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Unmasking the Microbial Signatures of Human Mastitis: From Bacterial Isolation to Biofilm Assessment

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Abstract

Background: Mastitis is a prevalent form of inflammation of the breast which occurs mostly when women are lactating. It has been noted that though *staphylococcus aureus* is commonly known to cause mastitis, the aspect of biofilm development in relation to the etiology of this disorder has received relatively little attention. **Methods:** Cross sectional and comparative study were done on 60 women with clinical diagnosis of mastitis and 20 normal females. The aseptically obtained swab samples of nipple areola complex of these patients were tested for culture sensitivity to identify isolates of *S. aureus* which were then screened for the presence of biofilm formation by microtiter plate technique and crystal violet staining. Statistical comparisons were performed using the Chi-square test and Fisher's exact test with statistical significance set at $p < 0.05$. **Results**: The presence of pathogens was observed in 91.7% of samples taken during mastitis infection; the predominant pathogens included *S. aureus* and coagulase-negative *staphylococci*. No pathogens were found in the control samples. Of the isolated strains of *S. aureus*, 55.6% showed a moderate to strong biofilm-forming capacity. Neither *S. aureus* nor biofilms were seen in the control samples. **Conclusion**: The occurrence of pathogenic bacteria along with biofilm formation was found to have a significant relation with mastitis, unlike that with

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healthy individuals who did not exhibit any such results. The isolation of *S. aureus* along with an analysis of its biofilm formation ability among the cases of mastitis patients has helped demonstrate the significance of biofilm assessment may improve microbiological characterization and assist in identifying isolates with increased virulence and persistence.

Keywords: Mastitis, *Staphylococcus aureus*, Biofilm formation, Lactating women, Microtiter plate assay, Crystal violet staining,

Introduction

Mastitis is an inflammation of the breast tissue, a condition primarily affecting lactating females, but sometimes seen in the non-lactating population as well. The disease typically arises due to milk stasis in combination with a secondary infection via micro abrasions at the nipple or duct rupture [1]. The typical presentation of mastitis consists of pain localized in the affected area, reddening, swelling, and, occasionally, systemic symptoms, like fever and chill. If left untreated, the disease can lead to a formation of a breast abscess in about 3–11% of the cases. Overall, the incidence of lactational mastitis varies across the globe, ranging from 2% to 20%, while it is maximal at early post-partum periods [2]. There is a variety of risk factors promoting the development of the disease. Nipple trauma caused by inappropriate breastfeeding technique represents one of the main risk factors for the disease development because damaged nipples provide easier access to bacteria to the interior of the breast tissues. Additional factors include milk engorgement, breast obstruction, hyperlactation, frequent use of breast pumps, and incomplete breast emptying. Stress of maternal origin, as well as lowered immunity, may also contribute to the disease development. Despite the natural antimicrobial nature of milk, its stagnation increases the chance of bacterial infection in the breast tissue [3].

According to microbiological analysis results, the spectrum of microorganisms found in human mastitis includes mainly opportunistic pathogens derived from the natural breast microbiome or normal skin flora. Most commonly isolated from the lesions is *S. aureus*. Other microorganisms causing mastitis include several species of *staphylococci* (coagulase-negative species, *S. epidermidis*); Group B *Streptococcus agalactiae*, as well as several other *streptococcal* strains; *Enterococcus faecalis*. At the same time, Gram-

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negative enteric bacteria, such as *E. coli* and *Klebsiella pneumoniae*, are rather rare causes of mastitis, accounting for less than 3% of the total number of lactational mastitis cases [4]. Among the factors promoting persistent infections, there is bacterial biofilm formation inside the milk ducts. In particular, bacterial biofilm is an aggregate of microorganisms embedded in an extracellular polymeric matrix protecting microflora from host immune systems and antimicrobials. During *S. aureus* infection, biofilm matrix is composed of polysaccharide, protein, and extracellular DNA (eDNA). Thus, biofilm enhances the adhesion of bacteria to the breast tissue and provides them with a favorable environment for chronic infection persistence. Consequently, assessing the biofilm-forming ability is critical when examining the possibility of recurrent mastitis cases [5][6].

