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ANALYSIS OF MILKING HYGIENE AND ITS ASSOCIATION TO STAPHYLOCOCCUS AUREUS CONTAMINATION IN FRESH COW MILK

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Abstract

Introduction: *Staphylococcus aureus foodborne disease is caused by the consumption of food contaminated with Staphylococcus aureus enterotoxins. Milk is a type of food that contains many nutrients but is susceptible to contamination by Staphylococcus aureus. Jember is one of the districts in East Java that produces cow milk. Previous research showed that the level of milk consumption in the community in 2018 was 3.1 kg/capita/year and 42% consumed pasteurized milk, which has lower quality and may still be contaminated with bacteria. The purpose of this study was to determine the relationship between milking hygiene and Staphylococcus aureus contamination.* ***Methods:*** *A cross-sectional study was conducted involving 36 cow milkers with traditional milking, selected by total sampling. The independent variable was milking hygiene and the dependent variable was Staphylococcus aureus contamination. Milking hygiene practice data were collected through direct observation using a questionnaire. Staphylococcus aureus contamination data were tested using Total Plate Count (TPC) and identified using Gram staining and catalase tests.* ***Results and Discussion:*** *The TPC test results showed that 61.1% of cow's milk fulfilled the Indonesian National Standard for Staphylococcus aureus contamination. There was a relationship between milking hygiene and Staphylococcus aureus contamination. The most influential milking hygiene was cage, udder, and teat hygiene.* ***Conclusion:*** *Staphylococcus aureus contamination did not meet the standards. Therefore, improving the sanitation and hygiene of cages, as well as udder and teat hygiene by dairy farmers, is necessary.*

INTRODUCTION

Staphylococcus aureus is a cocci-shaped gram-positive bacterium with a diameter of 1 µm and arranged in grape-like colonies. *Staphylococcus aureus* does not produce spores and has the highest resistance because it can survive under aerobic or anaerobic conditions. It is widely distributed in air, dust, water, waste, surfaces, animals, and humans, and has a crucial competitive advantage in Intermediate Moisture Food (1). *Staphylococcus aureus* usually inhabits and grows on the nose and skin of healthy humans as normal flora. However, this bacterium is also pathogenic if the number of bacteria is excessive compared to normal levels, the host's immunity is decreased, and the bacteria are not in their proper predisposition. These bacteria can cause diseases ranging from mild to severe (2). These bacteria cause foodborne diseases caused *Staphylococcus aureus* if they contaminate food.

Staphylococcus aureus foodborne disease is a condition that occurs because *Staphylococcus aureus* bacteria contaminate food and then produce *Staphylococcus enterotoxin* (SE) and *Staphylococcus enterotoxin*-like toxin (SEl) (3). *Staphylococcus* aureus infections contribute to significant morbidity and mortality in both developing and developed countries. This bacterium accounts for up to one-third of all foodborne gastrointestinal diseases. It is the leading cause of foodborne diseases, causing an estimated 241,000 illnesses annually in the United States. However, because the condition is self-limiting, the presentation rate to healthcare facilities is low (10%), resulting in under-reporting of the estimated 6 to 80 million cases that

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occur annually. However, foodborne illness surveillance systems in developing countries are poor. As a result, the incidence of staphylococcal-related foodborne diseases is estimated to be much greater than that recorded, with many cases unreported (4).

There are many variants of the *Staphylococcus enterotoxin*. At least there are a total of 26 serologically distinct SE and SEI types, namely: A (SEA), B (SEB), C1 (SEC1), C2 (SEC2), C3 (SEC3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J (SEIJ), K (SEIK), L (SEIL), M (SEIM), N (SEIN), O (SEIO), P (SEIP), Q (SEIQ), R (SER), S (SES), T (SET), U (SEIU), W (SEIW), V (SEIV), X (SEIX), and Y (SEIY) (5). The first five well-characterized SEs are SEA and SEE, which can cause vomiting. SEA is the most common serotype found in *Staphylococcus aureus* and is responsible for up to 78% of food poisoning outbreaks, followed by SED and SEB. The mechanisms involved in SEA-induced vomiting involve binding to submucosal mast cells and inducing mast cell degranulation. 5-hydroxytryptamine also plays an important role in the transmission of vomiting stimulation to the vagus afferent nerve or central nervous system. Type SED can form homodimers in the absence of Zn^{2+}, which facilitates binding to MHC II molecules on antigen-presenting cells and allows it to function as a superantigen. SEC has three different antigen sub-types, SEC1, SEC2, and SEC3, SEG, SEH, SEI, SER, SES, and SET also have potent emetic activity, while SEIJ, SEIK, SEIM, SEIN, SEIO, and SEIP have very low emetic activity (6–8).

