



## Isolation and Identification of Bacteria and Fungi Correlated with Mastitis in Breastfeeding Women in AI-Anbar Governorate/West of Iraq

Yasmeen Kateb Ahmed<sup>1\*</sup>, Abbas Obaid Al-janabi<sup>1</sup>, Amer Fakhri Mizban<sup>2</sup>

<sup>1</sup>Department of Microbiology, College of Medicine, University of Anbar, Iraq

<sup>2</sup>Department of Surgery, College of Medicine, University of Anbar, Iraq

\*Corresponding Author: [yas20m0018@uoanbar.edu.iq](mailto:yas20m0018@uoanbar.edu.iq) (orcidory000-0001-6533-3875)

**Citation:** Ahmed YK, Al-Janabi AO, Mizban AF. Isolation and Identification of Bacteria and Fungi Correlated with Mastitis in Breastfeeding Women in AI-Anbar Governorate/West of Iraq. Al-Kitab Journal for Pure Sciences. 2023 Oct 04;7(2):62-77. Available from: <https://doi.org/10.32441/kjps.07.02.p6>.

**Keywords:** Mastitis, Breastfeeding, Staphylococcus aureus, Candida spp.

### Article History

|                  |              |
|------------------|--------------|
| Received         | 15 Aug. 2023 |
| Accepted         | 30 Sep. 2023 |
| Available online | 04 Oct. 2023 |

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### Abstract:

Mastitis is a common phenomenon experienced by a lot of breastfeeding women. Lactational mastitis, commonly occurs because of prolonged milk duct engorgement, which allows bacteria to enter through breaks in the skin, resulting in an infection. Mastitis can occur at various stages of lactation, although it is most observed within the second and third weeks following childbirth. For isolation and identification of bacteria associated with mastitis, Blood agar medium, MacConkey agar, and mannitol salt agar were used, whereas Sabouraud Dextrose Agar (SDA) was used for fungal isolation. Different biochemical test has been performed for bacterial isolates including gram stain, catalase, coagulase, and Vitec 2 compact. For fungal isolates, KOH treatment and lactophenol cotton blue test were performed. Fungal DNA was extracted then PCR was used for the detection ITS Gene of candida spp. Using gel electrophoresis of PCR product on 2% agarose. The study revealed Staphylococcus aureus, Klebsiella pneumoniae, and Candida spp. as significant bacteria that are commonly related to mastitis in women. The prevalence of Staphylococcus aureus was seen to be high, with a notable manifestation of lesions. Additionally, both Staphylococcus aureus and Klebsiella pneumoniae were shown to be associated with the presence of redness. Candida albicans had a notable

prominence, displaying a heightened prevalence in the regions of the nipple. Furthermore, it demonstrated a correlation with the presence of lesions and redness. The presence of redness was observed as a notable sign in cases linked with *Candida tropicalis*. This study provides insights into the microbial diversity linked to mastitis mainly in breastfeeding women, highlighting the prevalence of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida spp.*

**Keywords:** Mastitis, Breastfeeding, *Staphylococcus aureus*, *Candida spp.*

## عزل وتشخيص البكتيريا والفطريات المرتبطة بالتهاب الضرع عند النساء المرضعات في محافظة الأنبار/غرب العراق

ياسمين كاتب أحمد<sup>١</sup>، عباس عبيد الجنابي<sup>١</sup>، عامر فخري مزبان<sup>٢</sup>

<sup>١</sup> قسم الأحياء الدقيقة، كلية الطب، جامعة الأنبار، العراق

<sup>٢</sup> قسم الجراحة، كلية الطب، جامعة الأنبار، العراق

[yas20m0018@uoanbar.edu.iq](mailto:yas20m0018@uoanbar.edu.iq)

