Adhesion of *Staphylococcus aureus* to the liner in relation to subsequent machine milkings in a lab test

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Abstract

Staphylococcus (*S.*) *aureus* is one of the main causes of bovine mastitis, and its transmission during milking is a significant challenge for herd management. This study investigates the persistence of *S. aureus* on teat liners after simulating the milking of a cow infected with *S. aureus*, testing two common liner materials: nitrile butadiene rubber (NBR) and silicone (SIL). A bucket milking system and a rubber udder were used to simulate the milking process, with an initial milking using *S. aureus*-contaminated milk, followed by six subsequent simulated milkings with uncontaminated ultra-high-temperature (UHT) milk. The presence of *S. aureus* on the teat liners was quantified by using a modified wet-dry swab method. Results demonstrated that *S. aureus* was consistently detectable on both liner materials throughout all six subsequent milkings, with a significant decrease in bacterial counts of approximately 85% for NBR and 78% for SIL. Importantly, NBR liners showed a higher risk of pathogen adherence compared to SIL liners. The findings reaffirm the risk of transmission of *S. aureus* through teat liners and highlight the importance of intermediate cluster disinfection between each milking to decrease this risk.

Key words: Staphylococcus aureus, mastitis, liner, transmission

Introduction

Staphylococcus (*S.*) *aureus* is one of the most common causes of both clinical and subclinical bovine mastitis [1, 2,3]. Infections with *S. aureus* cause elevated somatic cell counts (SCC) and significant production losses globally [4,5]. The challenges associated with these infections include low cytobacteriological cure rates following antibiotic treatment [3], intermittent shedding of *S. aureus*, and often frequently low shedding rates (<100 colony-forming units (cfu)/ml), which could complicate detection [6]. Intramammary infections (IMI) caused by *S. aureus* can last between 64 and 192 days [7,8,9]. Additionally, *S. aureus* has the ability to cause long-lasting chronic infections [10]. Shedding rates can vary widely [11,12] and the accurate infectious dose of *S. aureus* remains unclear. In experimental challenges, one study used 1 mL containing 1000 cfu to initiate an infection [13], while another challenged cows with 8 x 10^3 cfu [14]. It is possible that considerably lower numbers of bacteria can cause infections of the mammary gland. Many staphylococci can be isolated from the teat canal [15,16]. Especially *S. aureus* can be isolated from the milk, from the hands of the milker, the milking gloves, or the liner during milking [17,18]. It can also be isolated from the housing environment, such as bedding material, slatted flooring, or air samples [19]. Notably, *S. aureus* is predominant- ly located on surfaces that are in direct contact with the cow's teat skin [18].

To prevent the spread of *S. aureus*, enhancing milking hygiene is crucial. Regular changing of milking gloves and the use of pre-milking cups to prevent splashing are recommended [20]. Additionally, proper udder preparation, such as pre-milking, cleaning the teats, and using a new dry cloth for each cow can significantly reduce transmission [21].

Wilson et al. [22] suggested separating *S. aureus*-infected and non-infected cows to reduce transmission during milking. Thus, milking infected cows at the end of milking can be a preventive measure to reduce the risk of spreading [23]. However, this separation may not be feasible for every farm, and due to the challenges in detecting *S. aureus*, infected cows could remain undetected within the group of uninfected animals [6].

The milking liner is a critical factor in the transmission of *S. aureus*, as it is in regular contact with the teat and milk, exposing multiple cows to the same cluster during each milking session. The age of the liner significantly influences the risk of transmission. Heavily time-worn liners may increase the likelihood of pathogen adherence, thereby raising the risk of infection spread [24].

This highlights the importance of intermediate disinfection of the cluster during milking to prevent new infections and to enhance comprehension of transmission routes and vectors. Given the essential contact between the teat and the liner during milking, this study aims to explore the number of milkings for which *S. aureus* remains detectable on the teat liner after milking a cow infected with *S. aureus* without any intermediate disinfection. A laboratory trial was conducted to determine the number of subsequent milkings with *S. aureus*-free milk in which the pathogen remained detectable on the liner after an initial milking with *S. aureus*-contaminated milk.

