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Clove Oil Modulates Biofilm-Associated Gene Expression in Oral Staphylococcus Aureus Compared with Tetracycline

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ABSTRACT: Oral Staphylococcus aureus can persist through adhesion and biofilm-associated pathways, and antimicrobial exposure below the minimum inhibitory concentration may alter virulence-gene transcription without completely suppressing growth. This study evaluated the effects of commercial clove oil and tetracycline on the relative expression of fnbA, icaC, and icaD in clinical oral S. aureus. Two hundred oral specimens were cultured, 75 isolates were identified as S. aureus, and four isolates (1, 5, 7, and 8) were selected for MIC/sub-MIC and transcriptional analysis. MICs were determined by resazurin-based broth microdilution. Isolate-specific sub-MIC exposures were followed by one-step RT-qPCR using 16S rRNA as the reference transcript and the 2^(−ΔΔCt) method. Clove-oil exposure reduced all three targets across the four isolates, with mean fold changes of 0.531 for fnbA, 0.445 for icaC, and 0.560 for icaD. Tetracycline increased fnbA and icaD to 1.178- and 1.555-fold, respectively, while reducing icaC to 0.596-fold. Exact Friedman tests showed overall condition effects for fnbA and icaD (P=0.0046) and icaC (P=0.0417), whereas Holm-adjusted pairwise comparisons were not significant. Commercial clove oil therefore produced a consistent downward transcriptional pattern, while tetracycline produced gene-dependent responses. These findings support further investigation of clove-oil constituents as modulators of biofilm-associated virulence pathways.

1. INTRODUCTION

The oral cavity can serve as an independent reservoir for *Staphylococcus aureus*, including methicillin-resistant strains. A recent genomic study of dental patients recovered *S. aureus* from oral and nasal sites and demonstrated clinically relevant oral carriage, including an association with denture use (Kawayanagi et al., 2025). Biofilm formation further supports persistence on oral and prosthetic surfaces, as demonstrated by Mohammed and Al-Mathkhury (2023), who evaluated *S. aureus* growth and biofilm-associated gene expression on denture materials.

Biofilm development in *S. aureus* is a multigenic process involving polysaccharide-associated and protein-mediated adhesion pathways. Recent clinical studies have documented frequent detection of *ica* genes and adhesion determinants such as *fnbA* among biofilm-forming isolates, while RT-PCR studies have confirmed expression of *icaA* and *icaD* (Jomehzadeh and Emrani, 2025; Armoon et al., 2024). Al-Khazraji and Aburesha (2026) further showed that probiotic stress can alter the expression of adhesion-related genes in clinical *S. aureus*, supporting the use of transcript-level analysis when evaluating anti-biofilm interventions.

Natural products are increasingly investigated as sources of antimicrobial and anti-virulence compounds. Recent microarray work showed that sub-inhibitory eugenol exposure produces widespread gene-expression changes in methicillin-resistant *S. aureus* (Buru et al., 2022), while newer studies demonstrate that plant-derived compounds can suppress *S. aureus* biofilm regulatory pathways and that clove essential oil has measurable antibacterial and antibiofilm activity (Liu et al., 2024; Qureshi et al., 2025). This direction is also supported by Iraqi studies: Kadhim and Saadedin (2024) used resazurin microdilution to evaluate a plant extract against *S. aureus*, and Maha et al. (2024) documented antibacterial activity of *Myrtus communis* phenolic compounds against *S. aureus*.

Antibiotic exposure below the MIC can modify *S. aureus* physiology and virulence without producing a uniform transcriptional response. Liu et al. (2023) showed directly that sub-MIC tetracycline and streptomycin enhanced *S. aureus* biofilm formation and produced transcriptomic and RT-qPCR changes in biofilm-associated regulatory genes, while Omar et al. (2024) demonstrated altered biofilm and virulence-associated gene expression after sub-MIC oxacillin exposure. These observations support gene-specific interpretation of sub-MIC responses rather than assuming that all virulence pathways will move in the same direction.

