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# ISOLATION OF THE BACTERIA THAT CAUSE MASTITIS IN DAIRY COWS ON FARMS IN YOGYAKARTA

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## ABSTRAK

Mastitis merupakan penyakit yang disebabkan oleh bakteri dan pada umum menyerang ternak perah. Berdasarkan hasil penelitian sebelumnya, terdapat perbedaan jenis bakteri penyebab mastitis di setiap daerah. Mengidentifikasi bakteri penyebab mastitis perlu dilakukan agar penyakit ini dapat ditangani dengan cepat, tepat, efektif dan efisien. Tujuan penelitian ini adalah untuk mengidentifikasi bakteri yang berpotensi menyebabkan mastitis di salah satu peternakan sapi perah di Yogyakarta. Pengujian *California Mastitis Test* dilakukan sebelum pengambilan sampel susu. Pengujian CMT dilakukan pada 65 kuarter susu dari 17 ekor sapi perah laktasi. Sampel susu yang menunjukkan hasil positif tiga kemudian diambil untuk diisolasi bakteri yang ada di dalamnya. Sampel susu yang berasal dari sapi yang terkena mastitis di peternakan tersebut diinolulasikan ke dalam *bood agar*, isolat yang tumbuh kemudian diinokulasikan kembali untuk selanjutnya dilakukan beberapa pengujian, seperti pewarnaan Gram, uji katalase, uji motilitas dan identifikasi menggunakan PCR. Hasil penelitian menunjukkan bahwa dua isolat dominan yang dihasilkan menunjukkan ciri – ciri berwarna ungu, berbentuk bulat, hasil uji katalase negatif dan hasil uji motilitas juga negatif. Hasil identifikasi menggunakan PCR menunjukkan bahwa kemungkinan spesies bakteri yang berhasil diisolasi adalah *Streptococcus agalactiae*, *Bacillus cereus ,Bacillus thuringiensis, Bacillus wiedmannii,* dan *Endophytic bacterium*. *Streptococcus agalactiae* dan *Bacillus cereus* merupakan bakteri dominan yang berhasil diisolasi dan kedua bakteri tersebut berpotensi sebagai penyebab mastitis pada peternakan di daerah Yogyakarta.

**Kata kunci** : Bakteri penyebab mastitis, *Bacillus cereus, Streptococcus agalactie*, Yogyakarta.

## ABSTRACT

Mastitis is a bacterial disease that commonly affects dairy cattle. According to previous studies, different regions have different types of bacteria that cause mastitis. Identifying the bacteria that cause mastitis is necessary for treating this disease quickly, precisely, effectively, and efficiently. This study aimed to identify bacteria that could potentially cause mastitis on a dairy farm in Yogyakarta. California Mastitis Test was conducted before milk sampling. CMT testing was performed on 65 milk quarters from 17 lactating dairy cows. Milk samples showing three positive results were taken to isolate the present bacteria. The milk samples from cows

with mastitis were inoculated into blood agar. The growing isolates were inoculated again for further testing, including Gram staining, the catalase test, the motility test, and identification using PCR. The results showed that the two dominant isolates produced purple-colored, round shapes with negative results for the catalase and motility tests. PCR identification revealed that the isolated bacterial species were *Streptococcus agalactiae, Bacillus cereus, Bacillus thuringiensis, Bacillus wiedmannii,* and *Endophytic bacterium*. *Streptococcus agalactiae* and *Bacillus cereus* were the dominant bacteria isolated, and both were potential causes of mastitis on farms in the Yogyakarta.

**Keyword** : Bacteria causing mastitis, *Bacillus cereus, Streptococcus agalactie*, Yogyakarta

## INTRODUCTION

Mastitis is one of the most common diseases in the dairy industry and can result in significant economic losses. The most prevalent form is subclinical mastitis, which is not evident through clinical symptoms, but can lead to decreased milk production, elevated somatic cell counts and an increased likelihood of clinical mastitis during lactation. (Tommasoni *et al*., 2023). Mastitis has become a problem. This is especially true in cases of increasing milk production. (Nuraini *et al*., 2023). Subclinical mastitis caused by *Staphylococcus aureus* is a serious problem because it causes the milk-producing tissue to turn into non-functional fibrous tissue. Mastitis is characterized by an inflammatory reaction involving an increase in blood protein and white blood cells in the mammary glands and milk produced (Prasetyo et al., 2013). Research by Nuraini et al. (2023) shows that the estimated combined prevalence rate of mastitis in dairy animals in Indonesia is 59.44%. Subclinical mastitis has a significantly higher prevalence than clinical mastitis (58.24% vs 3.31%) based on the type of mastitis. The highest prevalence (66.62%) was found in Central Java, while the lowest prevalence (41.77%) was found in Yogyakarta. The prevalence of mastitis was 64.48% in West Java and 60.52% in East Java.

