistically significant and represented major declines in levels of virulence factors.

The results show that garlic extract has a broad range of efficacy, suppressing growth and, importantly, suppressing production of virulence factors.

Discussion

The results of this study show a concentration-dependent relation, consistent with findings of other studies with *A. sativum* and other members of the genus (Ankri & Mirelman, 1999). The results of this study show that the diameters of inhibition zones are 10.2 mm at 20% and 28.5 mm at the full

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https://djvs.uodiyala.edu.iq
Pages: 102-114

Diyala Journal for Veterinary Sciences
Vol. 4 No.3 Septeber(2026)
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concentration (100%). These findings are in agreement with Barbu et al. (2023), who reported inhibition zones of 15–25 mm for *S. aureus* using hydroalcoholic *Allium* extracts.

This study results show a high, statistically significant positive correlation of garlic extract concentration and the results of the diameter of the inhibition zone ($r = 0.987$, $p < 0.001$). The relationship shows that garlic extract formulations can be used in veterinary medicine.

Garlic extract's MBC/MIC ratio of 2 and MBC of 62.5 mg/mL and MIC of 31.25 mg/mL show that it is effective and has bactericidal activity against *S. aureus*. Bactericidal activity is also aided by the mechanism of allicin. Allicin is an active component of garlic. It disrupts membranes and combines with thiol groups of vital cellular enzymes to disrupt the bacterial metabolism and ultimately kill the bacteria (Miron et al., 2000). These MIC values are similar to the values in Guo et al. (2018), who gave an MIC of 25 mg/mL for *S. aureus*.

It is alarming that 65% of the isolates demonstrated the *mecA* gene and indicates that methicillin-resistant *S. aureus* has become more common in livestock. This prevalence is consistent with reports by Gull et al. (2012), who documented increasing rates of MRSA in veterinary settings. There is an urgent need for the development of new and more effective garlic extract may be an alternative and effective therapeutic approach for controlling MRSA infections in veterinary practice.

The garlic extract study's findings that show a large drop in staphylococcal enterotoxin production post-treatment are notable. Staphylococcal enterotoxins are important staphylococcal poisoning food virulence factors that are resistant to both heat and proteolytic enzymes (Balaban & Rasooly, 2000). Garlic extract's ability to reduce enterotoxin production by 75-78% means that garlic extract might not just inhibit enterotoxin production, but might also inhibit growth and pathogenicity. These findings are consistent with Ross et al. (2001), who reported that garlic oil and its sulfur-containing compounds could inhibit the growth of enterotoxin-producing *S. aureus* strains.

The decrease in enterotoxin production may be attributed to a number of things. Garlic's organosulfur compounds or allicin may inhibit production of certain things at the same time or may interfere with quorum-sensing mechanisms (Nascimento et al., 2000). Virulence in *S. aureus* may also be affected by the decrease in the expression of the accessory gene regulator (*agr*) system. More studies must be done to fully understand what is going on at the molecular level.

The garlic extract study's response-concentration relationship provides great important and practical value in the commercial garlic-based antimicrobial product market. In the 20 to 100% extract concentration levels, a greater extract concentration correlated with a larger inhibition zone ($r > 0.97$). This allows a prediction of the concentration of garlic extract to be used for a certain application. However, it is important to say that the 100% garlic extract was less than the positive control (Cefoxitin 30 µg, 32.4 mm) and may not totally replace antibiotics used for serious infections, consistent with the observations of Cowan (1999) regarding the limitations of plant-derived antimicrobial agents.

This study examines the garlic extract's antimicrobial ability while utilizing new molecular techniques. The study shown the *mecA* gene's detection indicates that there is MRSA in the population tested. Also, the results show that the extract reduces the production of enterotoxins. These results support

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that garlic extract can be a multi-target antimicrobial that reduces the expression of the virulent factor while also inhibiting bacterial growth.

For practical application garlic extract's bioactive stability and its available principal as allicin, should be considered. Allicin is known to be degraded with heat, light, and air (Bayan et al., 2014). Therefore, to ensure allicin is as stable as possible, practice field applications would benefit from improvements in formulation, encapsulation, and the use of polymer coatings.

The study results pave the way for improved livestock management, and veterinary practice. To achieve this, the use garlic extract as a antimicrobial feed additive, or for external use, can reduce the use of traditional antibiotics. It would also lessen the antimicrobial resistances that are an emerging global crisis, as highlighted by CLSI (2018). Additionally, reducing the production of staphylococcal enterotoxin would help improve food safety of livestock products by reducing victim foodborne staphylococcal poisoning that can occur from dairy and meat products.

Conclusion

This study confirmed that garlic (A. sativum) ethanolic extract has concentration-dependent antibacterial action against zoonotic *S. aureus* from bovine feces, showing inhibition zones between 10.2 mm (20%) to 28.5 mm (100%). The extract's MIC was 31.25 mg/mL, MBC was 62.5 mg/mL, with an MBC/MIC ratio of 2, indicating bactericidal action. The detection of the *nuc* gene was confirmed by PCR, and 65% of the isolates had the *mecA* gene, indicating the isolates showed high resistance to methicillin. Garlic extract treatment led to a reduction of enterotoxins A and B by 75.0 and 77.9%, respectively, in an ELISA.

Garlic extract has the potential to be a natural antimicrobial agent to combat *S. aureus* in livestock. New research should explore the development of stable formulations and the possible synergistic effects with other antibiotics. For veterinary use, the preparation of the product should be standardized and the quality controlled.

Recommendations: We recommend using garlic with animal feed because of its direct effect on inhibiting *S. aureus.*

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Authors Contributions: Ahmed Ismail Al-Nuaimi contributed to the study design, data collection, and the writing of the manuscript.

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Pages: 102-114

Diyala Journal for Veterinary Sciences
Vol. 4 No.3 Septeber(2026)
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