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Metagenomic Analysis of Bacterial Diversity in Milk of Mastitis Cows from Farms with Different Milking Management

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ABSTRACT

Most cases of mastitis are caused by bacteria that infect the udder through the milk ducts causing inflammation. The objective of this study was to identify the bacteria that cause mastitis in farms that apply different milking management. Mastitis testing using the California Mastitis Test (CMT) was conducted on 132 quarters of milk samples from 33 cows in three farms with different milking management practices. The 3 positive samples from each farm were taken and analyzed for metagenomics in the laboratory. The V1-V9 regions of the 16S ribosomal RNA gene were amplified using primers 27F and 1492R under specific polymerase chain reaction conditions for bacterial identification. DNA concentration was determined using both NanoDrop spectrophotometers and Qubit fluorometer. Library preparations were conducted using Kits from Oxford Nanopore Technology. Primary data were obtained using MinKNOW version 23.04.5. The results of metagenomic analysis of mastitis cow milk samples from farms with different milking management were dominated by bacteria from the Firmicutes and Proteobacteria phylum. *Streptococcus agalactiae* was the dominant bacteria causing mastitis in Kemiri farm and UPTD BPBPTDK, while *Lactococcus lactis* was the dominant bacteria causing mastitis in UPT farm. Kemiri farm had the highest diversity of bacteria in milk compared to the other two farms. The same 56 bacterial species were found on all three farms. Different milking management practices on the three farms showed different bacterial diversity and causes of mastitis.

Key words: *Lactococcus lactis*, Mastitis, Milking management, *Streptococcus agalactiae*

INTRODUCTION

Mastitis is the most common inflammation of the mammary gland in dairy cattle. It is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue. If left untreated, this inflammation can reduce milk production and quality, causing huge economic losses (Bhakat et al. 2020). Many studies have shown that the main cause of mastitis is bacteria that spreads among the herd. When one teat is infected with bacteria, it is possible for it to spread to healthy teats (Ruegg 2017).

Bacterial infection of the udder is a major cause of mastitis in dairy cows. Many species of pathogenic bacteria have been identified as causative agents of mastitis in dairy cows. The types of bacteria that infect the udder are classified into 2 types, namely pathogenic bacteria and bacteria originating from the surrounding environment of the barn. Mastitis spread in herds is thought to be transmitted from cow to cow during the milking process (Cheng and Han 2020). For decades, *Streptococcus agalactiae* and *Staphylococcus aureus* were considered the most dominant infectious pathogenic bacteria (Ruegg 2017). A total of 193 bacteria were isolated from 174 quarter milk samples from 151 cows with above-average SCC scores and 34 dairy cows infected with clinical mastitis in Tennessee, Kentucky and Mississippi. Of the 193 bacteria isolated, six bacterial species dominated, including *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli, Klebsiella oxytoca* and *Klebsiella pneumoniae*. *Staphylococcus aureus* is the dominant bacteria among the six bacteria (Abdi et al. 2021).

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The results of metagenomic analysis of bacterial genus isolated from subclinical mastitis milk from Cicurug, Sukabumi conducted by Kusumawati et al. (2021) were dominated by the genus Corynebacterium_1, Corynebacterium, Solibacillus, Romboutsia, Micrococcus, Acinetobacter, Aerosphaera, Ignavigranum, Lysinibacillus and Staphylococcus. The results of metagenomic analysis detected several bacteria such as *Corynebacterium variabile, Micrococcus lylae, Acinetobacter harbinensis, Acinetobacter soli* and *Acinetobacter ursingii.* Three factors play an important role in mastitis infection: pathogenic bacteria, livestock and the environment. In addition to these three factors, cow breed, udder anatomy, parity, nutritional status, immunity, lactation rate, productivity, pen density, climate, housing system, pen cleanliness, sanitation and milking methods also influence the incidence of mastitis (Sahoo et al. 2024).