### الخلاصة:

إن التهاب الثدي هو ظاهرة شائعة تصيب أغلب النساء المرضعات. تنتج هذه الإصابة من تورم في القنوات اللبنية والذي بدوره يسمح للبكتيريا بالدخول خلال الشغرات في الجلد ومن ثم حدوث العدوي قد تحدث تلك الإصابة خلال مراحل مختلفة من الرضاعة، ولكن يحدث غالباً في أول ثلاث أسابيع تلي الولادة. ولأجل عزل وتعريف البكتيريا المتعلقة بالتهاب الثدي تم استخدام وسائط مثل وسط الدم (*Blood agar*)، ووسط مأكونكي (*MacConkey agar*)، ووسط ملح مانيتول (*Mannitol salt agar*)، في حين تم استخدام وسط سابورود ديستروز أجار (*Sabouraud Dextrose Agar*) لعزل الفطريات. تم عمل اختبارات كيميائية مختلفة لعوامل البكتيريا المعزولة، بما في ذلك اختبار صبغة جرام (*Gram stain*)، واختبار الكاتالاز (*Catalase*)، واختبار الكواجيليز (*Coagulase*)، ونظام فيتيك ٢ كومباكت (*Vitek 2 compact*). أما بالنسبة لمجموعة الفطريات فقد تم إجراء اختبار هيدروكسيد البوتاسيوم (*KOH treatment*) واختبار اللاكتوفينول كوتون بلو (*Lactophenol cotton blue test*). ثم عمل استخلاص الحمض النووي للفطريات ثم عمل (*PCR*) لاكتشاف جين *ITS* لجنس الكانديدا (*Candida spp.*). وبعد ذلك تم عمل (*gel electrophoresis*) لفحص منتج *PCR* على ٢٪ جل أجاروز، أظهرت نتائج الدراسة ارتباط عدد من البكتيريا والفطريات بالتهاب الثدي. وكان أبرزها (*Klebsiella pneumoniae*) (*Staphylococcus aureus*) وأنواع من الفطريات من جنس الكانديدا (*Candida spp.*). كان *Staphylococcus aureus* مرتبطاً بتكون التقرحات، ولكن وجود *Staphylococcus aureus* مع *Klebsiella pneumonia* كان مرتبطاً بالاحمرار، ولكن وجود فطر *Candida albicans* كان مرتبطاً بأعراض الاحمرار والتقرحات وخاصة في منطقة الحلمة. ووجد أن وجود الاحمرار كان علامة بارزة على وجود فطر *Candida tropicalis*. تقدم هذه الدراسة رؤى مختلفة حول التنوع الميكروبي المرتبط بالتهاب الثدي خاصة في النساء المرضعات وتسلط الضوء على انتشار *Staphylococcus aureus*، و *Klebsella pneumoniae* وكذلك *Candida spp.*

**الكلمات المفتاحية:** التهاب الثدي، الرضاعة الطبيعية، بكتيريا ستافيلوكوكس، فصائل فطر الكانديدا.

## 1. INTRODUCTION:

Breastfeeding is critical in maintaining the proper development of the brain and in preventing the simultaneous appearance of malnutrition, infectious illnesses, and mortality [1]. Furthermore, regardless of the country's economic situation, it provides long-term benefits by lowering the chance of obesity and chronic illnesses during maturity [2]. Breastfeeding, in addition to providing passive immunity, promotes active communication between the immune systems of mothers and newborns [3]. Furthermore, women pass on microbial elements to their offspring via breast milk [4].

When infants under the age of 12 months and young children between the ages of 12 and 36 months receive breast milk from their mothers through the act of breastfeeding, they have the best chance of survival, growth, and optimal development [5]. This is partly because breastfeeding is a dynamic intervention activity, and breast milk contains unique living qualities that contribute to these favorable results [6,7].

Breastfeeding women often encounter a condition known as lactational mastitis, which manifests as inflammation in the breast tissue.[8] This discomfort arises due to factors like nipple fissures and milk stasis, which can disrupt the otherwise harmonious experience of breastfeeding [9]. Due to a decline in their defense ability, bacteria through the milk ducts retrograde into the mammary gland, leading to infection [10].