In order to assess the role of pathogenic bacteria and their biofilm formation potential, we decided to compare our case sample with a control group consisting of healthy lactating and non-lactating women and collected in the same way as the samples from mastitis patients. We will analyze differences in sociodemographic, age structure, breastfeeding status, presence or absence of bacterial cultures, types of species isolated, as well as the biofilm-forming ability of *S. aureus* isolates. To determine statistical significance between two groups, we will apply Chi-Square test and Fisher Exact test. Despite increasing evidence regarding the role of bacterial biofilms in persistent infections, limited information is available concerning the prevalence of biofilm-forming *S. aureus* among Iraqi women with mastitis. Therefore, this study aimed to investigate bacterial isolates recovered from mastitis patients and evaluate the biofilm-forming capacity of *S. aureus* isolates in comparison with healthy controls.

Materials and Methods

Study Population

This study recruited 60 patients that were clinically diagnosed with mastitis, characterized by the presence of breast pain, swelling, redness, and fever. Additionally, 20 healthy females free of breast disease (including both lactating and non-lactating women) without clinical evidence of breast inflammation/infection were recruited as controls. The control group was matched with the patient group regarding the demographic variable age.

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Written informed consent was taken from all participants before taking the sample. All subjects were recruited in the same geographical area during the same time frame [7].

Sample Collection

In order to collect breast samples, the following steps were performed. First, a sterile swabbing technique was applied to take samples from all recruited subjects' nipples. In case of patients with mastitis or breast abscess, the same sampling method was performed on expressed milk and purulent discharge. This allowed for the aseptic collection of superficial bacteria. The current study was performed between November 2023 and April 2025 and included women with the ages of 15-48 years old that were clinically diagnosed with either mastitis or breast abscess. All these patients were inpatients of Al-Imam Al-Sadiq Teaching Hospital in Babylon Province, Iraq. Samples of breast pus and milk were collected in aseptic manner from all inpatients using sterile swabs. Healthy control participants underwent the same aseptic sampling process. All swabs were immediately inoculated into transport medium and cultured within two hours after collection [7].

Inclusion criteria: Women aged 18–50 years clinically diagnosed with acute mastitis or breast abscess and who had not received systemic antibiotics within 72 h before sampling were included.

Exclusion criteria: autoimmune diseases, breast cancer, chronic inflammatory disease, antibiotic therapy, immunosuppression

Culture and Identification

Each collected sample was inoculated into standard culture media: blood agar and MacConkey agar, and incubated aerobically at 37 °C for 24-48 hours. After the completion of the incubation period, culture media were checked for bacterial growth and subculture to obtain pure cultures from distinct-looking colonies. Gram staining and microscopic examination served as preliminary identification tools. When required, further biochemical tests such as catalase, coagulase, and CAMP assays were carried out. Species identification of isolated microorganisms was performed with the help of VITEK® 2 system (bioMérieux) [8].

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Ethical Considerations

Written informed consent was obtained from all subjects (healthy control participants and patients) before collecting samples. Approval for the present study was taken from the Scientific Ethical Committee of College of Science, University of Karbala under No. 0033CSE.

Biofilm Assay

A semi-quantitative crystal violet staining microtiter plate biofilm assay was used to assess the ability of the bacterial isolate to produce a biofilm. At first, *Staphylococcus aureus* isolates were grown overnight in Tryptic Soy Broth (TSB) at 37 °C. The culture was then diluted in 1:100 ratio in TSB+1% glucose. The dilutions were plated on a 96-well flat-bottom sterile polystyrene microplates in duplicates. The plates were incubated statically for 24 hours at 37 °C. Wells containing only broth (negative controls) were included in each test set. After this time, the culture media were removed, and the plates were washed thrice with PBS solution. Wells were fixed with 2% sodium acetate solution for 5 minutes and then stained with 1% crystal violet solution for 15 minutes. Wells were washed again with distilled water and dried.