SE and SEI share four similar properties: structural similarity, superantigenicity, emetic activity, and resistance to heat and proteolytic enzymes. *Staphylococcus enterotoxins* are commonly heat-resistant (121°C for 10 min), and the toxin remains active after boiling for 30 min. *Staphylococcus enterotoxins* that are resistant to digestive proteolytic enzymes, such as trypsin and pepsin, can maintain activation in the digestive tract, causing food poisoning (3).

The quantity of toxin required to cause the disease is less than 1 µg (3). The incubation period and severity of symptoms vary widely according to the type and amount of *Staphylococcus enterotoxin* ingested and the susceptibility of each person. Symptoms may develop within 30 min to 8 h after the ingestion of contaminated food. Symptoms include fever, chills, dizziness, nausea, vomiting, abdominal pain, and watery diarrhea (2). Massive fluid loss can lead to dehydration and hypotension (4).

Staphylococcus aureus contaminates food and produces *enterotoxins* when conditions allow the growth of these bacteria in food. *Staphylococcus enterotoxins* are tolerant to heat, acids, and stomach enzymes. When contaminated food enters the digestive organs and reaches the small intestine, the enterotoxin penetrates the lamina propria through mucus-producing goblet or epithelial cells. *Staphylococcus enterotoxin* activates mast cells, which then stimulates the release of 5-hydroxytryptamine (5-HT) and histamine. Subsequently, the released 5-HT binds to 5-HT3 receptors expressed on enteric nerves and induces depolarization. Finally, depolarization of the vagus afferent nerve stimulates the brainstem emetic locus to initiate the gag reflex. T cells and neutrophils are also enabled, but their roles remain unknown (9-10).

Staphylococcus enterotoxins induce inflammation in the gastrointestinal tract with severe lesions in the jejunum and ileum. *Enterotoxins* cause intestinal epithelial cell damage, resulting in villous destruction, villous distension, lymphoid hyperplasia, and crypt elongation (6). Inflammation of the lower gastrointestinal tract caused by *Staphylococcus enterotoxins* is largely mediated by the super-effect of enterotoxin antigens on MHC class II-expressing macrophages, dendritic cells, and myofibroblast APC- and TCR-expressing CD^{4+} T cells. *Staphylococcus enterotoxin* can traverse the intestinal epithelial barrier in its intact form and bind to MHC class II molecules expressed in subepithelial myofibroblasts. This process results in the production of potent proinflammatory cytokines and chemokines such as MCP-1, IL-6, and IL-8. $TCD4^{+}$ cells and macrophages from gut-associated lymphoid tissue (GALT) to the site of SE-associated inflammation in the gastrointestinal mucosa. The interaction of MHC class II, *Staphylococcus enterotoxin*, and TCR can result in the hyperactivation of APCs and T cells, leading to excessive T cell proliferation and an uncontrolled burst of various pro-inflammatory cytokines and chemokines, leading to acute super-antigen-mediated inflammation and shock (6).

All food products, including cattle milk and dairy products, have the potential to be contaminated with *Staphylococcus aureus* enterotoxin, especially those rich in protein. Cattle milk is considered the only natural food that contains the most complete nutrients and is ideal for regular consumption. Many nutrients in cow milk also support microbial growth (11). This makes milk highly susceptible to pathogenic microbial contamination, thereby making it perishable. Therefore the maximum limit of microbial contamination of *Staphylococcus aureus* in fresh milk as 1x 102 colonies/ml to protect consumers (12).

Milk and dairy products can be a source of disease because of unhygienic collection and processing. Milking

hygiene is a series of activities to maintain and keep clean starting from the preparation, process, and post-milking carried out to minimize microbial contamination from farmers or the environment in cow's milk (13). The factors that need to be considered in milking hygiene are the personal hygiene of the milkers, cleanliness of the barn, cleanliness of the cows, and cleanliness of the containers used.