Materials and Methods

An application for a license for animal testing was not required because animals were not used in this experiment. This study was approved by the Animal Welfare Committee of the University of Veterinary Medicine Hannover, Foundation, Hannover, Germany (TVO-2023-V-57).

The milking of a cow was simulated using a rubber udder and a bucket milking machine. First, *S. aureus*-contaminated milk was passed through all quarters of the rubber udder to simulate the milking of an *S. aureus*-infected cow. Subsequently, the six milkings of cows without *S. aureus* infection were simulated using uncontaminated milk. The detection of *S. aureus* on the liner surfaces was carried out using the wet-dry swab method. Standardized excretion rates, liner surfaces, and milk quantities were used to achieve this.

Materials

A rubber udder in a wooden holder was used as a surrogate for the udder of a bovine animal. The milking process was conducted using a bucket milking system equipped with an Interpuls pulsator L80 (Interpuls S.p.A., Albinea RE, Italy), a vacuum pump (190 L/minute), and a milking cluster Classic 300 from GEA Farm Technologies GmbH (Boenen, Germany), which was equipped with two-piece liners, proper inspection glasses and new short milk tubes of nitrile butadiene rubber. Heavy weight shells with a length of 147 mm were used for the rear teats (7021-2721-090; 380 g) and light weight shells with the same length were used for the front teats (7021-2721-100; 235 g) (both from GEA Farm Technologies GmbH, Boenen, Germany).(Figure 1). The pulsator operated at a frequency of 65 pulses per minute and a pulse ratio of 60:40, as recommended in the literature as common practice [25]. The vacuum was set to 40 kPa. The study was performed with new silicone teat cup liner (Classic Pro 7029- 2725-000 with a barrel diameter of 25 mm and a mouthpiece opening of 22 mm) and new nitrile butadiene rubber (NBR) liners were used (Classic liner 7021-2725-220 with a barrel diameter of 27 mm and a mouthpiece opening of 23 mm). The sterile test milk used in this study was ultra-high temperature (UHT) milk with a fat percentage of 3.5 (Milbona, haltbare Vollmilch, 3.5%).

Preparation of *Staphylococcus aureus*-contaminated milk

S. aureus (ATCC 29213) was used to contaminate the sterile test milk.

Figure 1: Bucket milking system with milking cluster connected to the rubber udder. Figure 2: Simulations of milkings.

The isolated pathogen was kept in the laboratory of Hannover University of Applied Sciences and Arts, Hannover, Germany at -80 °C with the addition of glycerol until assayed.

A McFarland standard of 0.5 was then set, corresponding to a bacterial density of $1.5*10^8$ cfu/mL. This was diluted to 10^6 cfu/mL and 3 mL of this was added to 2.997 L UHT milk to obtain 10^3 cfu/mL, simulating the bacterial load typically found in intramammary infections [26]. The exact pathogen density of the test milk was 1,400 cfu/mL for the experiment.

Modified wet-dry swab sampling method

Sampling was performed with the modified wet-dry swab method (WDS) according to DIN 10113-1:1997-07. This methodology was also used in previous studies [27,28].

One-quarter strength sterile Ringer's solution (Merck KGaA, Darmstadt, Germany) was used for the swabs, and 3 mL was used per test tube. The swabs were packed sterile. Two swabs were always used for one sample. The first swab was moistened with the solution by dipping it in it for 5 sec. The excess liquid was squeezed out. The first swab was used to wipe the inner surface of the liner. Afterwards, a second dry swab was used to wipe the same surface. All swab samples were taken at 5 cm depth – measured from the teat liner opening- whereby the swab was passed in one rotation of 360° over the inner surface of the teat liner without losing contact to the liner until the swab reaches the starting point. The pressure was so strong that the wooden pin was bent. After taking both samples, both swabs were placed in the same tube. To prevent contamination of the swab medium through the samplers, the handles were broken off as they entered the tube. The complete sampling was performed by the same person. During sampling, another liner is always tested next to ensure that the bacterial load is not affected by the process.