Against this background, the present study asked whether isolate-specific sub-MIC exposure to commercial clove oil and tetracycline produces distinct transcriptional responses in the biofilm-associated genes *fnbA*, *icaC*, and *icaD* of clinical oral *S. aureus* isolates.

2. MATERIALS AND METHODS

2.1. Study design, specimen collection, and bacterial identification

A total of 200 oral specimens, including saliva, tongue-dorsum swabs, and dental-plaque samples, were collected aseptically from patients attending hospitals in Iraq between November 2025 and January 2026. Specimens were transported in sterile tubes for microbiological examination. Samples were inoculated onto nutrient agar, mannitol salt agar, and blood agar and incubated aerobically at 37°C for 24 h. Presumptive *Staphylococcus aureus* colonies were evaluated by standard biochemical methods and confirmed using the VITEK® 2 automated identification system. Seventy-five isolates were identified as *S. aureus*. Four isolates, designated 1, 5, 7, and 8, were used for the subsequent MIC/sub-MIC and RT-qPCR analyses. Laboratory procedures were performed at Al-Suwaira General Hospital and the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

Ethical approval for this study was granted by the Research Ethics Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq.

2.2. Preparation of tetracycline and clove oil

Tetracycline hydrochloride powder with a stated purity of 96% was obtained from Thermo Scientific Chemicals, China (catalogue B21408.14; lot 10252519; CAS 64-75-5). A primary solution of 25 mg/mL was prepared in sterile distilled water. A 32 µg/mL working stock was prepared by diluting 12.8 µL of the primary solution to a final volume of 10 mL with sterile distilled water.

Commercial clove oil was obtained from Hemani Herbal, Karachi, Pakistan. Dimethyl sulfoxide (DMSO) was used as a dispersing vehicle before serial dilution in Mueller-Hinton broth. One milliliter of clove oil was mixed with 1 mL DMSO, and the resulting dispersion was passed through a 0.22-µm syringe filter before use. Mueller-Hinton broth (Oxoid, England) was used in the MIC and sub-MIC exposure procedures.

2.3. MIC and sub-MIC determination

MICs were determined using a resazurin-based broth microdilution procedure, consistent with recent microtiter-resazurin testing applied to plant-derived antimicrobials against *S. aureus* (Kadhim and Saadedin, 2024). Fresh bacterial colonies were suspended to 0.5 McFarland and diluted 1:100 in double-strength Mueller-Hinton broth to obtain an approximate final inoculum of 1×10^6 CFU/mL after equal-volume mixing. Tetracycline was tested in two-fold serial concentrations from 16 to 0.5 µg/mL. Clove oil was tested at nominal final dilutions of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256.

Microplates were incubated aerobically at 37°C for 24 h. Resazurin (5 µL of 0.015% solution) was then added to each well, followed by incubation at 37°C for an additional 4 h. Blue or purple wells were interpreted as inhibited metabolic growth,

whereas pink wells indicated bacterial metabolic activity. The MIC was defined as the lowest tested concentration that prevented the resazurin color change. The next lower concentration was designated the sub-MIC used for transcriptional exposure.

2.4. Sub-MIC exposure

Standardized inocula were incubated statically in Mueller-Hinton broth at 37°C for 24 h under three experimental conditions: untreated control, isolate-specific sub-MIC tetracycline, and isolate-specific sub-MIC clove oil. Tetracycline exposure solutions were prepared in sterile distilled water, whereas clove-oil exposure medium was prepared using the clove oil-DMSO dispersion described above.

2.5. RNA extraction and quantification

Following exposure, bacterial cells were harvested and total RNA was extracted using the Presto™ Mini RNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's bacterial protocol. Bacterial pellets were treated with lysozyme-containing lysis buffer, followed by RB buffer and β-mercaptoethanol. After addition of 70% ethanol, lysates were loaded onto RB columns, washed, dried, and eluted with RNase-free water. RNA concentration was measured using the QuantiFluor® RNA System with a Quantus™ Fluorometer (Promega, USA). RNA samples were standardized to 20 ng/μL before one-step RT-qPCR analysis.