In the initial stage, patients commonly exhibit symptoms such as breast redness, swelling, tenderness, and inadequate milk discharge [11]. As the condition advances, the formation of a lump becomes more pronounced, accompanied by additional symptoms including fever, chills, fatigue, headaches, and other manifestations [12]. Most often, *S. aureus* colonizes the skin and is one of the most important causes of locational mastitis [13,14]. Methicillin-resistant *Staphylococcus aureus* (MRSA) may also become a risk factor for this infection [15,16].

Mastitis is intimately associated with bacterial growth, a state where the mastitis-causing microbiota increases while the normal mammary microbiota diminishes [13]. Also, *Klebsiella pneumonia* has been reported as one of the organisms related to mastitis [16,17]. Fungal infections affecting the nipple and breast may be experienced by breastfeeding women. It is caused by *Candida albicans*, a prevalent fungal organism responsible for various thrush infections [18,19]. Inflammatory processes and pain may be sparked by the co-existence of *Candida* spp. in the lactating nipple and breast, including *Staphylococcus aureus* (*S. aureus*), *Candida albicans*, and another *Candida* spp.

Another possibility is that this syndrome could be caused by multiple organisms. Co-infection with *Staphylococcus aureus*, *Candida albicans*, and other *Candida* spp. in the lactating nipple and breast may lead to inflammation and pain [20].

There are many organisms involved in mastitis in breastfeeding women, so this study aimed to isolate and identification of microorganisms related to mastitis in breastfeeding women, determine the relation between mastitis symptoms and organisms, and assess between microorganisms and mastitis source.

## 2 .Materials and Methods:

**2.1 .Study Design and Sampling:** It is a cross-sectional study consisting of 100 patients with skin infections with mastitis who were recruited from AL-Ramadi Teaching Hospital in Anbar Governorate, Iraq. From the mother and her child, the sample was collected between November 2022 and March 2023. Samples were obtained from a mother with mastitis and her baby. The step in collecting samples is to use sterile cotton swabs, where a swab is taken from the mother from the site of lesion or redness located on the nipple and breast, and a swab is also taken from the mother's milk and her baby's mouth.

**2.2 .Identification of Bacterial Isolates:** The blood Agar media used for isolation. 4.25-gram agar was dissolved in 100 ml dis.H<sub>2</sub>O and sterilized in an autoclave for 15 minutes at 121, cooled to 45 C then 10% V/V sterile defibrinated blood was added aseptically, plates were inoculated and incubated at 37 C for 48 hr [21].

Mannitol salt agar medium also was used to test the ability of the organisms to ferment mannitol, 11.1 gram of the media was dissolved in 100 ml dis H<sub>2</sub>O and sterilized in an autoclave for 15 minutes at 121 C. Cooled, poured in Petri dishes, and plated were inoculated then incubated for 48h at 37C [22].

Subculture on MacConkey agar was performed by dissolving 5.5 grams of medium in 100 ml dis. H<sub>2</sub>O was sterilized in an autoclave at 121C for 15 minutes poured onto plates, inoculum was spread on the surface of the plates, and incubated for 48h at 37 C. Gram stain test was carried out to characterize the isolates [23].

A 3% solution of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was created by diluting a stock solution (15%) in a dark bottle, then a few drops were added to culture on the slide and on blood agar to do catalase test [24] coagulase test was performed in a glass tube containing a few milliliters of citrated plasma, few drops of bacterial suspension were added, then the tube was incubated for 24 hours in the oven [25] and Vitek 2 were done by using (Gram-positive Vitek-2, Gram-negative Vitek-2) kits acquired from BioMerieux, France.

**2.3 .Identification of fungal isolates:** Sabouraud Dextrose Agar (SDA) (Oxide, United Kingdom) was prepared and inoculated by swabs and then incubated for 24 hr at 37C. For the KOH test, 10 grams of potassium hydroxide were dissolved in 80 mL dis.H<sub>2</sub>O. Drops of KOH solution were added to the specimen, a coverslip was placed over the specimen after 10 minutes [26]. Lactophenol cotton blue [27] and Germ tube tests were performed for fungal isolates [28].