Simulation of milkings

The study commenced with the assumption that the average daily milk yield from two milkings for an average cow would be 24 liters, equating to 12 liters per milking event. In the initial phase of the experiment, the rubber udder was connected to the milking cluster using new liners made of nitrile butadiene rubber (NBR). In the subsequent phase, silicone (SIL) liners were used. The equipment was cleaned before the start of the experiment with a surface cleaning agent, which was fully drained before starting. After drying, 70% ethanol was added, and the equipment was rinsed with distilled water afterwards.

At the beginning of the experiment, milking simulations were conducted using 12 liters of the sterile test milk contaminated with *S. aureus* (1,400 cfu/mL) to simulate the initial milking process of an infected cow. A WDS was taken from the first liner as a positive control sample (Figure 2, a). Following this, a milking simulation of a healthy cow was conducted, using an additional 12 liters of uncontaminated (sterile) milk, after which a WDS was obtained from the second liner (b). The procedure was continued and WDS were taken from the third and fourth liners (c/d). After sampling from the fourth liner(d) of the milking cluster, it was cleaned and disinfected in accordance with the above-described procedure.

The process of contamination was then repeated to enable a total of six milkings with 12 L of sterile milk, each time taking a new sample from a different liner (a/b/c/d) to prevent falsification of the results. The entire experiment was conducted three times. Afterwards, the process was repeated with a SIL liner, following the same methodology as described above (Figure 2).

Microbiological examination

The tubes were shaken with a vortex mixer before serial dilution was

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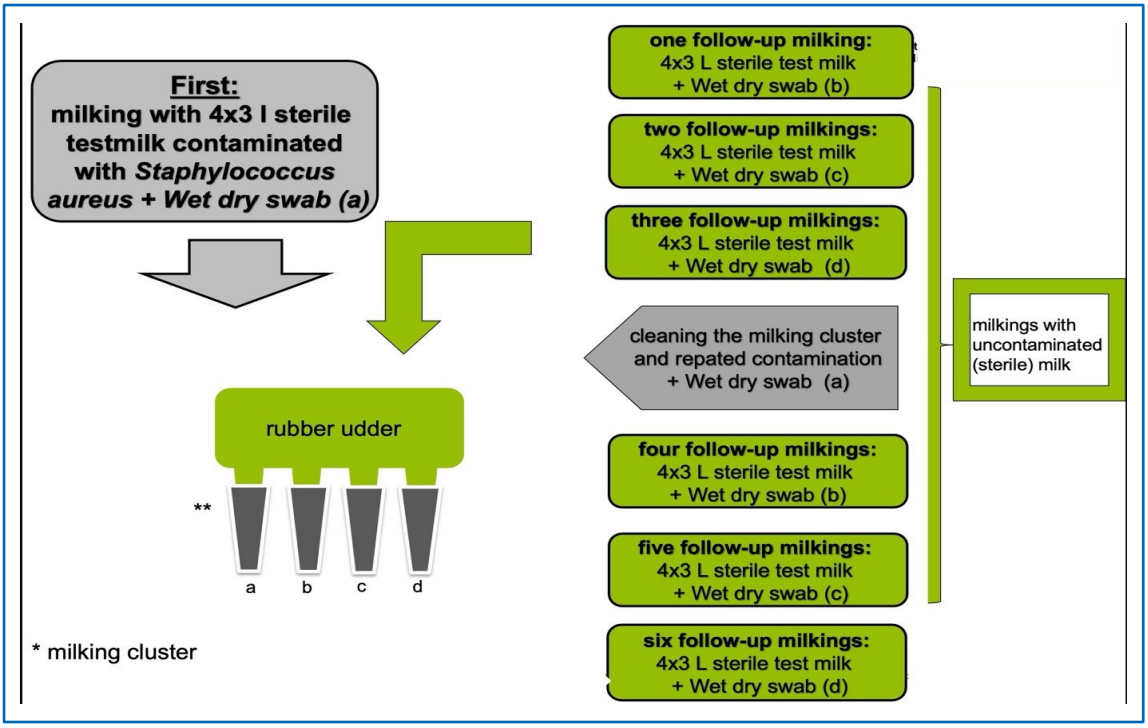