To measure optical density, each well was filled up with 200 µL of 95% ethanol to dissolve the dye. Optical density was quantified spectrophotometrically at 570 nm wavelength with the use of a microplate reader. The average absorbance in duplicate wells was recorded. Based on OD570, bacterial biofilm production was categorized as negative (OD570 < 0.120), weak (OD570 0.120-0.240), and strong (OD570 > 0.240) [7]. Each isolate was tested in triplicate, and the mean OD570 value was used for statistical analysis.

Data Recording

Systematic recording of both demographic and microbiological data was done on each patient and control participant. Recorded demographic parameters were age groups (<25; 26-30; 31-35; 36-40; >40), lactation status (lactating or non-lactating); recorded laboratory parameters were results of culture (positive culture or negative), species of isolated bacteria, and biofilm formation among *S. aureus* isolates. Data entry was performed

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manually by entering information twice, which ensured its accuracy. For statistical analysis, data was coded. Data consistency and quality assurance were done before performing statistical calculations. Laboratory results were linked to anonymized participant ID to allow further subgroup analyses according to their clinical status and biofilm producer status.

Statistical Analysis

To compare patient and control participants regarding various demographic and laboratory parameters, relevant statistics were applied. Specifically, for comparing categorical variables (bacterial isolation, particular bacteria, and lactation), the Chi-Square test or Fisher's Exact test (in case of small expected frequencies) were used. A result was considered statistically significant when $p<0.05$. Distribution in terms of age groups and lactation status was assessed using the Chi-Square test. Comparison of culture positivity between patients and controls, comparison of the proportion of *S. aureus* isolation and the frequency of biofilm producers between study groups were calculated using Fisher's Exact test (the presence of zeroes). Statistical analysis was performed with SPSS version 25.

Results

Participant characteristics

The population of those individuals who were found suffering from mastitis formed a cohort of 60 members while 20 individuals constituted the control group and both these populations were similar in terms of their age range. The exact age distribution of the two populations is shown in table no. 1 below: Patients' age range was from 22 years to 45 years having an average age of approximately 32 years while controls' age was between 22 years and 44 years having an average age of approximately 32 years. No statistical difference in age distribution was observed between these two populations (chi-square test, $p= 0.94$). The lactation status is mentioned in table no. 2, in the case of patients, 40 out of 60 (66.7%) were lactating, while 20 out of 60 (33.3%) were not lactating. On the other hand, in the case of the control group, 10 out of 20 (50%) were lactating and 10 out of 20 (50%) were not lactating.

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Table 1. Age distribution of mastitis patients and healthy controls.

Age group (years)	Patients, n (%)	Controls, n (%)
20–25	10 (16.7%)	2 (10.0%)
26–30	15 (25.0%)	6 (30.0%)
31–35	20 (33.3%)	6 (30.0%)
36–40	10 (16.7%)	4 (20.0%)
>40	5 (8.3%)	2 (10.0%)
Total	60 (100.0%)	20 (100.0%)

Table 2. Lactation status in patients and controls.

Lactation status	Patients, n (%)	Controls, n (%)
Lactating	40 (66.7%)	10 (50.0%)
Non-lactating	20 (33.3%)	10 (50.0%)
Total	60 (100.0%)	20 (100.0%)

Bacterial culture results

The cultures results showed notable differences between patients and control subjects in the analysis carried out. Out of 60 swabs obtained from patients, the vast majority contained bacteria, with 55 patients (91.7%) being positive to one or several bacteria. On the other hand, 5 patient swabs (8.3%) showed absence of any bacterial growth. All 20 swabs from the control subjects (100%) were negative. These results are presented in Table 3 below, summarizing the results obtained from bacterial isolates. The highest proportion of isolates found in patients was *S. aureus*, coagulase-negative *staphylococci*, and *streptococci*. The distribution of isolates between cases of mastitis and the controls is shown in Figure 1, which pertained to 18 patients (30.0%). Coagulase-negative *staphylococci* accounted for 15 patients (25.0%), while *streptococci* accounted for 10 patients (16.7%). Other Gram-negative rods included *E. coli* (5 patients; 8.3%) and others (5 patients; 8.3%). As required by definition, there were zero isolates from the control

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subjects for all species, resulting in a rate of 100% of no growth. There was a statistically significant difference in culture positivity between the groups (55/60 in patients vs 0/20 in controls), using Fisher's exact test ($p<0.001$). More importantly, *S. aureus* was identified in 18 patients, with no similar isolates identified in the control subjects ($p \approx 0.005$ by Fisher's exact test).