In dairy livestock management, hygiene is of utmost importance, especially hygiene at the farm level. It greatly affects the quality and safety of the milk produced, besides maintaining cleanliness is indirectly able to reduce losses during production and post-harvest. Cleanliness is not only about the cleanliness of the animal (especially the udder), but also the cleanliness around the barn, hygienic milking and milk harvesting methods, and the cleanliness of milk storage containers. There is a correlation between hygienic milking management and the incidence of mastitis caused by pathogenic bacteria. Milking management plays an important role in maintaining udder health from mastitis. Routine teat dipping, dry cow therapy and mastitis treatment at the farm level can be done to reduce somatic cell counts and maintain milk production (Kashongwe et al. 2017). Hygiene is very important in the maintenance of dairy cattle. Improving sanitation such as improving milking hygiene, do teat dipping after milking, milking machine maintenance are common measures that should be taken to prevent new cases of mastitis (Cheng and Han 2020). Given the importance of milking management and hygiene at the farm level, the research objective was to determine the diversity of bacteria that cause subclinical mastitis in farms that apply different milking management.

MATERIALS AND METHODS

Ethical approval

This research was approved by the Local Ethics Committee Ethics Committee for Animal Experimentation, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Number: 122/EC-FKH/Eks./2023).

Study period and location

The research took place from June to October 2023. Mastitis milk samples were conducted in 3 farms, namely Kemiri farm owned by Kemiri cattle group (located in Kemiri, Purwobinangun Village, Pakem District, Sleman Regency, Yogyakarta, Indonesia), UPTD BPPTDK owned by the Yogyakarta Provincial Livestock Service Office (located in Kaliurang District, Sleman Regency, Yogyakarta, Indonesia) and UPT farm owned by the Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta (located in Depok District, Sleman Regency, Yogyakarta, Indonesia). The three farms were chosen because they apply different milking management. Kemiri farm implements milking with a milking machine and routinely performs teat dipping after milking. UPTD BPPTDK implements manual milking by hand without applying teat dipping after milking and UPT farm implements milking with a milking machine and does not perform teat dipping after milking.

Determining SCM in dairy cows

The determination of cows affected by mastitis was made by performing the CMT. CMT testing was conducted on 33 dairy cows across the three farms. The first step was to take 2ml of fresh milk from each teat of the sample cow. The milk was placed on the CMT paddle in a vertical position. CMT solution was added to each part of the paddle then the CMT paddle was rotated, so that the milk and CMT solution were mixed. The mixing process took no more than 10 seconds. The results seen in the CMT paddle are read quickly because the visible reaction will break down within 20 seconds. Assessment of this reaction is visual. The more gel formed, the greater the value. Negative results (-) are characterized by no lumps and very dilute, trance (T) is characterized by the presence of lumps and this reaction will disappear if the paddle is turned again, positive 1 (+) is characterized by a solution that thickens but does not tend to gel, if the paddle is turned for more than 20 seconds the viscosity disappears, positive 2 (++) is characterized by clumps and forms a light gel, the mixture will clump in the center of the paddle and will coat the bottom when removed and positive 3 (+++) is characterized by the formation of a very thick gel and difficult to move.

Sample collection

The milk samples used were milk samples from cows that showed positive 3 (+++) test results when testing for mastitis with the CMT test. A 50ml sample of milk was taken, put into cooler box, and taken directly to the laboratory for analysis.

Determine the concentration of DNA

Milk samples from each farm were taken as much as 10ml and then centrifuge at 2500 rpm/g for 10 minutes. The pellet obtained was washed with saline, then rinsed with sterile distilled water. The pellet was used for genomic DNA extraction using ZymoBIOMICS DNA Miniprep Kit D4300 (Zymo Research, Cambridge, UK). Determination of DNA concentration was done using NanoDrop spectrophotometer and Qubit fluorometer (Thermo Fisher, Waltham, MA, USA). Assessment of DNA quality was done through agarose gel electrophoresis and subsequent visualization using Gel-Doc EZ imaging (Bio-Rad, CA, USA).

Amplification of the 16s rRNA

The V1–V9 regions of the 16S rRNA gene for the identification of bacterial species were amplified using 27F and 1492R primers.

