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Prevalence, Biofilm Formation, Antimicrobial Resistance, and Immune Response of *Staphylococcus* in the Milk of Dairy Cattle

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Abstract | Raw milk remains a critical vector for public health threats due to its susceptibility to microbial contamination, particularly by pathogenic *Staphylococcus* species. This cross-sectional study assessed the prevalence, biofilm-forming ability, antimicrobial resistance patterns, and host immune response profiles of *Staphylococcus aureus* and *S. epidermidis* isolated from 40 raw milk samples collected from commercial dairy farms. Microbiological analysis revealed a high prevalence of *S. aureus* (62.5%) and *S. epidermidis* (25%), with *S. aureus* isolates demonstrating significantly greater biofilm production (70%) compared to *S. epidermidis* (30%) ($\chi^2 = 8.3, p < 0.01$). Antibiotic susceptibility testing revealed that *S. aureus* exhibited a 42% resistance rate to ciprofloxacin, despite the presence of measurable inhibition zones. In contrast, resistance to gentamicin was notably lower (5%), with the difference being highly significant (ANOVA, $F = 45.6$, $p < 0.001$). Immunological profiling indicated that *S. aureus*-contaminated samples induced markedly elevated IL-6 (42.5 ± 3.2 pg/mL) and IL-10 (15.3 ± 1.8 pg/mL) levels compared to both *S. epidermidis*-contaminated samples and controls ($p < 0.001$, Tukey's post-hoc test), reflecting a robust pro- and anti-inflammatory host response. These findings underscore the urgent need for enhanced hygiene protocols, routine microbial screening, and prudent antibiotic stewardship in dairy production systems. Limitations of this study include the relatively small sample size and reliance on phenotypic rather than molecular identification methods. Future research should employ larger, geographically diverse sample sets and incorporate molecular typing and expanded cytokine profiling to elucidate resistance mechanisms and improve detection accuracy. Implementation of these recommendations will be essential to safeguard dairy safety and public health within a One Health framework.

Keywords | *Staphylococcus*, Raw milk, Antibiotic resistance, Biofilm, Cytokines

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INTRODUCTION

Milk is a cornerstone of human nutrition, providing essential proteins, fats, vitamins, and minerals, and serving as the basis for numerous dairy products consumed worldwide (Mekouar, 2021). However, its physicochemical properties including high water activity and neutral Ph render it highly vulnerable to microbial contamination at every stage, from production to consumption (Pajohesh *et al*., 2022; Di Domenico *et al*., 2018). This contamination not only compromises product quality and shelf life but also poses significant health risks, particularly to infants, older people, and individuals with compromised immune systems (Chung *et al*., 2015). Among the diverse microbial hazards associated with raw milk, *Staphylococcus aureus* and *Staphylococcus epidermidis* are particularly concerning. *S. aureus* is a primary causative agent of bovine mastitis, resulting in significant economic losses in dairy herds and posing a potential risk for the transmission of enterotoxins and antibiotic-resistant strains to humans through the food chain (Eidaroos *et al*., 2025; Niedziela *et al*., 2021). The ability of staphylococci to form biofilms further enhances their persistence in dairy environments, complicating sanitation efforts and facilitating the survival and dissemination of resistant clones (Singh *et al*., 2023; Qiu *et al*., 2022; Lianou *et al*., 2021). Recent global trends indicate a worrying rise in antimicrobial resistance among foodborne pathogens, including staphylococci isolated from livestock, which threatens the efficacy of critical antibiotics such as fluoroquinolones and aminoglycosides (Lianou *et al*., 2021; Pajohesh *et al*., 2022; Ali *et al*., 2024; Al-Sailawi *et al*., 2024).