According to data from the Central Statistics Agency, East Java Province has the largest dairy cattle population in Indonesia in 2021, with a total of 301,780 cows. Jember is one of the districts with dairy cows, with a total population of 1,546 cows in 2019. These dairy cows are spread across 17 districts in the regency (14-15). The purpose of this study was to identify the association between milking hygiene and *Staphylococcus aureus* contamination in cow's milk in the Jember Regency.

METHODS

Study Design

This was an analytical observational study with a cross-sectional design. This study measured the dependent and independent variables simultaneously only once at the same time. Observations of milking hygiene and milk sampling were conducted on dairy farms spread across the Jember Regency. The analysis of contamination of cow milk samples was carried out at the Microbiology Laboratory of the Faculty of Medicine, University of Jember. The study was conducted between August 2022 and March 2023. The population in this study was all cow milkers on farms spread across the Jember Regency. The study sample consisted of 36 milkers selected using the Total Sampling method with inclusion and exclusion criteria. The inclusion criteria were milkers from farms with lactating cows and milkers from farms that provided milk for public consumption. The sample exclusion criterion was milkers from farms whose milking processes used machines.

Data collection

The data of this study were primary data obtained from observations of milking hygiene and laboratory tests for *Staphylococcus aureus* contamination. The dependent variable was *Staphylococcus aureus* contamination. The independent variable in this study was the milking hygiene. The milking hygiene observed in this study was cleanliness of the barn, cleanliness of the cow, washing hands before milking, wearing personal protective equipment, wearing clean clothes, cleanliness of the milk collection container, tying the cow's tail, cleanliness of the udder and teats, foremilk removal, and teat dipping.

Fresh milk samples were collected once per milker, resulting in 36 cow milk samples. Milk sampling was performed by placing 100 ml of cow's milk in sterile bottles from each milker as a research subject. Cow milk samples were tagged and placed in a Cooler Box to prevent bacterial growth.

The examination of *Staphylococcus aureus* colonies was performed using the spread plate counting method on the surface of the media. The first step was to dilute the sample, and 1 ml of cow's milk (100 µL) was transferred into 9 ml of distilled water to obtain a dilution of 10-1 dilutions of 10-2, and 10-3 were made. A total of 15–20 ml of MSA medium was poured into each cup and allowed to solidify. One milliliter of suspension was pipetted from each dilution. The suspension was leveled on the surface of the agar media and allowed to absorb the suspension. The medium containing the suspension was incubated at 35°C for 45–48 hour. *Staphylococcus aureus* positive media showed a yellowish color change. The petri dish selected was a cup containing 25 to 250 colonies. In the petri dish with the lowest dilution of <25 or >250 colonies, colonies were counted in Petri dishes with higher dilutions.

The identification test for *Staphylococcus aureus* was a Gram stain test and catalase test. Gram staining serves to observe the nature of the gram and the morphology of the bacteria. The preparations were made on a glass object and fixed on a bunsen. The preparations were stained with crystal violet and waited for one–two minutes. The remaining crystal violet was removed and the cells were rinsed with running water. The entire preparation was stained with Lugol solution and allowed to stand for 30 seconds. The Lugol solution was discarded and the cells were rinsed with running water. The preparation was diluted with 96% alcohol until all the dye was washed off, and then immediately washed with running water. The preparations were stained with safranin and waited for two minutes. Safranin was rinsed under running water and allowed to dry. The preparations were observed under a microscope with 100x objective lens magnification using an emersion. Bacteria such as *Staphylococcus aureus* are gram-positive bacteria that form clustered cocci.

The last identification test was the catalase test, which distinguished the genera *Staphylococcus sp.* and *Streptococcus sp.* The H_2O_2 liquid was dripped onto the object glass. One ounce of inoculum from MSA was placed in the H_2O_2 droplet and stirred evenly. Catalase-positive cells showed gas bubbles (O_2) produced by *Staphylococcus*.

Data analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) 26.0. A univariate analysis was performed on each research variable to describe its characteristics and determine its distribution. Bivariate analysis was performed using the chi-square test or Fisher's exact test to test the research hypothesis by proving the association between *Staphylococcus aureus* contamination and milking hygiene. Multivariate analysis in this study used logistic regression analysis to analyze the highest risk factors for *Staphylococcus aureus* contamination.