Library preparation and sequencing

Nanopore sequencing was operated by MinKNOW software version 23.04.5. Basecalling was performed using Guppyversion 6.5.7 with high-accuracy model (Wick et al. 2019). The quality of FASTQ files were visualized using NanoPlot, and quality filtering was performed using NanoFilt (De Coster et al. 2018; Nygaard et al. 2020).

Bioinformatics analysis

Filtered reads were classified using centrifuge classifier (Kim et al. 2016). Bacteria and archaea index was built using NCBI 16SRefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Downstream analysis and visualizations were performed using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and R Studio using R version 4.2.3 (https://www.R-project.org/).

RESULTS

Microbiome in milk influences the pathophysiology of bovine mastitis (Hoque et al. 2019). The microbiota in milk is known to be very complex due to contamination from various sources (Addis et al. 2016). In milk from mastitis-infected cows, the microbiota consists of various genera with great microbial diversity (Taponen et al. 2019). The relative abundance of milk samples from the three farms can be seen in Fig. 1. The bacteria that dominate milk from Kemiri farm and UPTD BPPTDK is *Streptococcus agalactiae*. While the bacteria that dominates the milk from the UPT farm is *Lactococcus lactis*.

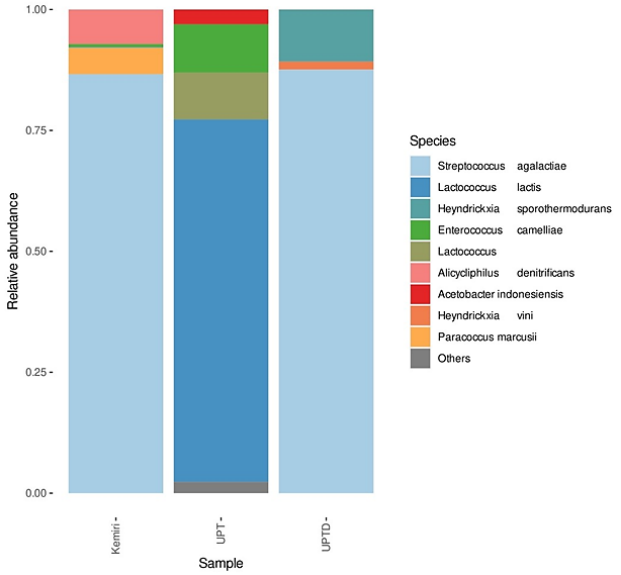

Fig. 1: The relative abundance of milk samples from the three farms

The total number and relative abundance of bacteria in the three farms at the phylum level are presented in Table 1. Based on the table above, the Actinobacteria, Firmicutes and Proteobacteria were the dominant phylum in the three farms. In Kemiri farm, the dominant bacterial phylum was Proteobacteria (41.73%). At UPT farm and UPTD BPPTDK, the dominant bacterial phylum was Firmicutes (74.52 and 99.80%). Bacterial composition of milk samples from the three farms can be seen in Fig. 2, 3 and 4.

Table 1: Total number and relative abundance of bacteria in the three farms at the phylum level

No	Phylum	Kemiri farm	UPT farm	UPTD BPPTDK
1	Abditibacteriota	2	0	0
2	Acidobacteriota	326	24	1
3	Actinobacteria	7540	3902	36
4	Aquificota	62	0	0
5	Armatimonadota	15	2	0
6	Firmicutes	29735	63718	91094
7	Bacteroidetes	1273	2285	7
8	Balneolota	68	7	0
9	Bdellovibrionota	68	28	0
10	Campylobacterota	657	19	6
11	Chlamydiota	2	0	0
12	Chlorobiota	12	0	0
13	Chloroflexota	91	13	0
14	Cyanobacteriota	296	24	4
15	Deinococcota	458	597	0
16	Elusimicrobiota	50	0	0
17	Euryachaeota	3	0	0
18	Fibrobacterota	6	0	0
19	Fusobacteriota	33	1	0
20	Gemmatimonadota	151	0	0
21	Ignavibacteriota	105	1	0
22	Kiritimatiellota	212	0	0
23	Lentisphaerota	55	1	1
24	Mycoplasmatota	210	3	1
25	Myxococcota	139	6	0
26	Nitrospinota	1	0	0
27	Nitrospirota	449	3	0
28	Planctomycetota	1270	1	4
29	Proteobacteria	31463	14840	120
30	Rhodothermota	5	0	0
31	Spirochaetota	172	1	0
32	Synergistota	8	0	0
33	Thermodesulfobacteriota	2	1	0
34	Thermomicrobiota	2	1	0
35	Thermotogota	159	3	0
36	Verrucomicrobiota	300	20	0