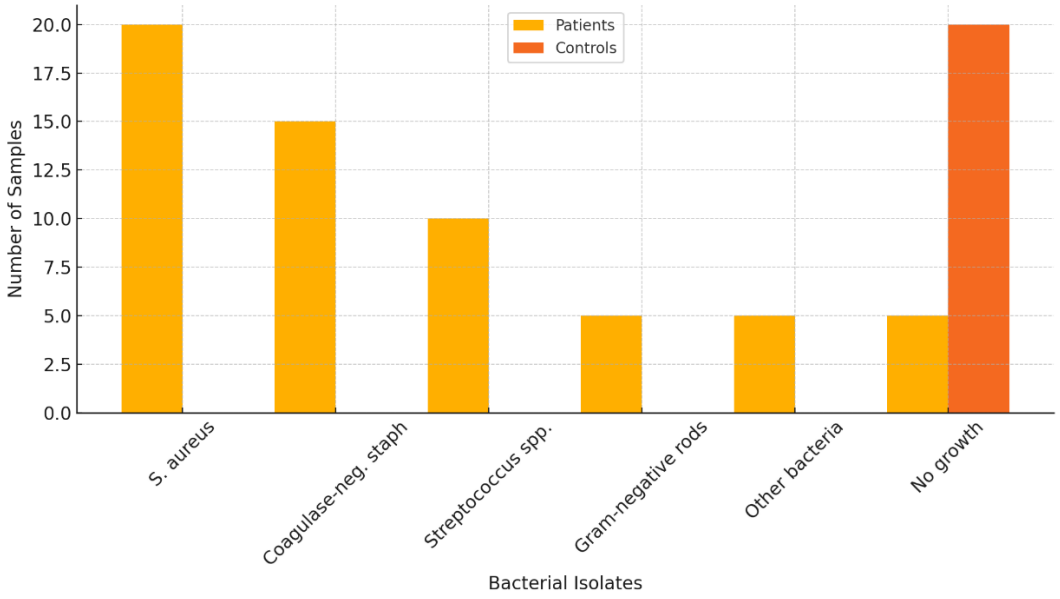

Figure 1. Distribution of bacterial isolates among mastitis patients and control participants. All bacterial isolates were obtained exclusively from mastitis cases.

Table 3. Bacterial isolates from mastitis patients and controls. *All control swabs yielded no growth.*

Bacterial isolate	Patients, n (%)	Controls, n (%)
Staphylococcus aureus	20 (33.3%)	0 (0%)
Coagulase-negative staphylococci	15 (25.0%)	0 (0%)
***Streptococcus* spp.**	10 (16.7%)	0 (0%)
Gram-negative rods (e.g., *E. coli*)	5 (8.3%)	0 (0%)
Other bacteria (mixed/rare isolates)	5 (8.3%)	0 (0%)
No growth	5 (8.3%)	20 (100.0%)
Total	60 (100.0%)	20 (100.0%)

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Biofilm production by *S. aureus* isolates

Eighteen isolates of *S. aureus* were collected, with each isolate originating from a different patient. In those patients' samples of isolates, 10 of the 18 (55.6%) were biofilm producers. As depicted in Figure 2, a majority of the *S. aureus* strains produced biofilm. Specifically, out of the 18 strains of *S. aureus* isolates, 6 were identified as strong biofilm producers, while 4 were moderate biofilm producers. Of the 18 isolates of *S. aureus*, 8 isolates (44.4%) were not biofilm-producing or only produced weak biofilm (Table 4). On the other hand, there were no isolates of *S. aureus* found in any control sample (0/20). Therefore, biofilm was produced exclusively by isolates from the patient group. All 20 of the control samples were found not to produce *S. aureus* or biofilm and were therefore labeled as "non-biofilm" (Table 4). Because there were no *S. aureus* isolates in the control group, there cannot be a direct comparison made regarding biofilm rates; however, biofilm *S. aureus* production occurred solely in the patient samples.