RESULTS

The respondents of this study were 36 cow milkers in the Jember Regency. The sociodemographic characteristics of milkmaids in the Jember Regency were mostly aged 15-64 years or productive age (97.2%). Among the milkers, 91.7% were male. The majority of milkers had a high school education level (44.4%). Ten milkers had a working experience of more than 15 years. The respondents'sociodemographic characteristics are presented in Table 1.

Table 1. Sociodemographic Characteristics of Respondents

Variable	Frequency	%
Age		
<15 years	0	0
15-64 years	35	97.2
>64 years old	1	2.8
Gender		
Male	33	91.7
Female	3	8.3
Education Level		
No School	6	16.7
primary school	8	22.2
Junior secondary school	6	16.7
Senior high school	16	44.4
Working Periode		
≤5	10	27.8
6-15	16	44.4
>15	8	27.8

Table 2. Characteristics of Milking Hygiene

Milking Hygiene	n	%
Good	29	80.6
Poor	7	19.4
Total	36	100

The analysis of milking hygiene in Table 2 shows that the majority of milkers in Jember District had good milking hygiene practices (80.6%). Milking hygiene was considered good if the milkers fulfilled more than five of the assessed components.

Milking hygiene is categorized into 10 components: hygiene of the barn, wearing clean clothes, washing hands before milking, hygiene of the cow, wearing personal protective equipment, hygiene of milk containers, hygiene of udder and teats, binding the cow's tail, and teat dipping. Table 3 shows that all milkers in Jember Regency did not wear personal protective equipment (100% bad).

Table 3. Distribution of Milking Hygiene Components

Milking Hygiene	Good		Bad		Total	
	n	%	n	%	n	%
The hygiene of the barn	30	83.3	6	16.7	36	100
Use of clean clothes	27	75	9	25	36	100
Habits of washing hands before milking	28	77.8	8	22.2	36	100
The hygiene of cow	32	88.9	4	11.1	36	100
Use of Personal Protective Equipment	0	0	36	100	36	100
The hygiene of milk container	30	83.3	6	16.7	36	100
The hygiene of udder and teat	30	83.3	6	16.7	36	100
Cow tail tying	12	33.3	24	66.7	36	100
Removing foremilk	30	83.3	6	16.7	36	100
Teat Dipping	12	33.3	24	66.7	36	100

The distribution of *Staphylococcus aureus* contamination in all the samples is shown in Table 4. The results showed that *Staphylococcus aureus* contamination in cow's milk that met the SNI standard was present in 22 samples, and 14 samples did not meet the standard.

Table 4. Distribution of *Staphylococcus aureus* Contamination

Staphylococcus aureus contamination	n	%
Meet the national standard	22	61.1
Does not meet the national srandard	14	38.9
Total	36	100

Fisher Exact Test analysis revealed that *Staphylococcus aureus* contamination was significantly associated with the hygiene of the barn ($p = 0.024$), washing hands before milking ($p = 0.036$), hygiene of milk containers ($p = 0.024$), and hygiene of udder and teat ($p = 0.024$).

A logistic regression analysis revealed that in milking hygiene, the primary risk factors for *Staphylococcus aureus* contamination were barn hygiene and udder and teat hygiene (Odds Ratio = 10.908). The Beta (B) value of both is 2.390, so if barn hygiene and udder and teat hygiene increase by 1% (getting poorer), there will be a 2.390 higher risk of *Staphylococcus aureus* contamination in milk.

Table 5. Distribution of the Association between Milking Hygiene Components and *Staphylococcus aureus* Contamination