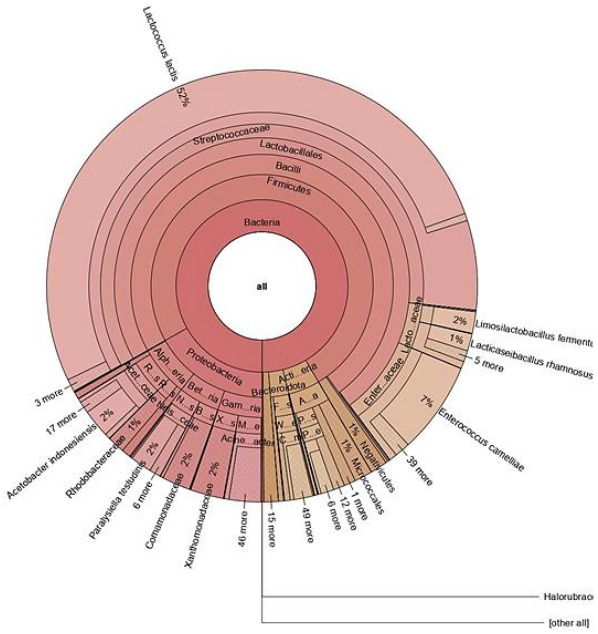

Fig. 2: Bacterial composition of milk samples from UPT farm

The Venn diagram (Fig. 5) shows the relationship between the presence of bacteria on the three farms from which milk samples were taken. A total of 56 bacterial species were present in the three farms.