2.6. One-step RT-qPCR

One-step reverse-transcription quantitative PCR was performed on a qTOWER³ real-time PCR system (Analytik Jena, Germany) using Luna® Universal One-Step RT-qPCR reagents (New England Biolabs, USA). Relative expression of *fnbA*, *icaC*, and *icaD* was evaluated using 16S rRNA as the reference transcript (Aziz and Ghaima, 2025). Primer sequences and amplicon sizes are shown in Table 1. Reaction composition and cycling conditions are shown in Tables 2 and 3.

Table 1. Primers used for one-step RT-qPCR.

Target	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon (bp)
fnbA	TCATGCAGGTCAAAGAGCGG	CAGTAGCCATTACGACAGAGCC	167
icaC	GCGTTAGCAAATGGAGACTATTGG	TGCGTGCAAATACCCAAGATAACA	89
icaD	TCGCTATATCGTGTGTCTTTTGGA	CGCGAAAATGCCCATAGTTTCA	101
16S rRNA	CACCTTCGGATACGGGTACC	GTTGACTGCCGGTGACAAAC	372

Table 2. One-step RT-qPCR reaction components.

Component	Volume	Final concentration
Luna Universal One-Step Reaction Mix (2×)	10 μL	1×
Luna WarmStart® RT Enzyme Mix (20×)	1 μL	1×
Forward primer (10 pmol/μL)	0.8 μL	0.4 μM
Reverse primer (10 pmol/μL)	0.8 μL	0.4 μM
RNA template (standardized to 20 ng/μL)	As required	—
Nuclease-free water	To 20 μL	—

Table 3. One-step RT-qPCR amplification conditions.

Step	Temperature	Time	Cycles
Reverse transcription	55°C	10 min	1
Initial denaturation	95°C	1 min	1
Denaturation	95°C	10 s	40
Annealing/extension	60°C	30 s	40
Melt-curve analysis	60–95°C	0.5°C increments/15 s	1

2.7. Relative expression analysis

Relative transcript levels were calculated by the comparative cycle-threshold approach and interpreted with reference to current quantitative real-time PCR guidance (Bustin et al., 2025). For each sample, ΔCt was calculated as Ct(target) − Ct(16S rRNA). For each treated isolate, ΔΔCt was calculated as ΔCt(treated) − ΔCt(untreated control), and relative expression was calculated as 2^(−ΔΔCt). Fold-change values above 1 were interpreted as relative upregulation and values below 1 as relative downregulation.

2.8. Statistical analysis

Fold-change values were summarized descriptively as mean ± standard deviation (SD), while inferential testing was performed on ΔCt values. Because the same four biological isolates were examined under untreated-control, tetracycline, and clove-oil conditions, paired non-parametric procedures were used separately for each target gene. Exact Friedman permutation P values were obtained by exhaustive enumeration of within-isolate condition permutations, and Kendall's W was reported as effect size. Pairwise comparisons used exact two-sided Wilcoxon signed-rank tests with Holm adjustment for the three within-gene comparisons. Statistical significance was set at $P<0.05$. The analyses were treated as exploratory because the biological sample comprised four isolates.

3. RESULTS AND DISCUSSION

3.1. MIC and sub-MIC profiles

The selected isolates showed two MIC/sub-MIC profiles (Table 4). Isolate 1 had a tetracycline MIC of 2 µg/mL and a sub-MIC of 1 µg/mL, whereas isolates 5, 7, and 8 had tetracycline MICs of 1 µg/mL and sub-MICs of 0.5 µg/mL. For clove oil, isolate 1 had a nominal MIC dilution of 1/16 and a sub-MIC dilution of 1/32; isolates 5, 7, and 8 had MIC and sub-MIC dilutions of 1/32 and 1/64, respectively. Representative resazurin microdilution plates are shown in Figure 1. The color transition provided a practical metabolic endpoint for selecting concentrations that permitted continued growth while imposing antimicrobial stress, which was essential for the subsequent transcriptional comparison.

Table 4. Isolate-specific MIC and sub-MIC exposure concentrations.