Variable	*Staphylococcus aureus* Contamination						p-value
	Standard Compliant		Not up to standard		Total		
	n	%	n	%	n	%	
The Hygiene of the Barn							
Good	21	70	9	30	30	100	0.024*
Poor	1	16.7	5	83.3	6	100	
Total	22	61.1	14	38.9	36	100	
Use of Clean Clothes							
Good	19	70.4	8	29.6	27	100	0.111
Poor	3	33.3	6	66.7	9	100	
Total	22	61.1	14	38.9	36	100	
Habits of Washing Hands Before Milking							
Good	20	71.4	8	28.6	28	100	0.036*
Poor	2	25	6	75	8	100	
Total	22	61.1	14	38.9	36	100	
The Hygiene of Cow							
Good	21	65.6	11	34.4	32	100	0.277
Poor	1	25	3	75	7	100	
Total	22	61.1	14	38.9	36	100	
Use of Personal Protective Equipment							
Good	0	0	0	0	0	100	-
Poor	22	61.1	14	38.9	36	100	
Total	22	61.1	14	38.9	36	100	
The Hygiene of Milk Container							
Good	21	70	9	30	30	100	0.024*
Poor	1	16.7	5	83.3	6	100	
Total	22	61.1	14	38.9	36	100	
The Hygiene of Udder and Teats							
Good	21	70	9	30	30	100	0.024*
Poor	1	16.7	5	83.3	6	100	
Total	22	61.1	14	38.9	36	100	
Cow Tail Binding							
Good	7	58.3	5	41.7	12	100	1.000
Poor	15	62.5	9	37.5	24	100	
Total	22	61.1	14	38.9	36	100	
Removing Foremilk							
Good	19	63.3	11	36.7	30	100	0.658
Poor	3	50	3	50	6	100	
Total	22	61.1	14	38.9	36	100	
Teat Dipping							
Good	9	75	3	25	12	100	0.292
Poor	13	54.2	11	45.8	24	100	
Total	22	61.1	14	38.9	36	100	

Table 6. Multivariate Analysis of Milking Hygiene Components

Variable	B	p-value	OR	95% C.I. for EXP(B)	
				Lower	Upper
The hygiene of the barn	2.390	0.050	10.908	1.001	118.847
The hygiene of udder and teats	2.390	0.050	10.908	1.001	118.847

DISCUSSION

The distribution of respondents' ages was classified based on working age: unproductive age, productive age, and non-productive age. The observation showed that 97.2% of the respondents were of productive age, and the others were of non-productive age. This is in agreement with the results of research conducted in the Tutur Sub-district, Pasuruan Regency; these milkers are dominated by productive age (16).

Hand milking requires a higher amount of energy; therefore, more male milkers are needed than females. Males and females have the same work habits; however, the difference is in muscle strength. Sex differences in muscle strength are more prominent in the upper body muscles than in the lower body muscles, and in concentric than eccentric contractions. The greater strength of males than females is not due to higher voluntary activation, but to greater muscle mass and type II fiber area (17-18). Therefore, the number of male respondents in this study was higher than that of female respondents.

The majority of the respondents' education level in the study area (44.4%) was high school. According to a study in Sleman Regency in 2021, a low level of education has a 1.909 greater risk of poor hygiene practices when compared to respondents who have a secondary education level and higher (19). The level of education of milkers can be related to their knowledge of milking hygiene. From this knowledge, it is expected that milkers can implement good milking hygiene practices. It can also be associated with other issues such as an increased incidence of contamination, production levels, and milk quality.

There were respondents with a working period of more than 15 years. The working period affects many experiences gained, so that knowledge and practices regarding milking hygiene are improving. There were 61.1% of cow milk samples that met the set standard for *Staphylococcus aureus* contamination, while the others did not meet the standard. The presence of *Staphylococcus aureus* in food can cause foodborne diseases with mild-to-severe symptoms. The presence of milk that does not meet the standard of *Staphylococcus aureus* contamination can be caused by other factors that have not been studied, such as milking techniques, sanitation of the barn environment, and sanitation of the container. According to research conducted in Tutur District, Pasuruan Regency in 2018, the sanitation of the cage environment consists of five components: cage location, technical requirements for cage buildings, cage

direction, cage cleanliness, and handling of livestock waste (20). Therefore, farmers must pay attention to all aspects to prevent microbial contamination of cow milk.

The study results showed a significant association ($p < 0.050$) between milking hygiene and *Staphylococcus aureus* contamination in cow's milk. These results are in agreement with research in Malang Regency, 2021, which found that milking hygiene is associated with microbial contamination (21). In this study, 80.6% of milkers were in the good category for practicing milking hygiene. Milking hygiene is an important factor that affects the quality of cow milk to minimize *Staphylococcus aureus* contamination from milkers, cows, and containers.