**2.4 .Molecular study of fungal isolates:** Fungal DNA was extracted by using the Fungi/Yeast Genomic DNA Isolation Kit Provided by Norgen Biotech, PCR was used for the detection ITS Gene of candida spp. The primers were designed based on the National Centre for Biotechnology Information (NCBI) and provided by the BioOneer Company **Table (1)** . To detect the ITS gene of Candida spp., PCR protocol was employed in **Table (2)**.

**Table 1: Sequence of PCR primer [29]**

| Sequence of forward and reverse |                       |
|---------------------------------|-----------------------|
| F                               | /TCCGTAGGTGAACCTGCGG  |
| R                               | /TCCTCCGCTTATTGATATGC |

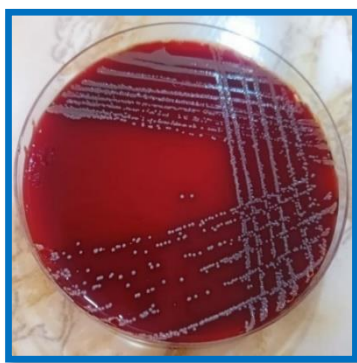
After PCR amplification, gel electrophoresis of PCR product was done on 2% agarose. The investigation encompassed a statistical analysis of the data concerning the types of microorganisms found in each sample, together with the observed variances in symptoms linked with them. The analysis was properly carried out using SPSS Version 25.0 software. The data underwent extensive preprocessing and validation procedures to guarantee its quality and dependability.

**Table 2: PCR amplification protocol [30]**

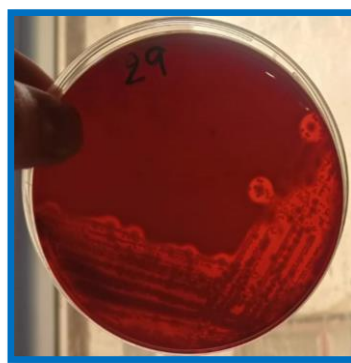
| Step                 | Temperature | Time  | Cycle |
|----------------------|-------------|-------|-------|
| Initial denaturation | 94          | 5min  | 1     |
| Denaturation         | 94          | 45 S  | 35    |
| Annealing            | 52          | 1 min |       |
| Extension            | 72          | 1 min |       |
| Extension            | 72          | 7min  | 1     |
| Hold Temperature     | 4           | 4min  | ----  |

### 3. Results and discussion:

**3.1 Bacterial isolation and biochemical identification:** For isolation of Staphylococcus aureus, the differential media blood agar was used, and the colonies appeared on the plate after 24 h (**Figure 1. A**). Gram stain test revealed that it is gram-positive bacteria which is a main character of Staphylococcus aureus. Also, it produced blurred edges and completely clear zones which indicates that beta-hemolysis took place. **Figure 1. B**



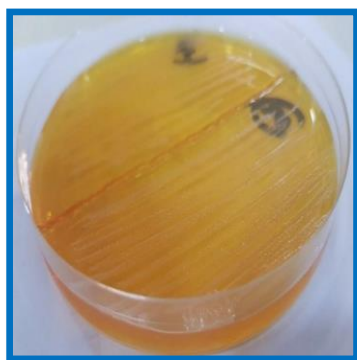
A) Growth of *Staphylococcus aureus*.