Isolate	Tetracycline MIC (µg/mL)	Tetracycline sub-MIC (µg/mL)	Clove-oil MIC*	Clove-oil sub-MIC*
1	2	1	1/16	1/32
5	1	0.5	1/32	1/64
7	1	0.5	1/32	1/64
8	1	0.5	1/32	1/64

**Nominal final dilution ratios in the assay/exposure medium.*

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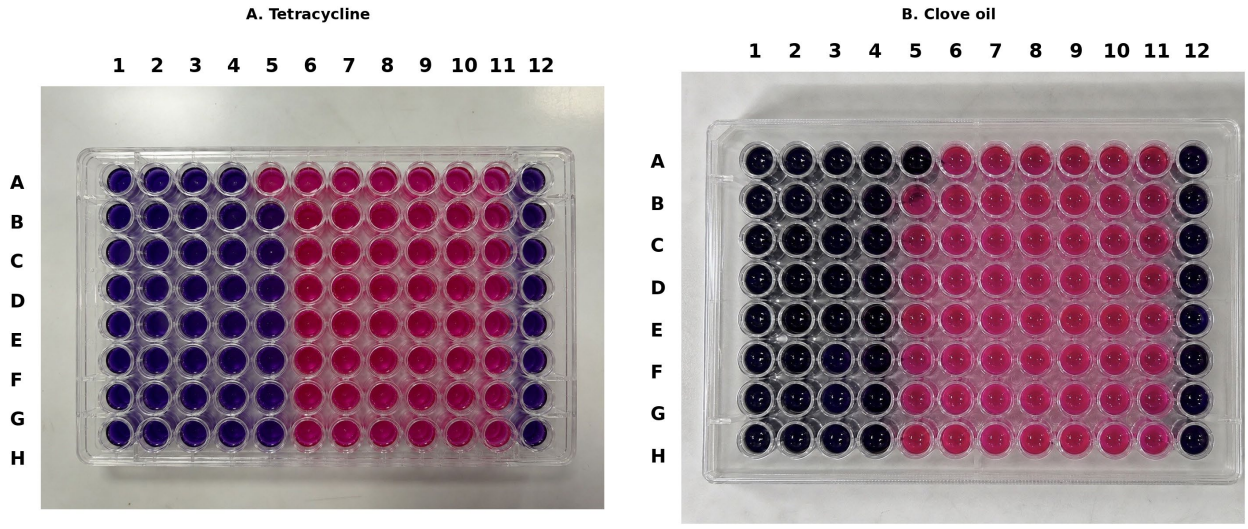

Figure 1. Representative resazurin-based broth microdilution plates for tetracycline (A) and commercial clove oil (B) against clinical oral Staphylococcus aureus isolates. Well coordinates are shown as columns 1–12 and rows A–H for orientation; the original plate photographs and well contents were not altered. Serial concentrations ran from left to right. For tetracycline, columns 1–6 correspond to 16, 8, 4, 2, 1, and 0.5 μg/mL. For clove oil, columns 1–7 correspond to nominal dilutions 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256. Purple/blue wells indicate inhibited metabolic activity, whereas pink wells indicate bacterial growth/metabolic activity.

3.2. Transcriptional response to clove oil and tetracycline

Commercial clove-oil exposure produced a uniform directional response: *fnbA*, *icaC*, and *icaD* were downregulated in every tested isolate. Mean fold changes were 0.531 ± 0.092 for *fnbA*, 0.445 ± 0.045 for *icaC*, and 0.560 ± 0.083 for *icaD* (Table 5). The consistency of the response across three functionally distinct biofilm-associated targets is compatible with a broader transcriptional effect rather than an isolated single-gene change. In $\log_2$ space, all clove-oil values were negative (Figure 2), emphasizing both the direction and magnitude of the response.