Up to 80% of cases of subclinical mastitis are caused by the bacteria *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus dysgalactiae, Streptococcus agalactiae* and *Streptococcus uberis*, as well as coliform bacteria, particularly *Escherichia coli* and Klebsiella (Nurhayati and Martindah, 2015). According to Supar and Ariyanti (2008), *Streptococcus agalactiae*, *Staphylococcus aureus* and *Staphylococcus epidermidis* account for 91.5% of cases, while *Streptococcus dysgalactiae*, *Streptococcus uberis*, coliform and others account for the remaining 8.5%. A study by Abdi et al. (2021) showed that the bacteria causing clinical mastitis in Tennessee, Kentucky, and Mississippi were *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli, Klebsiella oxytoca*, and *Klebsiella pneumoniae*. *Staphylococcus aureus* was the most commonly isolated cause of mastitis. A study by Kusumawati et al. (2021) found that the bacterial species responsible for subclinical mastitis in Cicurug, Sukabumi, were *Corynebacterium variabile, Micrococcus lylae, Acinetobacter harbinensis, Acinetobacter soli* and *Acinetobacter ursingii*. Conversely, Oikonomou et al. (2012) found that *Trueperella pyogenes* was the most common bacterium causing mastitis in the United States, followed by *Streptococcus dysgalactiae* and *Staphylococcus aureus*. In the south-eastern region of Brazil, the most frequently isolated bacteria from mastitis milk samples were *Escherichia coli* (6.6% of total cultures), *Streptococcus uberis*

(6.1%) and *Streptococcus agalactiae* (5.9%) (Tomazi et al., 2018). Bi et al. (2016) found that the most common pathogenic bacteria in milk samples in China were *Staphylococcus spp., Streptococcus agalactiae, Streptococcus dysgalactiae, Arcanobacterium pyogenes* and the staphylococcal β-lactamase gene.

Based on the other studies, it can be concluded that there are differences in the bacteria that cause mastitis in different locations. Identifying the microorganisms that cause infection is very important. This affects the accuracy of therapy, the prevention of transmission and the prevention of antibacterial resistance. Conventional identification of the causative microorganisms is carried out through culture methods, followed by examination of their physiological and biochemical characteristics (Rinanda, 2011). The treatment of mastitis should consider identifying the responsible microorganisms (Royster and Wagner, 2015). Therefore, it is important to identify the bacteria causing mastitis in order to treat it effectively and efficiently.

## MATERIALS AND METHODS

The study was conducted from January to March 2024. CMT testing and milk sampling were carried out on cows suspected of having mastitis at a dairy farm in the Kaliurang, Yogyakarta. A total of 65 quarters from 17 lactating dairy cows were tested using the CMT method.

***California Mastitis Test* (CMT)**

California Mastitis Test was carried out according to the method described by Mellenberger and Roth (2000). First, 2 mL of milk was taken from each teat of the sample cows. This milk was then placed in a CMT paddle in a vertical position. California Mastitis Test solution was added to each section of the paddle, and it was rotated to mix the milk and solution. This process takes no more than 10 seconds. The results visible on the paddle must be read quickly, as the visible reaction deteriorates within 20 seconds. This reaction is assessed visually. The higher the value, the more gel formed. According to Mellenberger and Roth (2000), the CMT test results are determined as follows: Negative (not infected): no clots and a very thin consistency. Transient (very mild and possibly infected): the presence of clots that disappear when the paddle is rotated again. Positive 1 (mild infection): a thickening solution that does not form a gel. If the paddle is rotated for more than 20 seconds, the thickness disappears. Positive 2 (infected): characterised by the formation of clots and a light gel. The mixture will clot in the centre of the paddle and coat the bottom when discarded. Positive 3 (strongly infected) is characterised by the formation of a very thick gel that is difficult to move.