Table 4. *Staphylococcus aureus* biofilm production among isolates from patients and controls. No *S.* aureus was isolated from any control; all control specimens were classified as non-biofilm.

Group	*S. aureus* isolates (n)	Strong biofilm (n, %)	Moderate biofilm (n, %)	Weak/no biofilm (n, %)
Patients	18	6 (33.3%)	8 (44.4%)	4 (22.2%)
Controls	0	0 (0%)	0 (0%)	20 (100%)*

Note: Controls had 20 individuals tested but 0 *S.* aureus isolates; "20 (100%)" indicates all controls were biofilm-negative (no isolate).

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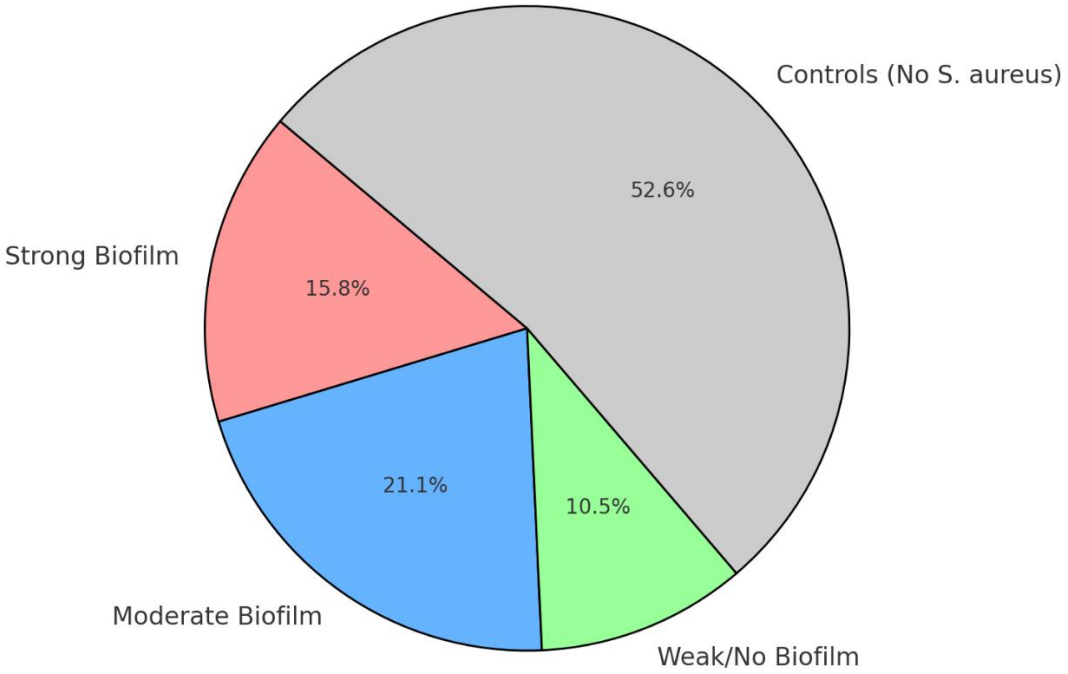

Figure 2. Proportional breakdown of *S. aureus* isolates by biofilm formation capacity among mastitis patients. No *S. aureus* was isolated from controls.

Statistical comparisons.