Figure 2: Simulations of milkings.

performed by using method L 00.00-54 of §64 LFGB (German Food and Feed Code). The samples were mixed again before the sample fluids were spread in several dilution steps (10^{-0} and 10^{-1}) in duplicate on plate count skimmed milk agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). After incubation at 37 °C for 24 h and 48 h, the plates were evaluated by counting all grown colonies, considering all plates with growth between 1 and 300 colonies for the microbial count. During the experiment, a positive result was assumed by counting one colony of *S. aureus*. Due to the defined colony morphology of *S. aureus*, any contamination with other microorganisms was easily detected. The colonies in cfu/mL from both dilution levels were converted to cfu per square centimeter (cfu/cm²). For the calculation, the circumference of the liner was multiplied by the length of the contact area of the swabs.

Statistics

The data were collected in Microsoft Excel, and analyzed by using the SPSS 29.0 program, SPSS, Inc. (Chicago, IL, USA). The outcome variable cfu/cm² was transformed by applying log10 /10cm² to approximate a normal distribution. An outlier that was more than four standard deviations above the mean of the other tests was removed from the data set before testing the normal distribution. The normal distribution was tested with the Kolmogorov-Smirnov test. The average of the respective sub-sample was calculated for descriptive purposes using Huber's M estimator.

The influence of the fixed factors teat liner material (NBR vs. SIL) and milking (repeated measurements) on the development of the bacterial count on the teat liner surface was calculated using a linear mixed model. Estimated mean values were calculated and a post-hoc analysis using Fisher's least significant difference (LSD with Bonferroni correction) test was performed. To improve clarity, the logarithmized bacterial counts were back-transformed in the figures in cfu/cm². Statistical significance was defined as $p < 0.05$.

Table 1: Huber M-Estimator: *Staphylococcus aureus* load on nitrile butadiene rubber (NBR) and silicone (SIL) liners (n=4) in cfu/cm² (n=42).

cfu/cm²	Huber M-Estimator	Huber M-Estimator
Milking	NBR[1]	SIL[2]
0[3]	6.11	4.71
1	5.85	3.06
2	2.8	0.76
3	1.66	1.02
4	1.66	2.29
5	1.66	1.79
6	0.89	1.02

[1] NBR = nitrile butadiene rubber
[2] SIL = silicone
[3] 0 = positive control

Results

The study involved 48 wet-dry swab samples (3 replicates x 2 liner materials (from both nitrile butadiene rubber (NBR) and silicone (SIL)) x 8 (2 x positive control, after 1-6 milkings with uncontaminated milk).

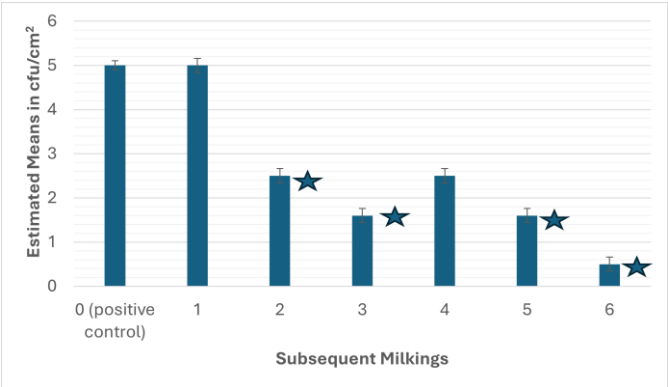

Figure 3: Estimated means: *Staphylococcus aureus* load on nitrile butadiene rubber liners (NBR) in (cfu/cm²); ★ = Significant reduction of bacterial load on liners compared to the positive control (= 0).

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The collected results did not show a normal distribution and were therefore transformed to log10 cfu/10cm².