**Sample collection and storage**

Milk samples were taken from dairy cows that had previously tested positive for CMT, showing three positive results. Approximately 50 ml of milk was collected and placed in a sterile glass bottle. This bottle was then stored in a cooler box at 4°C and taken to the laboratory for further testing.

**Isolation of the bacteria that causes mastitis.**

One drop of milk from a case of mastitis was aseptically streaked onto the surface of a blood agar medium, then incubated at 37°C for 24 hours. The resulting growth was streaked onto a new blood agar plate and incubated at 37°C for a further 24 hours. The growing isolates were then subjected to several tests, including Gram staining, the catalase test, and the motility test.

**Identification by Gram staining**

A single bacterial colony grown on a blood agar medium is streaked onto a

microscope slide. This preparation is then stained with Gram A stain for one minute and rinsed with water. The remaining water is then discarded, and the slide is stained with Gram B stain, left for one minute, rinsed with water and stained with Gram C stain for 10 to 20 seconds. After rinsing again with water, the slide is stained with Gram C for 10–20 seconds, rinsed again with water, and finally stained with safranin (Gram D) for 10–20 seconds. The preparation is then dried and observed under a binocular microscope at 1000x magnification to examine cell morphology. Gram-positive bacteria appear purple, while Gram-negative bacteria appear red.

**Catalase Test**

Gram-positive colonies were collected using a sterile loop, streaked onto a glass slide and dripped with 3% hydrogen peroxide ($H_2O_2$). The catalase test showed a positive result when bubbles formed; a negative result was indicated by the absence of bubbles.

**Motility Test**

Bacterial isolates are aseptically inoculated into MRS broth containing 0.5% agar using a straight-tipped inoculating needle and then incubated at 36°C for 18 to 24 hours. If the bacterial growth spreads, the bacteria are motile; if it does not spread (forming lines), they are non-motile.

**Identification of bacteria using PCR.**

Isolates suspected to be the dominant cause of bacterial mastitis were then identified by extracting their genomic DNA using the Quick-DNA Fungal/Bacterial Miniprep Kit, amplifying it with 2x Dream Taq PCR Master Mix and performing bidirectional sequencing.

**Data analysis**

The data collected were analysed descriptively.

## RESULT AND DISSCUSSION

A total of 62 out of 65 quarters of 17 lactating dairy cows were affected by mastitis. This equates to 95.4% of the farm's cow population being affected by the disease. A sample cow's milk suspected to be infected with mastitis was inoculated onto blood agar and incubated to obtain several bacterial colonies. The bacterial colonies that grew on the surface of the blood agar from the milk of the cow with mastitis were then re-inoculated on blood agar and incubated. Two dominant isolates (SSA II and SSA IV) were then taken. Several tests were then performed on these two isolates, including Gram staining, the catalase test, and the motility.

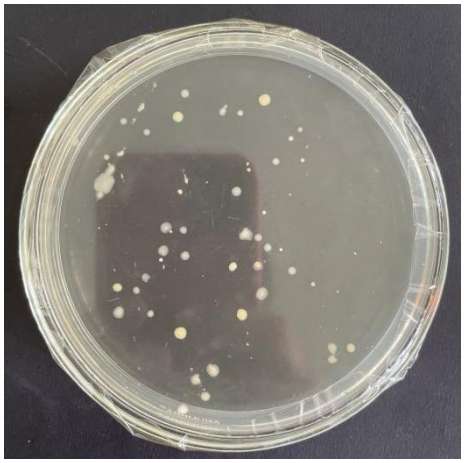

Figure 1. A colony of bacteria is suspected of causing mastitis.

**Gram staining**

Gram staining is one of the simplest and cheapest methods of quickly diagnosing bacteria. It is much faster than bacterial culture and provides an initial guideline for deciding on antibiotic therapy before definitive evidence of the specific bacteria causing the infection becomes available. The disadvantage of this method is that it can only determine the size and shape of bacteria, and view their internal structure, using dye alone (Bulele *et al*., 2019).