In Iraq and similar settings, inconsistent mastitis control protocols and limited laboratory resources may exacerbate this trend, yet local data on resistance patterns and biofilm prevalence remain scarce. Beyond direct infection risks, staphylococcal contamination of milk can trigger robust host immune responses, as reflected by elevated levels of cytokines such as interleukin-6 (IL-6) and interleukin-10 (IL-10), which serve as biomarkers of inflammation and immune modulation (Bochniarz *et al*., 2017; Jesse *et al*., 2017; Wang *et al*., 2000). Understanding these immunological profiles is crucial for developing diagnostic and intervention strategies, particularly within the One Health framework, which recognizes the interconnectedness of animal, human, and environmental health (Abdullahi *et al*., 2021; Algammal *et al*., 2020). Despite the recognized public health importance of staphylococcal contamination in raw milk, there is a paucity of region-specific data on the prevalence, biofilm-forming capacity, antimicrobial resistance, and immunological impact of these pathogens in Iraq. Addressing this gap is essential for informing evidence-based interventions and guiding policy decisions. Therefore, this study was designed to assess the prevalence, biofilm production, antimicrobial resistance profiles, and cytokine response patterns of *S. aureus* and *S. epidermidis* isolated from raw milk in commercial dairy farms in Babylon Governorate, Iraq.

We hypothesize that raw milk in this setting serves as a reservoir for pathogenic, biofilm-forming, and antibiotic-resistant staphylococci, which are capable of eliciting significant immune responses and thus pose a multifaceted risk to dairy safety and public health. The findings are expected to inform local and regional surveillance, hygiene practices, and antimicrobial stewardship policies, aligning with the principles of One Health.

MATERIALS AND METHODS

Sample collection and preparation

Forty raw milk samples were aseptically collected from commercial dairy farms in Babylon Governorate, Iraq, using sterile 50 mL conical tubes (Falcon™). Before sampling, cow teats were disinfected with 70% ethanol to minimize external contamination. Samples were immediately stored at 4°C and transported to the laboratory within 4 hours to preserve microbial integrity. Each sample was gently inverted ten times before to analysis to ensure homogeneity (Middleton *et al*., 2014).

Microbiological analysis

Bacterial isolation

For primary isolation, 100 µL aliquots from each milk sample were streaked onto Mannitol Salt Agar (MSA; Oxoid CM0085) and Congo Red Agar (CRA), the latter supplemented with 0.8 g/L Congo red dye (Kateete *et al*., 2010). Plates were incubated aerobically at 37°C for 24–48 hours.

Bacterial identification

Presumptive *Staphylococcus* colonies were identified using a three-step protocol. First involved an assessment of colony morphology, with mannitol-fermenting *S. aureus* appearing yellow on MSA and non-fermenters such as *S. epidermidis* as pink/red. The second step involved Gram staining using Hucker's method to confirm Gram-positive cocci, while the third one; included biochemical testing, including catalase (3% H_2O_2) and tube coagulase (rabbit plasma) assays (Rai *et al*., 2023). For definitive identification, the API Staph system (bioMérieux, France) was used according to the manufacturer's instructions.

Biofilm formation assessment

Biofilm production was first screened qualitatively on CRA plates. After 48 hours of incubation at 37°C, biofilm-producing strains were identified by the formation of

black, dry, rough colonies, while non-producers formed smooth red colonies (Kaiser *et al.*, 2013). For quantitative assessment, a microtiter plate assay was performed: overnight cultures were diluted 1:100 in tryptic soy broth supplemented with 1% glucose, inoculated into sterile 96-well polystyrene plates, and incubated at 37°C for 24 hours. Wells were washed with phosphate-buffered saline (PBS), fixed with methanol, stained with 0.1% crystal violet, and the bound dye was solubilized with 95% ethanol. Optical density (OD) was measured at 570 nm using a microplate reader (BioTek ELx808). Isolates were categorized as strong, moderate, weak, or non-biofilm producers based on OD570 values relative to negative controls.

Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was performed on Mueller-Hinton agar (Oxoid CM0337) according to CLSI guidelines (Rai *et al.*, 2023). Bacterial suspensions were adjusted to a 0.5 McFarland standard (~1×10^8 CFU/mL). Antibiotic disks (Oxoid) included ciprofloxacin (5 µg), gentamicin (10 µg), colistin (10 µg), and doxycycline (30 µg). Plates were incubated at 37°C for 24 hours. Inhibition zones were measured in millimeters using digital calipers, with each test performed in triplicate. Results were interpreted as susceptible or resistant according to CLSI breakpoints.