B) Beta-hemolysis of *Staphylococcus aureus* on blood agar

**Figure 1: *Staphylococcus aureus* on blood agar medium.**

Small yellow colonies appeared on mannitol salt agar due to mannitol fermentation by producing an acid that causes the red color of phenol to turn yellow and this is a character of *Staphylococcus aureus*. **Figure (2).**



**Figure 2: positive mannitol fermenter *Staphylococcus aureus*.**

*Staphylococcus aureus* is characterized by being catalase positive as it transforms hydrogen peroxide ( $H_2O_2$ ) to water and oxygen this test was done on both blood agar and on the slide. **Figure (3).**



A. On blood agar



B. On slide

**Figure 3: Catalase test positive of *staphylococcus aureus***

*Staphylococcus aureus* cells clumped together within 10 seconds in comparison to the control because of *staphylococcus aureus* being coagulase positive. **Figure (4).**

Vitek 2 was done and with all results biochemical tests showed that the isolate was *Staphylococcus aureus*.



Figure 4 Coagulase test positive for *staphylococcus aureus*

The identification of *Klebsiella pneumoniae* using MacConkey agar is based on the ability of this organism to ferment lactose as *Klebsiella pneumoniae* is a lactose-fermenting bacterium so pink mucoid colonies are present on the MacConkey agar plate. **Figure (5)**. Gram stain test of the isolate showed that it is a gram-negative bacterium, which is consistent with *Klebsiella pneumoniae*. The Vitek 2 compact system confirmed the identification of the isolate as *Klebsiella pneumoniae*.



Figure 5: *Klebsiella pneumonia* growing on MacConkey agar medium

**3.2 .Biochemical identification of fungi:** *Candida* spp. grew on SDA medium which is differential media for pathogenic fungi including different species of *Candida*. **Figure (6)**.

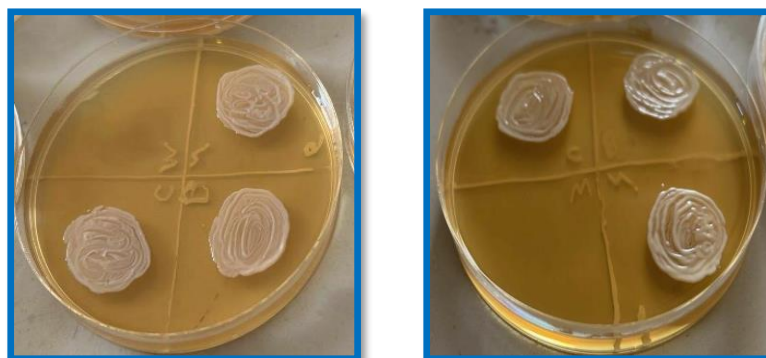


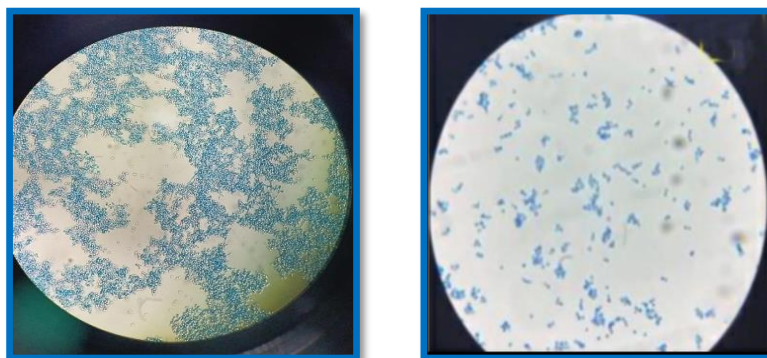
Figure 6: Macroscopic examination for *candida* spp. on SDA

Upon microscopic examination of isolates treated with potassium hydroxide (KOH), the result may exhibit a combination of hyphae, pseudohyphae, and budding yeast cells **Figure (7)**.



**Figure 7: Direct microscopic examination of candida spp using 10% KOH for diagnosis of candida spp**

Morphology of *Candida* spp. become obvious after LPCB staining. LPCB staining is a useful method for the visualization and identification of fungal structures and spores, including those of *Candida* spp **Figure (8)**.



**Figure 8 Microscopic examination of fungal spores and other fungal structures with lactophenol cotton blue (candida)**

Germ tubes appeared after 3, 6, and 9 hours indicating that the *Candida* spp. isolates are likely *Candida albicans* **Figure (9)**.