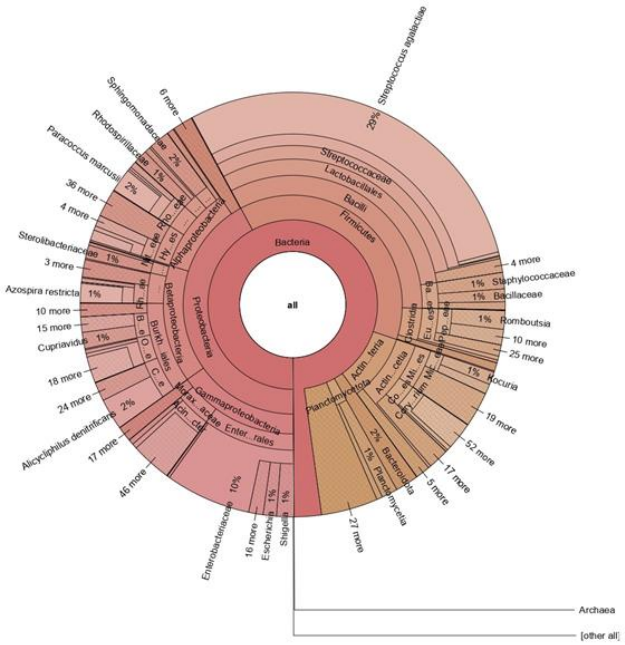

Fig. 3: Bacterial composition of milk samples from Kemiri farm

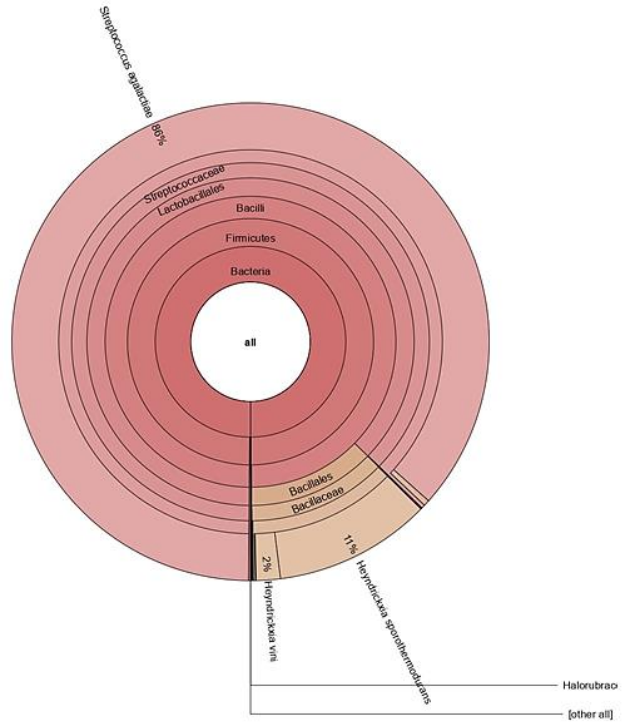

Fig. 4: Bacterial composition of milk samples from UPTD BPPTDK

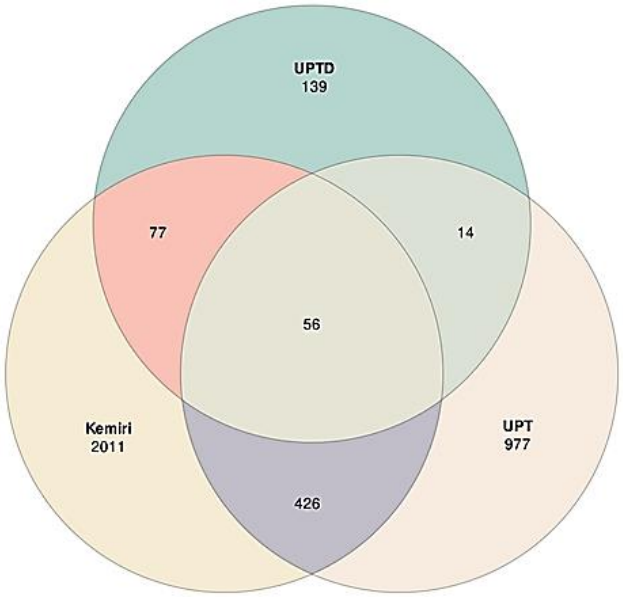

Fig. 5: Venn diagram of microorganisms from metagenomic milk samples

DISCUSSION

Mastitis is the most common disease found in dairy herds and causes huge economic losses. Subclinical mastitis has a direct impact on milk quantity and quality. One way to reduce the percentage of mastitis infection is regular mastitis examination followed by good milking hygiene and management. Environmental conditions, animal factors (age, parity and lactation) immune status, differences in milking practices, seasonal variations and hygienic conditions are factors that influence the prevalence of subclinical mastitis (Ragab et al. 2023).

The prevalence of mastitis in dairy cows is high, impairing milk production efficiency. Many *Streptococcus agalactiae* bacteria were recovered from cows infected with subclinical mastitis. This indicates that subclinical mastitis is associated with unhygienic milking practices and poor hygiene management (Furgasa et al. 2019). The onset of mastitis is due to three factors: the pathogen, the cow and the farmer's management. It is better to prevent mastitis than to treat it (De Vliegher et al. 2018).

Streptococcus agalactiae is a pathogenic bacterium that dominates milk from UPTD BPPTDK. As is known, UPTD BPPTDK applies hand milking without do teat dipping after milking. *Streptococcus agalactiae* is a Gram-positive pathogenic bacterium that causes infectious mastitis. Besides being found in the gastrointestinal tract of cows, it is also found around the environment of dairy cows. Transmission by this bacterium can be through milking machines as well as through contaminated drinking water. Avoiding infection by *Streptococcus agalactiae* bacteria can be done by keeping the udder clean and practicing hygienic milking. However, it is not enough to control *Streptococcus agalactiae* infection, fecal management and cleanliness of the environment around the barn must also be considered (Jørgensen et al. 2016).