Cytokine profiling

Whey fractions were obtained by centrifuging milk samples at 3000×g for 15 minutes, followed by filtration through 0.45 µm membranes. Concentrations of interleukin-6 (IL-6) and interleukin-10 (IL-10) were determined using commercial bovine-specific ELISA kits (Thermo Fisher Scientific; IL-6: ESS0029, IL-10: ESS0030), following the manufacturer's protocols. Absorbance was read at 450 nm using a BioTek ELx808 microplate reader. Cytokine concentrations were calculated from standard curves, and all samples were assayed in triplicate.

Statistical analysis

Data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Descriptive statistics (mean ± standard deviation) were used for continuous variables. Group comparisons for cytokine levels and antibiotic inhibition zones were performed using one-way ANOVA followed by Tukey's post-hoc test. Categorical variables, such as prevalence and biofilm formation rates, were compared using the chi-square test. Statistical significance was set at $p < 0.05$ (Motulsky, 2011).

RESULTS

Prevalence and identification of *Staphylococcus* isolates

Out of 40 raw milk samples analyzed, *Staphylococcus aureus* was identified in 25 samples (62.5%), while *S. epidermidis* was detected in 10 samples (25%). The remaining five samples (12.5%) were negative for staphylococcal growth. The difference in prevalence between the two species was statistically significant ($\chi^2 = 15.2, p < 0.001$) (Table 1, Figure 1).

Table 1: Prevalence of *Staphylococcus* isolates in raw milk samples.

Species	Number of positive samples (%)	Total samples (n)
S. aureus	25 (62.5%)	40
S. epidermidis	10 (25.0%)	40
Negative	5 (12.5%)	40

Chi-square test showed a significant difference in prevalence between *S. aureus* and *S. epidermidis* ($p < 0.001$).

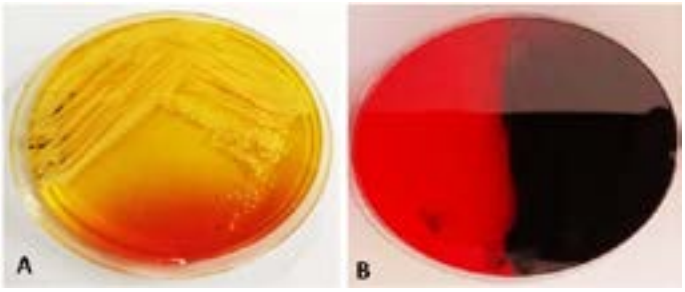

Figure 1: Representative images of *Staphylococcus* growth on selective media. A: *S. aureus* showing yellow colonies on MSA due to mannitol fermentation. B: Biofilm-forming *S. aureus* with black, dry colonies on CRA; non-biofilm producers appear as smooth red colonies.

Biofilm formation capacity

Biofilm production was observed in 70% of *S. aureus* isolates (18/25) and 30% of *S. epidermidis* isolates (3/10), as determined by Congo Red Agar. The difference in biofilm-forming capacity between the two species was statistically significant ($\chi^2 = 8.3$, $p = 0.004$) (Table 2, Figure 1).

Table 2: Biofilm formation by *Staphylococcus* isolates.

Species	Biofilm producers n (%)	Non-producers (%)	Total isolates
S. aureus	18 (72.0%)	7 (28.0%)	25
S. epidermidis	3 (30.0%)	7 (70.0%)	10

Chi-square test revealed a significantly higher proportion of biofilm producers among *S. aureus* compared to *S. epidermidis* ($p = 0.004$).

Antibiotic susceptibility patterns

Antibiotic susceptibility testing demonstrated marked differences in resistance profiles. *S. aureus* isolates exhibited a 42% resistance rate to ciprofloxacin, while resistance to gentamicin was only 5%. The mean inhibition zones for gentamicin (25.4 ± 1.2 mm) were significantly larger than those for ciprofloxacin (18.2 ± 0.9 mm), colistin (15.8 ± 1.1 mm), and doxycycline (20.6 ± 1.4 mm) (ANOVA $F = 45.6$, $p < 0.001$; Tukey's post-hoc test) (Table 3, Figure 2).