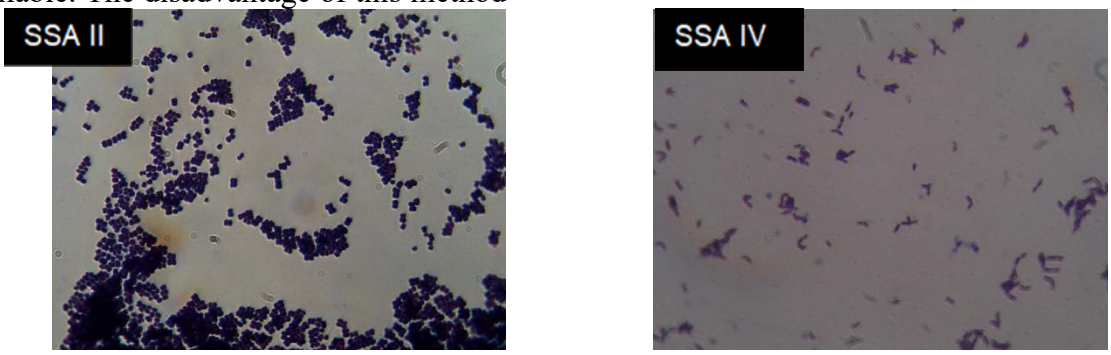


Figure 2. Gram staining results on two bacterial colonies identified as causing mastitis.

Both isolates were purple, indicating that the two bacterial isolates suspected of causing mastitis are Gram-positive. Gram-positive bacterial colonies are purple, whereas Gram-negative ones are red. This colour difference is due to variations in bacterial cell wall structure and teichoic acid content between Gram-positive and Gram-negative bacteria. Gram-positive bacteria contain peptidoglycan with teichoic acid, which allows them to avoid decolourisation by alcohol when crystal violet is present. In contrast, Gram-negative bacteria lack teichoic acid, resulting in decolourisation of crystal violet by alcohol and the formation of a red colour upon staining with safranin (Aristyawan *et al*., 2018).

**Catalase Test**

The catalase test is used to detect the presence of the catalase enzyme in a bacterial species. This enzyme breaks down hydrogen peroxide, releasing water and oxygen. This test is useful for characterising most bacteria. This enzyme protects bacteria from oxidative damage. It helps to separate catalase-positive organisms, such as staphylococci, from catalase-negative species, such as streptococci (Khatoon et al., 2022).

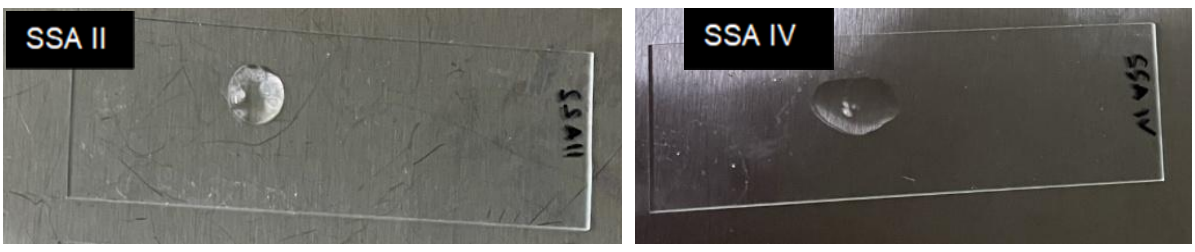


Figure 3. Catalase test results for two bacterial isolates suspected to be causing mastitis.

Based on the results of the catalase test, both bacterial isolates were found to be catalase-negative. Neither isolate produced bubbles when $H_2O_2$ was added. This is because these bacteria do not produce the catalase enzyme, which is required to

hydrolyse hydrogen peroxide into water and gas bubbles (Permatasari et al., 2022).

**Motility Test**

Bacteria can move thanks to their flagella. These are organelles that enable bacteria to move. The presence or activity of flagella in bacteria can be visualized by observing the microbes' ability to move in the growth medium. Bacteria are said to be motile if the area around them appears cloudy after incubation (Jain et al., 2020). The test results showed that neither isolate had flagella, as the area around the puncture did not appear cloudy.


Figure 4. Results of motility tests for both bacterial isolates suspected of causing mastitis.