These findings are consistent with recent natural-product studies targeting *S. aureus* biofilm biology. Buru et al. (2022) demonstrated broad transcriptomic responses of methicillin-resistant *S. aureus* to sub-inhibitory eugenol exposure. Liu et al. (2024) showed that quercetin can attenuate MRSA biofilm formation through the SarA regulatory pathway, and Qureshi et al. (2025) documented antibacterial and antibiofilm activity of clove essential oil in a broader pathogen panel. Recent Iraqi work also supports the antibacterial potential of chemically characterized plant preparations against *S. aureus* (Kadhim and Saadedin, 2024; Maha et al., 2024; Abbas and Majeed, 2025; Nusrat and Al-Azawi, 2025). The present results extend this natural-product context to simultaneous assessment of *fnbA*, *icaC*, and *icaD* in oral isolates. The commercial clove oil was evaluated as the complete preparation, so the observed transcriptional effect is attributed to the preparation as a whole rather than to a quantified eugenol fraction.

Tetracycline produced a contrasting gene-dependent pattern. Mean *fnbA* expression increased modestly to 1.178 ± 0.049-fold and *icaD* increased to 1.555 ± 0.329-fold, whereas *icaC* decreased to 0.596 ± 0.112-fold. The highest individual response was the 2.042-fold *icaD* increase in isolate 1. This non-uniform behavior is consistent with recent evidence that sub-MIC antibiotic exposure can alter *S. aureus* biofilm and virulence programs in an antibiotic- and strain-dependent manner (Alkhafajy and Al-Mathkhury, 2023). Liu et al. (2023) directly demonstrated enhanced biofilm formation under sub-MIC tetracycline and identified associated transcriptomic and RT-qPCR changes, while Omar et al. (2024) demonstrated qRT-PCR changes in biofilm- and virulence-associated genes after sub-MIC oxacillin. The direction of individual genes therefore cannot be assumed a priori, which is consistent with the heterogeneous tetracycline response observed here.

Table 5. Relative expression (2^(−ΔΔCt)) by isolate and treatment.

Gene	Treatment	Isolate 1	Isolate 5	Isolate 7	Isolate 8	Mean ± SD
fnbA	Tetracycline	1.141	1.173	1.248	1.149	1.178 ± 0.049
fnbA	Clove oil	0.521	0.616	0.406	0.582	0.531 ± 0.092
icaC	Tetracycline	0.693	0.616	0.435	0.642	0.596 ± 0.112
icaC	Clove oil	0.420	0.395	0.470	0.493	0.445 ± 0.045
icaD	Tetracycline	2.042	1.357	1.464	1.357	1.555 ± 0.329
icaD	Clove oil	0.674	0.507	0.570	0.490	0.560 ± 0.083

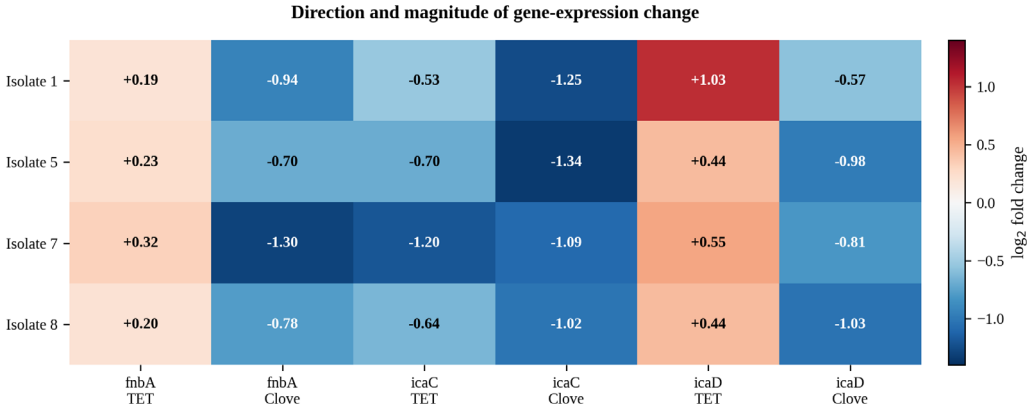

Figure 2. Heatmap of $\log_2$ fold-change values for fnbA, icaC, and icaD following tetracycline and clove-oil exposure. Positive values indicate relative upregulation and negative values indicate relative downregulation versus the corresponding untreated control.