Table 3: Antibiotic susceptibility of *Staphylococcus* isolates.

Antibiotic	Mean inhibition zone (mm) ± SD	Resistance rate (%)	CLSI Breakpoint (mm)	Interpretation
Gentamicin	25.4 ± 1.2	5	≤ 15	Susceptible
Ciprofloxacin	18.2 ± 0.9	42	≤ 21	High resistance
Colistin	15.8 ± 1.1	30	≤ 11	Moderate resistance
Doxycycline	20.6 ± 1.4	18	≤ 14	Moderate resistance

A one-way ANOVA revaled significant differences among antibiotics ($p < 0.001$). Tukey's post-hoc test indicated gentamicin was significantly more effective than all other antibiotics ($p < 0.05$ for all pairwise comparisons).

Cytokine response profiles

Immunological analysis revealed that three concentrations of IL-6 and IL-10 were significantly elevated in milk samples inoculated with *S. aureus* compared to those with *S. epidermidis* and the control group. Mean IL-6 levels in the *S. aureus* group were 42.5 ± 3.2 pg/mL, compared to 28.9 ± 2.7 pg/mL for *S. epidermidis* and 10.2 ± 1.5 pg/mL for controls. Similarly, IL-10 levels were 15.3 ± 1.8 pg/mL for *S. aureus*, 10.4 ± 1.2 pg/mL for *S. epidermidis*, and 6.8 ± 0.9 pg/mL for controls. One-way ANOVA followed by Tukey's post-hoc test confirmed these differences were highly significant ($p < 0.001$ for all comparisons) (Table 4).

Table 4: Cytokine concentrations in milk samples.

Group	IL-6 (pg/mL) Mean ± SD	IL-10 (pg/mL) Mean ± SD
S. aureus infected	42.5 ± 3.2	15.3 ± 1.8
S. epidermidis	28.9 ± 2.7	10.4 ± 1.2
Control	10.2 ± 1.5	6.8 ± 0.9

One-way ANOVA with Tukey's post-hoc test showed significant elevations in both cytokines for *S. aureus*-infected samples compared to *S. epidermidis* and controls ($p < 0.001$).

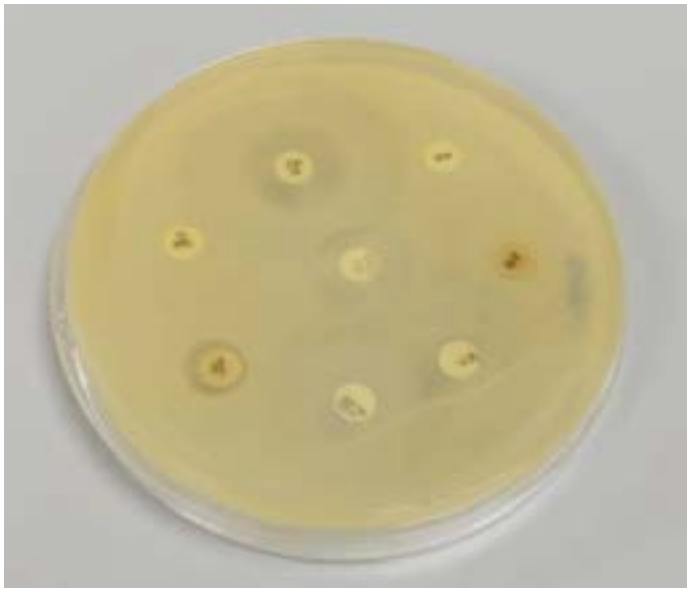

Figure 2: Antibiotic susceptibility testing of a *Staphylococcus* isolate from raw milk, showing inhibition zones for Ciprofloxacin, Colistin, Doxycycline, and Gentamicin on Mueller-Hinton agar.