Barn hygiene was significantly associated with *Staphylococcus aureus* contamination. These results are in agreement with those of Mukaturi and Sululta Town, Oromia Region, Ethiopia, 2019, which showed an association between the hygiene of barns and *Staphylococcus aureus* contamination of milk (11). This was caused by inadequate hygiene of the milking area, cows milked in dirty and muddy milking areas, and sewerage full of feces, which increased milk contamination and supported the proliferation and transmission of *Staphylococcus aureus* to the udders of cows. Based on observations, milkers always clean the barn before milking, and milking is performed twice daily. This can decrease the number of *Staphylococcus aureus* contaminations in cow's milk.

The habit of washing hands before milking was significantly associated ($p < 0.050$) with *Staphylococcus aureus* contamination. These results are in agreement with research in Bahir Dar City, Ethiopia, 2019, which showed an association between the hand hygiene of milkers and *Staphylococcus aureus* contamination (22). The majority of milking in Jember Regency uses the hand milking method, so hand hygiene of milkers is very important. Hand milking carries a high risk of transferring bacteria from milkers to whole milk.

Container hygiene was significantly associated with *Staphylococcus aureus* contamination. These results are in agreement with research in Holeta, Central Ethiopia, 2022, which showed an association between the hygiene of milk containers and *Staphylococcus aureus* contamination (23). Another study from another city in Ambo and Bako towns, Oromia, Ethiopia, conducted in 2022 also made a similar point (24). Repeated use of dairy containers without sufficient cleaning may increase *Staphylococcus aureus* contamination. Observations during milking preparation showed that some milkers did not wash the containers or only washed them using clean water without soap, because they considered the milk collection containers to be clean. A few milkers also use plastic containers that are more difficult to clean and do not have the standards for milk containers set by the standard.

Hygiene of the udder and teats before milking was significantly associated ($p < 0.050$) with *Staphylococcus aureus* contamination. These results are in agreement with the research by Kenya, 2022, who found that the hygiene of the udder and teat was significant for *Staphylococcus aureus* contamination in cow's milk (25). The udder and teat are parts of the cow's body located near the anus and on the lower side. Therefore, udder and teat are easily exposed to cow feces and are contaminated with bacteria. Therefore, it is important to clean these parts to minimize contamination of cow milk.

The multivariate analysis results showed that the most influential milking hygiene with *Staphylococcus aureus* contamination was the hygiene of the barn and the hygiene of the udder and teats. The Beta value for both is 2.390, which means that if the hygiene of the barn and the hygiene of the udder and teats increases by 1% (poorer), there will be a 2.390 times higher risk of *Staphylococcus aureus* contamination in milk. An OR value of 10.098 means that if cage hygiene or udder and teat hygiene is poor, it has a risk of 10.098 times compared to barn hygiene or good udder and teat hygiene. These results are in agreement with research in Asella Ethiopia, 2023, which states that cage hygiene is a risk factor for *Staphylococcus* aureus contamination (26). All cow activities, such as sleeping, eating, drinking, and defecating, were conducted in the barn, making it the most significant source of contamination. Dirty barns cause the cow's body, especially the udder and teats that are in direct contact with the barn floor, to become dirty. The cow's udder and teats are surfaces in direct contact with milk; therefore, they are also the most significant sources of contamination. Dirty udders and teats contain many microbes, especially *Staphylococcus aureus*. When the milking process is carried out with dirty udders and teats, the attached *Staphylococcus aureus* is carried into milk.

This study was limited in scope to determine the association between milking hygiene and *Staphylococcus aureus* contamination in cow's milk. Other contamination factors, such as milking techniques, udder and nipple health, cage sanitation, and container sanitation, were not investigated in this study. This study also did not examine consumer habits in processing cow's milk before consumption because based on the results of observations and interviews during the preliminary study,

it was found that people deliberately consumed fresh cow's milk without going through proper processing.

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CONCLUSION

Statistical analysis showed that *Staphylococcus aureus* contamination was significantly associated with stable hygiene, hand washing before milking, milk container hygiene, and udder and teat hygiene. The components of milking hygiene that became the main risk factors were the hygiene of barns and udders and teats. It is recommended that dairy farmers maintain good milking hygiene practices and enhance the sanitation hygiene of barns and cow teat health. In addition, consumers need to process cow's milk before consumption.

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