3.3. Statistical interpretation

Exact Friedman testing detected overall condition effects for all three targets: *fnbA* (Q=8.000, exact P=0.0046, Kendall W=1.000), *icaC* (Q=6.500, exact P=0.0417, W=0.813), and *icaD* (Q=8.000, exact P=0.0046, W=1.000) (Table 6). Pairwise exact Wilcoxon comparisons yielded a Holm-adjusted P value of 0.375 for each comparison, above the 0.05 significance threshold. This combination of significant omnibus tests and above-threshold corrected pairwise P values is compatible with the very small paired biological sample: consistent rank ordering across three conditions can produce a strong Friedman statistic, while exact two-condition tests at n=4 have limited attainable P values. The statistical results therefore support interpretation at the level of the observed exploratory transcriptional pattern rather than a superiority claim.

Table 6. Exact Friedman tests across untreated-control, tetracycline, and clove-oil conditions.

Gene	Friedman Q	Exact P	Kendall W	Holm-adjusted pairwise P
fnbA	8.000	0.0046	1.000	0.375
icaC	6.500	0.0417	0.813	0.375
icaD	8.000	0.0046	1.000	0.375

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3.4. RT-qPCR amplification profiles and study context

Amplification curves were obtained for *fnbA*, *icaC*, *icaD*, and the 16S rRNA reference transcript. Derivative melt-curve profiles showed a dominant peak for each primer set under the reported run conditions (Figure 3). These profiles support the internal consistency of the generated Ct measurements, while the expression results remain appropriately interpreted at the level of the four biological isolates.