To summarize, statistical analyses showed that mastitis group had significant differences from the control group in terms of the microbiological results, whereas the demographic characteristics were similar. In particular, 91.7% of patients and 0% of the control group were characterized by positive bacterial growth ($p < 0.001$, Fisher's exact test). *Staphylococcus aureus* was isolated in 30.0% of patients and 0% of controls ($p \approx 0.005$). However, there were no significant differences regarding age and lactation ($p > 0.05$ for both); thus, the variables can be considered to be well-matched. Hence, the main difference between the groups is that only the mastitis group had the pathogen and biofilms (Tables 3–4). The differences were found to be statistically significant from the point of view of the microbiological analysis of the samples. The main differences between the groups concerning the microbiology of mastitis are shown in Figure 3.

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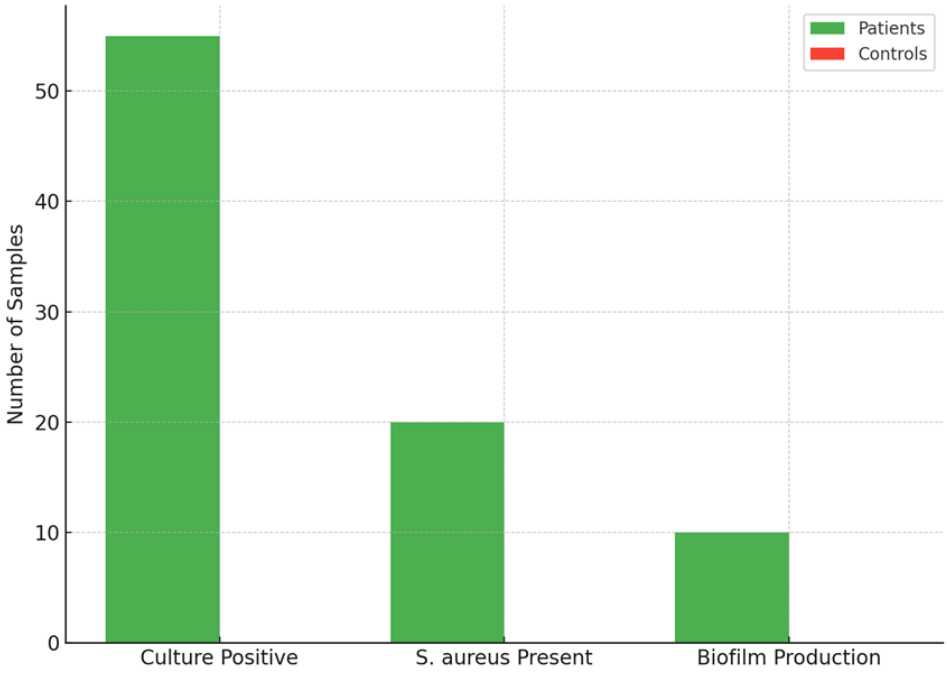

Figure 3. Comparison of key microbiological findings between mastitis patients and controls, highlighting significant differences in culture positivity, *S. aureus* isolation, and biofilm production.

Discussion:

The present study demonstrated that *S. aureus* was the predominant bacterial species isolated from mastitis patients and that more than half of these isolates exhibited moderate-to-strong biofilm-forming ability. As it turned out from this research, the presence of the healthy control group helped identify the microbial features that are characteristic only of mastitis. First of all, the absence of any culturable pathogens from the breast surface in the samples obtained from healthy women, who were tested using the same protocol as patients, allowed us to conclude that microorganisms found in patients with mastitis really represented the actual pathogens rather than commensals. Hence, all samples taken from the healthy group were negative in terms of the cultivation of bacteria (Table 3). In contrast, the vast majority of patients with mastitis had pathogen-positive cultures, were predominated different species of *staphylococci*. This difference was statistically significant ($p < 0.001$). Such results are consistent with previously published data about

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significantly higher rates of the colonization of breast tissue with *Staphylococcus epidermidis* and *Staphylococcus aureus* in infected women compared with healthy subjects. Earlier, the scientists noted that *S. epidermidis* was the most frequent strain isolated in cases of mastitis, which also has much higher bacterial titers in milk samples than in healthy women. Additionally, mastitis strains of this species produced larger amounts of biofilms and possessed increased levels of antibiotic resistance [9,10].