**Identification of bacteria using PCR.**

The 16S rRNA gene is one of the most well-characterised genes and is widely used for identifying microorganisms (Rinanda, 2011). The figure below shows the top 10 HIT BLAST results and the phylogenetic tree for both isolates.

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Streptococcus agalactiae strain SSH_2104020158.D967-1-13.16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | OQ680013.1 |
| Streptococcus agalactiae strain 149 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670748.1 |
| Streptococcus agalactiae strain 148 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670747.1 |
| Streptococcus agalactiae strain 146 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670745.1 |
| Streptococcus agalactiae strain 144 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670743.1 |
| Streptococcus agalactiae strain 142 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670741.1 |
| Streptococcus agalactiae strain 139 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670738.1 |
| Streptococcus agalactiae strain 109 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670708.1 |
| Streptococcus agalactiae strain 107 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670706.1 |
| Streptococcus agalactiae strain 038 16S ribosomal RNA gene, partial sequence | 2623 | 2623 | 100% | 0.0 | 100.00% | MZ670637.1 |

Figure 5. A list of the top ten HIT BLAST matches for SSA II samples against the NCBI database, excluding sequences from uncultured samples.

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Bacillus cereus strain F5 16S ribosomal RNA gene, partial sequence | 2621 | 2621 | 100% | 0.0 | 99.86% | MK088304.1 |
| Bacillus thuringiensis strain NS9 16S ribosomal RNA gene, partial sequence | 2621 | 2621 | 100% | 0.0 | 99.86% | ON045795.1 |
| Bacillus wiedmannii strain NY-19 16S ribosomal RNA gene, partial sequence | 2615 | 2615 | 100% | 0.0 | 99.79% | MF988727.1 |
| Endophytic bacterium strain B6 16S ribosomal RNA gene, partial sequence | 2615 | 2615 | 100% | 0.0 | 99.79% | OM938272.1 |
| Bacillus cereus strain ML208 16S ribosomal RNA gene, partial sequence | 2615 | 2615 | 100% | 0.0 | 99.79% | KC692193.1 |
| Bacillus thuringiensis strain Gaoshi-1 16S ribosomal RNA gene, partial sequence | 2615 | 2615 | 100% | 0.0 | 99.79% | GU201858.1 |
| Bacillus cereus strain QD232 16S ribosomal RNA gene, partial sequence | 2615 | 2615 | 100% | 0.0 | 99.79% | EF488087.1 |
| Bacillus cereus strain OOA 16S ribosomal RNA gene, partial sequence | 2614 | 2614 | 100% | 0.0 | 99.79% | OR702892.1 |
| Bacillus thuringiensis strain PS3 chromosome, complete genome | 2614 | 36481 | 100% | 0.0 | 99.79% | CP130743.1 |
| Bacillus cereus strain CP1 16S ribosomal RNA gene, partial sequence | 2614 | 2614 | 100% | 0.0 | 99.79% | OR660382.1 |

Figure 6. A list of the top ten most significant matches between SSA IV samples and the NCBI database (excluding sequences from uncultured samples).