Streptococcus agalactiae is one of the bacteria infectious pathogens that can adapt well around the udder and can spread from one cow to another through the milking process (Bi et al. 2016). If a farm does not do teat dipping after milking, the cows are more likely to contract mastitis (usually caused by infectious pathogens) than cows that are milked and do teat dipping as part of milking management (De Vliegher et al. 2018). UPTD BPPTDK and UPT farm do not do teat dipping after milking, so the dominance of pathogenic bacteria in them is more than 50%. While Kemiri farm applies teat dipping after milking as part of milking management, the number of dominant pathogenic bacteria is less than 30%. The total population of *Streptococcus agalactiae* bacteria at UPTD BPPTDK was 89%, the large population of pathogenic bacteria indicates that this farm pays less attention to the sanitation

of pen, milkers and milking equipment so that pathogenic bacteria are easily spread during the milking process..

Prevention of subclinical mastitis in dairy cows can be done by performing teat dipping before and after the milking (Tanbayeva et al. 2024). This is supported by a statement from Andrew et al. (2021) which states, improving milk quality is inseparable from maintenance management and cleanliness of milking equipment, but to improve milk quality, you must also pay attention to care before and after milking, namely by doing teat dipping. It is very important to perform udder care after milking. Teat dips after milking should be done immediately as the teat canal remains open for 30 minutes to 1 hour after milking, so it is necessary to protect the teats from pathogenic bacterial contamination until the next milking (Komarov 2016).

The dominant pathogenic bacteria causing mastitis in the UPT farm is *Lactococcus lactis. Lactococcus lactis* is one of the potential causative agents of mastitis in dairy cows (Wu et al. 2023). Research by Rodrigues et al. (2016) showed that Lactococcus was the dominant genus in mastitis milk samples, while in healthy milk samples it was relatively low. This suggests that Lactococcus bacteria could be a potential etiological agent in mastitis. *Lactococcus lactis* is increasingly being found as a cause of infection in humans or animals (especially mastitis cases in cows) due to an increase in the identification of infective microorganisms (Plumed-Ferrer et al. 2013).

Many factors play a role in the occurrence of mastitis infection, such as bacteria, farmers (management) and the cattle themselves. A cow (of a certain age, at a certain stage of lactation, with a different level of immunity from other cows), managed by a farmer (who feeds it certain nutrients and applies certain milking management) in a certain environment (a certain type of barn, barn hygiene that may be different from other barn environments, exposed to a diversity of mastitis pathogenic bacteria and with different virulence traits) can cause disease. When the balance is tipped in favour of the pathogen, mastitis occurs (De Vliegher et al. 2018). In addition to the factors mentioned above, differences in the bacteria that cause mastitis in each farm can also be caused by differences in farm location. This is in line with Bi et al. (2016) which states that the prevalence of bacteria that cause mastitis in dairy herds differs between provinces. This is due to geographical variations. Quality milk and dairy products come from continuously improved cow management hygiene, such as animal health and care, stable cleanliness, udder cleaning, cleaning and disinfection of milking machines, equipment and so on. Therefore, hygiene training for farm workers is important to be conducted on a regular basis (Goksoy et al. 2020).

Conclusion

Metagenomic analysis of mastitis cow milk samples from farms with different milking management revealed a diversity of bacterial families and genera, especially from the Firmicutes and Proteobacteria phylum. The main bacteria observed and detected as causing mastitis in the three farms were *Streptococcus agalactiae* and *Lactococcus lactis*. Milk samples of mastitis cows from farms with different milking management showed different bacterial diversity and richness.

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Author's Contribution: Theresia Ika Purwantiningsih: conceived and designed the research, collected data, analyzed data, and drafted the manuscript. Budi Prasetyo Widyobroto, Yustina Yuni Suranindyah and Wayan Tunas Artama: designed the research, supervised the research, and proofread the draft manuscript. All authors revised the manuscript and read and approved the final manuscript.

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