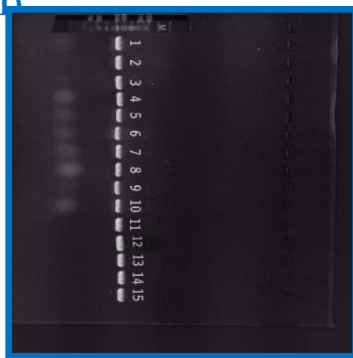
**Figure 9: Germ tube test for candida identification, it tested after 3 h, tested again after 6 h and after 9 h.**

**3.3 .Molecular study of fungi:** Gel electrophoresis has been performed on genomic DNA extracted from *Candida* spp and the presence of DNA bands on the gel indicates the presence of genomic DNA in the sample **Figure (10)**.



**Figure 10 Gel electrophoresis of genomic DNA extraction, 1% agarose gel.**

PCR was used for the detection of the ITS Gene of *Candida* spp. After PCR amplification, gel electrophoresis of PCR product was done on 2% agarose. The target gene was found, and its band size is 550 bp **Figure (11)**.



**Figure 11 Gel electrophoresis showing PCR product with a band size of 550 bp. N: DNA ladder (1000 plus)**

**3.4 .The association between the type of organism and the mastitis occurrence and symptoms in women:** Percentage of various organisms (*Staphylococcus aureus*, *Klebsiella pneumonia*, and *Candida albicans*) found in various sources (nipples, breast, and kid oral cavity) among women with mastitis. The presence of *Candida albicans* in different sources with a higher percentage than *Staphylococcus aureus* & *Klebsiella pneumonia* **Table (4)**.

**Table 4: Type of microorganisms and mastitis occurrence**

|                   | <i>Staphylococcus aureus</i> | <i>Klebsiella pneumonia</i> | <i>Candida albicans</i> | $\chi^2$ | P value |
|-------------------|------------------------------|-----------------------------|-------------------------|----------|---------|
| Nipples           | 38 %                         | 20 %                        | 90%                     | 3.324    | 0.1897  |
| breast            | 50%                          | 20%                         | 73%                     |          |         |
| Nipples           | 38 %                         | 20 %                        | 90%                     | 0.009    | 0.995   |
| Child oral cavity | 37%                          | 20%                         | 90%                     |          |         |
| Breast            | 50%                          | 20%                         | 73%                     | 3.661    | 0.16    |
| Child Oral Cavity | 37%                          | 20%                         | 90%                     |          |         |

Different microorganisms are linked to distinct mastitis symptoms. *Staphylococcus aureus* and *Candida albicans* appear to be linked to the symptom of lesions, whereas *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Candida albicans* appear to be linked to redness. Cases involving *Candida albicans* alone, on the other hand, have a higher prevalence of the combination of redness and lesions. Finally, *Candida tropicalis* is associated with redness as a symptom **Table (5)**.

**Table 5: Type of microorganisms and symptoms of mastitis correlation**

|                                                                                      | Symptoms |         |                   |
|--------------------------------------------------------------------------------------|----------|---------|-------------------|
|                                                                                      | Redness  | Lesions | Redness & Lesions |
| <i>Staphylococcus aureus</i> & <i>Candida albicans</i>                               | 8%       | 30%     | 0%                |
| <i>Staphylococcus aureus</i> & <i>Klebsiella pneumonia</i> & <i>Candida albicans</i> | 32%      | 0%      | 0%                |
| <i>Candida albicans</i>                                                              | 0%       | 0%      | 20%               |
| <i>Candida tropicalis</i>                                                            | 10%      | 0%      | 0%                |

#### 4. Discussion:

The bacterial isolate was *Staphylococcus aureus* as resulted from the biochemical tests performed which included growth on blood agar and making beta-hemolysis, and mannitol fermentation as these are characteristics of *Staphylococcus aureus* as reported [31]. Also gram-positive, catalase-positive [32], and the positive result of the coagulase test [31] support that it is *Staphylococcus aureus*. Vitek 2 results align with other biochemical tests and the demonstrated characteristics of *Staphylococcus aureus* [33].