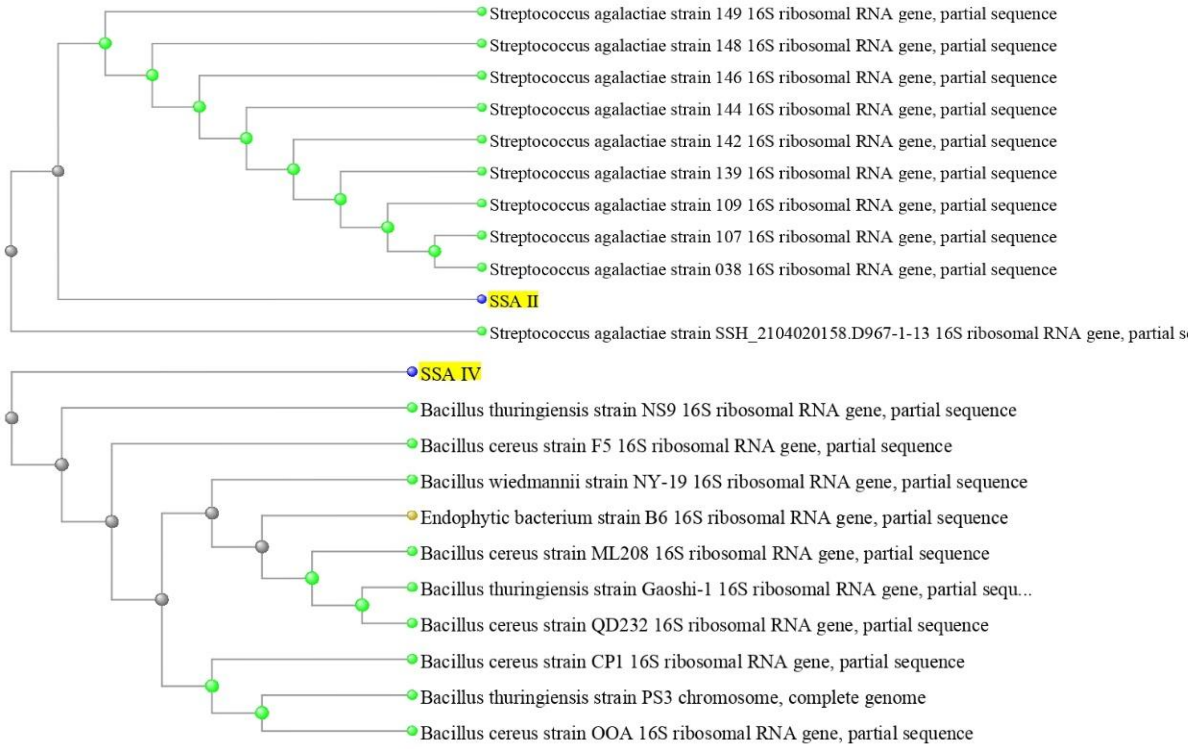


Figure 7. Phylogenetic tree of SSA II and SSA IV samples

Based on the test results, isolate SSA II is identified as Streptococcus agalactiae. Several bacterial species were identified in isolate SSA IV, including Bacillus thuringiensis, Bacillus wiedmannii, Endophytic bacterium and Bacillus cereus. Bacillus cereus are possible bacteria in isolate SSA IV.

Streptococcus agalactiae, also known as group B streptococcus (GBS), is an infectious bacterium that causes mastitis in dairy cows. This disease results in considerable economic losses (Lin et al., 2021). Streptococcus agalactiae is purple, round, and forms chain-like colonies. Biochemical tests showed negative catalase test results (no bubbles appeared after adding 3% hydrogen peroxide) and positive CAMP test results (Han et al., 2022). Streptococcus agalactiae is an udder pathogen transmitted directly from cow to cow during milking. Bacterium infects udder, causing irritation, swelling, and subclinical mastitis. Cows with subclinical mastitis produce low milk yields and have high somatic cell counts (Kibebew, 2017). The bacterium Bacillus cereus causes several diseases in humans and animals (Baldwin, 2020). Bacillus cereus is a rod-shaped, Gram-positive, facultatively anaerobic, endospore-forming pathogen that causes mastitis in cows and severe food poisoning in humans (Swartz et al., 2016). The Bacillus cereus strains isolated from cases of subclinical bovine mastitis showed high rates of resistance to most of the tested antibiotics due to the presence of antibiotic-resistant and virulence genes (Eid et al., 2023). The most common contaminants on dairy farms are soil, straw, and other fodders. Bedding used for indoor housing of cows during winter is also a potential contaminant. Contaminated udders can result in the presence of Bacillus cereus in raw milk (Bennett and Belay, 2001). Streptococcus agalactiae and Bacillus cereus are bacteria that can potentially cause mastitis. However, no literature studies show that Bacillus thuringiensis,

Bacillus wiedmannii, or Endophytic bacteria can cause mastitis in dairy cattle.

## CONCLUSION

The bacteria successfully isolated from the milk of cows with mastitis on farms in the Yogyakarta area were *Streptococcus agalactiae, Bacillus cereus, Bacillus thuringiensis, Bacillus wiedmannii,* and *Endophytic bacteria*. *Streptococcus agalactiae* and *Bacillus cereus* are bacteria that have the potential to cause mastitis on farms in Yogyakarta.

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