Liners made of nitrile butadiene rubber Materials (Table 1)

Figure 3 shows the mean values for the NBR teat liners. The positive control had the highest value at 5 cfu/cm². The results for NBR showed that *S. aureus* could be detected on the liner after all six subsequent milkings with uncontaminated milk (Figure 3). The bacterial load in the second, third, fifth, and sixth subsequent milking was significantly reduced compared to the positive control. In the first milking, there was no numerical reduction in bacteria on the teat liner. Furthermore, in contrast to the other subsequent milkings, there was a distinct numerical increase in bacterial load in the fourth subsequent milking (Figure 3).

Silicone liners (Table 1)

In contrast to the bacterial count on the NBR liners, the bacterial load on the silicone liners was reduced by half a power of ten (approx. 67%) (Figure 4). Nevertheless, *S. aureus* was detectable on the liners until the last subsequent milking with the uncontaminated milk. The decrease in the detectable bacterial count on the liners from one subsequent milking to the following subsequent milking was comparable to that of NBR liners. The bacterial load in the second, third, fifth, and sixth subsequent milking was significantly reduced compared to the positive control. In the first milking, there was no numerical reduction in bacteria on the teat liner (Figure 5). Furthermore, the fourth subsequent milking with silicone liners demonstrated a notable increase of 1.2 cfu/cm² in bacterial growth in comparison to the other subsequent milkings (Figure 5).

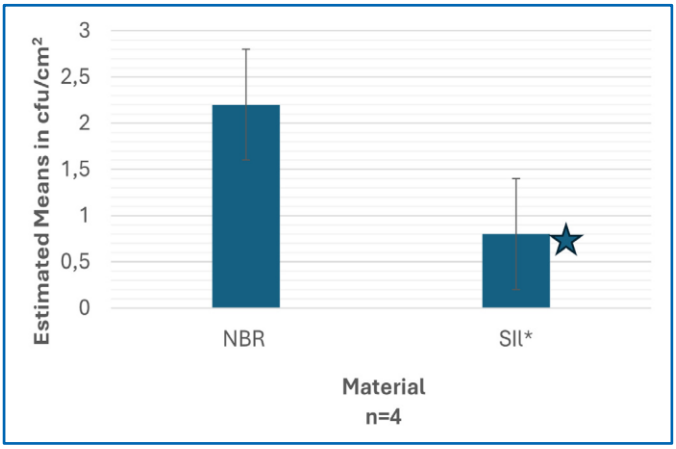

Figure 4: Estimated Means: bacterial load on the nitrile butadiene rubber liners (NBR) and silicone liners (SIL) in comparison in cfu/cm² (n=42); ★ = The bacterial load on the SIL liner is significantly different from the NBR liner.

Discussion

This study aimed to quantitatively investigate how long *S. aureus* is detectable on the teat liner after milking a cow infected with *S. aureus* without performing an intermediate disinfection. For this purpose, the quantitative wet-dry swab method (WDS) in accordance with DIN 10113-1:1997-07 was used, which was already proven to be very efficient and accurate in similar studies [27, 28, 29, 30].

Sampling and testing are highly standardized so that repeatable and valid results can be obtained [27]. Sterile swabs and a sterile swab solution were used for sampling and the moisture of the swab was always the same. During sampling, the same area of the teat liners surface was sampled with the same pressure [27].

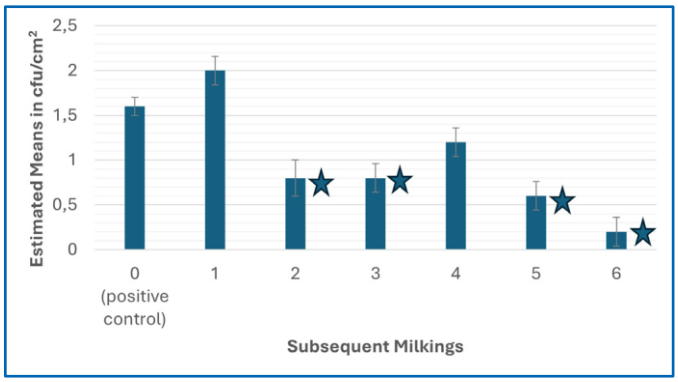

Figure 5: Estimated means: *Staphylococcus aureus* load on silicone (SIL) teat liners in (cfu/cm²); ★ = Significant reduction of bacterial load on liners compared to the positive control (= 0).