For *Klebsiella pneumoniae*, the identification tests conducted are going with characters of it which had mentioned in previous studies. Vitek2 [33], lactose fermentation on MacConkey agar [34], being gram-negative [35]. According to the Clinical and Laboratory Standards Institute (CLSI), gram stain testing is a rapid and reliable method for the preliminary identification of bacterial isolates [36].

In the context of fungi, *Candida* spp. characters found in previous studies are the same results obtained from the biochemical tests. Growing on Sabouraud Dextrose Agar (SDA) which contains antibiotics such as chloramphenicol and cycloheximide that inhibit the growth of bacteria and other fungi, thereby allowing for the selective growth of *Candida* spp. [37]. The presence of a combination of hyphae, pseudohyphae, and budding yeast cells after KOH treatment [38] and Morphology of *Candida* spp. after LPCB staining [39, 40] are recognized as characteristic attributes frequently linked to *Candida* spp. Germ tube is also used for the

Identification of a *Candida albicans* or non-*albicans* species depending on the presence or absence of germ tubes [41]. A molecular study was also done to detect the ITS gene of *Candida* using PCR and a single band of 550 bp was found [42]. The ITS region (Internal Transcribed Spacer) is a highly conserved region found in the rDNA gene of fungi, including *Candida* spp. This region has been widely used as a target for molecular identification of fungal species using PCR-based techniques, including PCR amplification and agarose gel electrophoresis [43].

Many studies have studied the relation between type of microorganisms and mastitis. One study reported that *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* spp. are the most common pathogens that cause mastitis [44]. A study in 2022 demonstrated that *Staphylococcus aureus* and *Klebsiella pneumoniae* were found at lower proportions in mastitis cases. *Staphylococcus aureus* is a common pathogen in cases of mastitis, but it may not be as common as *Candida albicans* [45]. In a case-control study, women with mastitis were more likely than women in the control group to have *Staphylococcus aureus* in their breast milk [46]. According to research performed in Germany, the pathogens most found in mastitis instances can differ. Coliform infections like *E. coli* were discovered to be the most common pathogens in severe episodes of mastitis [47].

A lot of studies investigated the relation between the mastitis symptoms' and microorganism's type. *Staphylococcus aureus* and *Candida albicans*: an investigation reported that these germs are linked to lesions. Women with *Staphylococcus aureus* plus *Candida albicans* in their breast milk may have more lesions than those with *Candida albicans* [46]. Both bacteria have been linked to the symptoms of breast tissue lesions. These bacteria and their link to the development of breast lesions emphasize the necessity of appropriate cleanliness and efficient preventive measures during breastfeeding [48].

*Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans*: This combination of bacteria is linked to redness as a mastitis symptom according to a study performed in China [49]. Redness is associated with *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans* [50].

***Candida albicans*:** Cases involving *Candida albicans* alone had a higher frequency of redness and lesions due to an investigation [51,52]. When *Candida albicans* causes an infection without the presence of other microbes, it may generate a more prominent inflammatory response, including redness [50].

***Candida tropicalis*:** This bacterium has been related to redness as a mastitis symptom as mentioned [53]. When *Candida tropicalis* infects breast tissue, it can cause mastitis or breast

inflammation. The affected area may exhibit redness, swelling, and discomfort as part of the inflammatory response [54].

## 5 .Conclusion:

This study utilized a range of microbiological and molecular methodologies to identify the microorganisms accountable for mastitis in women. The investigation also analyzed the correlation between bacteria and the incidence and manifestations of mastitis. *Candida albicans* had a significant prevalence, namely in the nipple. The findings of the research demonstrated that various microorganisms exhibit unique symptom profiles.

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