DISCUSSION

This study highlights significant microbial contamination in raw milk, with *Staphylococcus aureus* (62.5%) and *S. epidermidis* (25%) prevalence underscoring the contamination risks associated with hygiene lapses. Selective media (MSA and CRA) confirmed biofilm formation in 70% of *S. aureus* isolates, enhancing its persistence despite sanitation efforts (Yambise *et al*., 2020; Alam *et al*., 2020). The increased *S. aureus* prevalence surpasses European reports (De Vliegher *et al*., 2012), aligning instead with challenges in developing regions (Nickerson *et al*., 2009), such as limited diagnostics and inconsistent mastitis protocols in areas like Babylon/Iraq. While *S. epidermidis* signals environmental contamination, *S. aureus* poses greater risks due to production of enterotoxins (Marza *et al*., 2017; Abed *et al*., 2024). The observed antibiotic resistance patterns revealed a critical paradox, where the apparent paradox between inhibition zone sizes and resistance rates becomes clear upon examining CLSI breakpoints. The 42% resistance to ciprofloxacin, despite measurable inhibition zones (18.2 ± 0.9 mm), directly reflects the stringent breakpoint criteria (≤21 mm) for this critically important antimicrobial (Humphries *et al*., 2019), and mirrors the growing global concern about fluoroquinolone resistance in livestock-associated staphylococci (Hooper, 2021, Al-Isawi *et al*., 2020). In contrast, the maintained susceptibility to gentamicin (5% resistance) suggests that it may still serve as a viable treatment option in this region. However, though its use should be guided by regular susceptibility testing given the potential for aminoglycoside resistance gene transfer. The immunological findings demonstrate a robust host response to *S. aureus* infection, with IL-6 levels (42.5 ± 3.2 pg/mL) exceeding those in *S. epidermidis* infections by nearly 50%, supporting the concept of pathogen-specific immune activation patterns (Guan *et al*., 2020; Bochniarz., 2017; Chung *et al*., 2015). However, the simultaneous elevation of IL-10 (15.3 ± 1.8 pg/mL) suggests a concurrent anti-inflammatory response that may contribute to the establishment of chronic infection. This phenomenon warrants further investigation through longitudinal studies. These collective findings carry essential practical implications: The high prevalence of biofilm necessitates revised hygiene protocols that incorporate

biofilm-disrupting agents, the resistance patterns require immediate antimicrobial stewardship programs focusing on fluoroquinolone restriction, and the cytokine profiles suggest potential for developing immune-based mastitis monitoring tools. While providing valuable insights, this study also highlights knowledge gaps that future research should address, particularly regarding the molecular mechanisms driving the observed high resistance rates and the potential for strain-specific variations in virulence factors across different dairy production systems.

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NOVELTY STATEMENT

This study provides the first comprehensive assessment of *Staphylococcus aureus* and *S. epidermidis* prevalence, biofilm formation, antimicrobial resistance, and immune response profiles in raw milk from dairy farms in Babylon Governorate, Iraq. The findings highlight region-specific challenges, such as high ciprofloxacin resistance and robust cytokine responses, which are critical for tailoring interventions in similar resource-limited settings.

AUTHOR'S CONTRIBUTION

Ali Dhiaa Marza conceptualized the study, developed the methodology, performed data analysis, and prepared the original draft of the manuscript. Amer Jebur Obayes AL-Isawi was responsible for sample collection, conducted laboratory analyses, and contributed to the review and editing of the manuscript. Noor R. Abady carried out the microbiological analyses and interpreted the resulting data. Ahmed Hassan Salh performed the immunological assays and handled statistical analysis. Faez Firdaus Jesse Abdullah supervised the research, secured funding, and finalized the manuscript. All authors reviewed and approved the final version of the manuscript. Financial Disclosure Statement.

Animal rights statement

Not applicable.

Ethical considerations

Sample collection was conducted as part of routine farm management procedures, without causing harm or requiring invasive intervention. Informed consent was obtained from all farm owners before participation.

Conflict of Interest

The authors have declared no conflict of interest in the publication of this manuscript.

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