The results showed that *S. aureus* was quantitatively detected on both NBR and silicone liners after all six subsequent milkings, with the bacterial load decreasing significantly over time. *S. aureus* adheres to the liner [18]. Therefore, the probability of infection increases if pathogens adhere to the liner over a longer period. This underlines the importance of intermediate disinfection. Various other measures can be taken to reduce the risk of transmission through the milking process, e.g., wearing milking gloves, cleaning the animals with disposable udder wipes or reusable wipes with the use of one wipe per animal and milking time, post-dipping after milking with a disinfectant, or forming a separate group of *S. aureus*-positive animals and milking these animals at the end of a milking period [31]. The study found that contamination occurred after the first milking of an infected cow with a typical shedding of *S. aureus*, suggesting that the subsequent cow in the milking sequence may already be at risk of infection. These results show that intermediate cluster disinfection after each milked cow in milking systems can be useful.

Despite a general decrease in bacterial load from milking to milking, the data showed an increase in bacterial load after four subsequent milkings, even though the same cleaning and disinfection procedures were used after three subsequent milkings and the same concentration of the test milk (1,400 cfu/mL) was the same as at the beginning of the experiment. This increase cannot be clearly interpreted; it may be artificial due to the effectiveness of the cleaning procedure prior to contamination. The bacterial load of the fifth milking was again notably reduced, indicating a lower influence of the increased bacterial load on the teat liner after four subsequent milkings.

The studies show that the adhesion of this pathogen to NBR liners is considerably better than to silicone liners. However, it cannot be directly inferred from this that silicone would be better suited to prevent cow-to-cow transmission, as this study only determined adhesion after milk contact and not detachment after skin contact. Previous research has indicated that older liners made of NBR present a higher risk of transmission due to their susceptibility to pathogen adhesion [32,33]. Regular testing of teat liners is recommended to assess their condition, as compromised liners pose significant challenges for effective cleaning and disinfection [34].

It is recommended that a limit of 1,200 hours of usage time be observed during raw milk production if the teat liner is cleaned and disinfected regularly [35]. Nevertheless, the manufacturers give their own

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recommendations for the respective service life of liners. These findings suggest that the likelihood of transmission is diminished when liners are replaced on a regular basis.

Subsequently, it was found that the number of replicates was sufficient to work out significant influences of the liner material and the milkings with uncontaminated milk. Nevertheless, the effects would have been more apparent with a larger sample. Further milkings would also have been useful to determine the exact milking at which microorganisms could no longer be detected. Although the test was carried out under standardized laboratory conditions, it is not easily transferable to the practice. On the one hand, because of the difference between UHT milk and raw milk with various fat content, on the other hand because of the shedding rates of *S. aureus*, which can vary widely from animal to animal. In this case the main objective was to show whether *S. aureus* was still detectable and what influence this could have on the following cows regarding transmission. The influence of the liner on transmission could also be transferred into practice under these conditions because the results show that the pathogen was still present after six milkings, which implies that the risk of transmission is likely to be considerable. Therefore, intermediate disinfection of the milking clusters is useful for reducing the risk of infection.

Conclusion

S. aureus was quantitatively detected on liners made of NBR and SIL after one milking with contaminated and six subsequent milkings with uncontaminated milk. The results show that even new liners carry a certain risk of transmission, although it is important to note that many factors contribute to infection. To prevent infections, it is necessary to control several different vectors, such as the milker's hands, liners, but also measures like post-milking disinfection and grouping of the animals in the milking process. Nevertheless, the liner poses a considerable risk, suggesting that intermediate disinfection may be an efficacious strategy to mitigate this, thereby contributing to a reduction in transmission during milking.

Conflicts of interest

The authors declare no potential conflicts of interest.

Compliance with Ethical Standards

This study has been conducted in compliance with ethical standards.

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