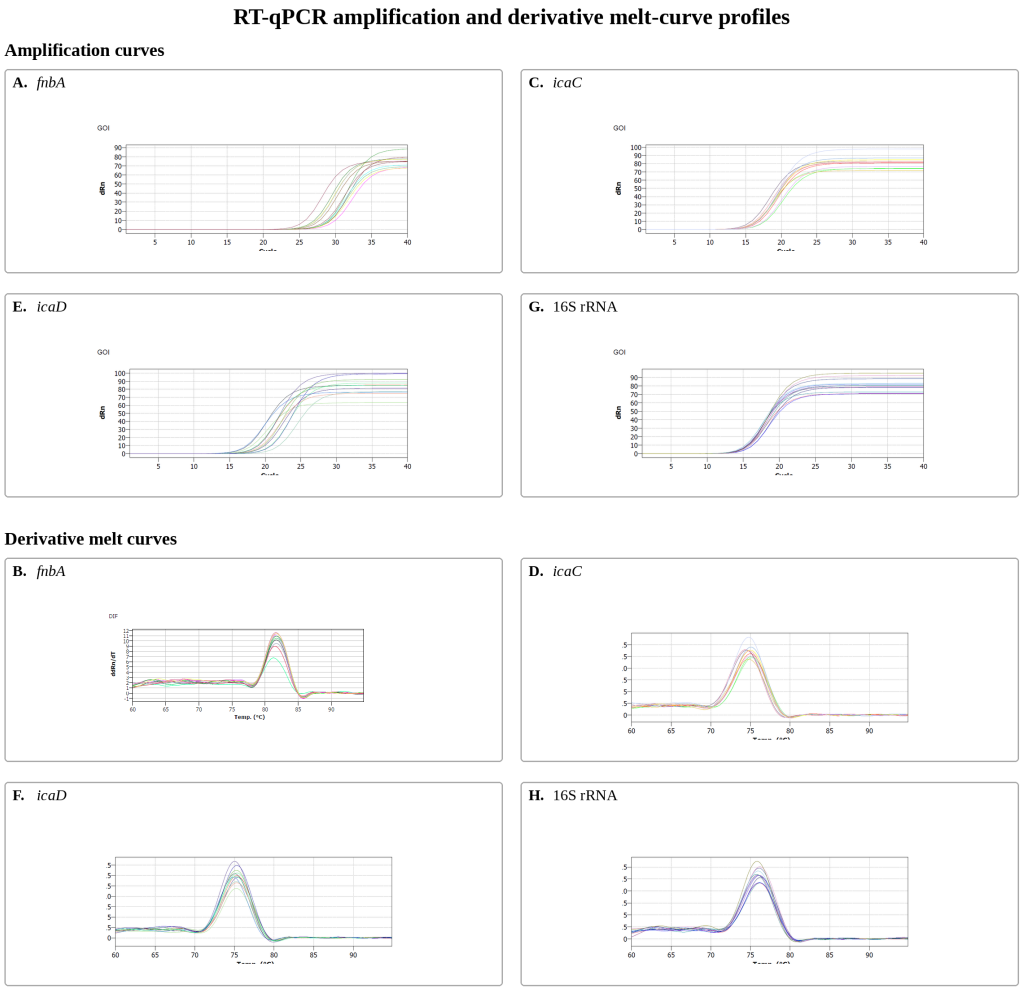

Figure 3. RT-qPCR amplification curves (A, C, E, and G) and derivative melt curves (B, D, F, and H). A–B, fnbA; C–D, icaC; E–F, icaD; G–H, 16S rRNA reference transcript. The panels are presented without alteration of the plotted instrument data.

The Iraqi findings fit a recent local research context. Mohammed and Al-Mathkhury (2023) demonstrated *S. aureus* biofilm formation and *ica* gene expression on denture materials, while Al-Khazraji and Aburesha (2026) quantified stress-associated changes in *S. aureus* adhesion-gene expression by real-time PCR. Kadhim and Saadedin (2024) evaluated a phytochemically analyzed plant extract against *S. aureus* using MIC/MBC and resazurin microdilution, whereas Maha et al. (2024) demonstrated antibacterial activity of phenolic compounds against *S. aureus*. The present study complements these independent local lines of work by combining isolate-specific MIC/sub-MIC exposure with RT-qPCR analysis of *fnbA*, *icaC*, and *icaD*.

Several design features define the scope of interpretation. The expression panel comprised four biological isolates, so inference is restricted to an exploratory isolate-level analysis. The experimental endpoint was transcriptional response after sub-MIC exposure; phenotypic biofilm biomass was outside the study endpoint. Commercial clove oil was evaluated as the complete

preparation dispersed in DMSO, so the findings apply to the tested preparation rather than to a quantified eugenol fraction. RT-qPCR interpretation was based on the reported Ct values, melt-curve profiles, and 16S rRNA normalization. Confirmatory work should extend the isolate panel, include chemical profiling of the oil and vehicle-specific comparison, and provide primer-efficiency assessment and expanded assay-control validation in line with MIQE 2.0 recommendations (Bustin et al., 2025).

4. CONCLUSION

Sub-MIC commercial clove oil was associated with consistent downregulation of *fnbA*, *icaC*, and *icaD* in the four tested oral *Staphylococcus aureus* isolates, whereas tetracycline produced a gene-dependent response characterized by increased *fnbA* and *icaD* and decreased *icaC* expression. The directionally uniform clove-oil pattern supports further evaluation of clove-oil constituents as potential modulators of biofilm-associated virulence pathways. Confirmation should include larger isolate panels, chemical characterization of the oil, a documented vehicle-control arm, fully validated RT-qPCR efficiency and negative-control reporting, and parallel post-treatment phenotypic biofilm measurements.

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AUTHOR CONTRIBUTIONS

H.K.I.: investigation, methodology, data curation, formal analysis, visualization, and writing—original draft. I.A.A.-H.: supervision, methodology, validation, and writing—review and editing. A.M.S.: supervision, methodology, validation, and writing—review and editing. All authors reviewed and approved the final manuscript and accept responsibility for the integrity of the work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

Ethical approval for this study was granted by the Research Ethics Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq.

DATA AVAILABILITY STATEMENT

The numerical data supporting the findings are presented in the manuscript. Additional study data can be provided by the corresponding author upon reasonable request.

GENERATIVE AI DISCLOSURE

OpenAI ChatGPT (GPT-5.6 Sol, accessed August 2026) was used solely for English-language editing, manuscript organization, and journal-style formatting. Experimental data and original laboratory photographs were generated and retained by the authors. The authors verified the scientific content, analyses, references, and final manuscript.

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