Like in the case with *S. epidermidis*, the results of the study conducted in the present case demonstrated that only the strains isolated from patients were capable of producing biofilms. All examined *S. aureus* strains isolated from patients exhibited moderate and high levels of biofilm-forming capability (Table 4). Meanwhile, there were neither *S. aureus* nor any signs of biofilm formation among the control group members. Though it was not possible to conduct comparative statistical analysis due to the absence of isolates from the control group, this observation strongly suggested that biofilm formation was one of the key virulence traits of *S. aureus* in the development of mastitis [11]. It should be noted that there were no significant differences between the groups regarding their demographic features. As expected, the most frequent cases of mastitis occurred in the age group of 26-35 years old, which can be explained by the fact that this age corresponds to active child-bearing period. Such an association with age has been revealed in previous studies as well [16]. While the current study did not assess other predisposing factors such as nipple trauma, breastfeeding techniques, etc., the literature suggests that these issues are highly associated with the development of mastitis [17].

Additionally, there were no significant differences between the study groups concerning age ($p = 0.94$) and lactation status ($p = 0.18$). Therefore, these factors can be excluded as possible contributors to the microbiological differences revealed between groups. Equally distributed groups regarding lactating and non-lactating women also indicated that lactation itself could not affect the obtained results as all samples from lactating healthy women remained negative for the presence of any bacteria. Thus, the data obtained from the control group also allowed identifying the importance of the isolation of specific microbiota for diagnosing mastitis [11]. While the results from the control group clearly demonstrate that all isolates identified in patients and the expression of biofilm formation

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trait are closely associated with mastitis, there is one caveat that should be mentioned here. The lack of healthy controls can lead to the underestimation of low-abundance microflora. Indeed, previously published studies showed that healthy breast milk samples contain *staphylococcal species*, but at much lower titers than in case of mastitis. In the current study, all control samples were negative for pathogen growth. However, this can be partly explained by the sample collection method or conditions used to isolate bacteria [11,12]. Anyway, these results are helpful for distinguishing healthy microflora from the microbiota of mastitis patients under the particular circumstances described above. Further, PCR or NGS can be used for identification of low-bacteria content in healthy breasts.

Summing up, it should be emphasized that the use of the healthy control group made it possible to state that only mastitis patients possess culturable pathogen and biofilm-forming strains of *Staphylococcus*. This conclusion helps prove the initial hypotheses that bacterial infection and biofilm formation play key roles in the pathogenesis of mastitis. Furthermore, these conclusions were confirmed statistically based on the chi-square and Fisher's test. Thus, comparing mastitis patients and carefully chosen healthy controls, we came to the understanding of the importance of biofilm and pathogenic microflora for this disease. Biofilm-producing isolates may contribute to treatment failure because biofilms reduce antibiotic penetration and facilitate bacterial persistence. One of the most significant findings obtained in this study was the biofilm-forming capabilities of all examined *S. aureus* isolates. About half of the isolates (57.1%) were categorized as strong biofilm producers based on the OD measured using a microtiter plate biofilm formation assay (Table 4). In fact, the ability to produce large amounts of biofilms may help explain chronic and recurrent nature of mastitis as it creates high tolerance to antibiotics and the host immune system [13,14,15].

Conclusion

There was a correlation found between mastitis and the ability of these microorganisms to produce biofilms and cause infection, while no correlation was found with regards to healthy controls. These results clearly demonstrate that these factors are unique to patients with mastitis and are absent in healthy women who were tested under the same conditions.

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Therefore, we can conclude that in the clinical setting, the presence of the mentioned bacteria and their ability to produce biofilms provides substantial evidence of mastitis caused by an infection. At the same time, the absence of any positive findings for the control group allows assuming that the colonization of healthy individuals with the mentioned flora is not misdiagnosed.

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Authors' Contributions

All authors contributed equally to the conception, study design, data collection, microbiological analysis, and manuscript writing. All authors read and approved the final version of the manuscript.

Declaration of Originality

This manuscript is original, has not been published before, and is not currently being considered for publication elsewhere.

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