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Biofilm-forming ability of *Staphylococcus aureus* on materials commonly found in milking equipment surfaces

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ABSTRACT

The presence of biofilms on milking equipment on dairy farms can be a source of bulk tank milk contamination, as well as a potential source of intramammary infections for cows. The biofilm-forming ability of bacteria, including *Staphylococcus aureus*, may differ depending on factors such as the intrinsic ability of bacteria to form biofilms, as well as the roughness and type of material of the surfaces. We investigated the ability of *S. aureus* to form biofilms on coupons made of stainless steel, nitrile (or Buna-N) rubber, ethylene propylene diene monomer (EPDM) rubber, silicone rubber, borosilicate glass, polycarbonate, and polyvinyl chloride, which are materials commonly used to manufacture pieces of milking equipment. Three *S. aureus* strains isolated from biofilms naturally formed in milking equipment on dairy farms, and previously characterized for high, medium, or low adherence ability by microtiter plate assay, were analyzed to assess their ability to form in vitro biofilms using a CDC Biofilm Reactor (Biosurface Technologies, Bozeman, MT). Bacterial counts of suspended biofilms and scanning electron microscopy were performed on coupons of each material. The highest bacterial counts were observed in Buna-N surfaces for high-adherence (L1-1171, mean = 4.54 $\log_{10}$ cfu/mL), medium-adherence (L1-030, mean = 4.18 $\log_{10}$ cfu/mL), and low-adherence (L1-256, mean = 3.71 $\log_{10}$ cfu/mL) *S. aureus*. The biofilm-forming ability for the same *S. aureus* strain, regardless its adherence abilities, was not significantly different among all tested surface materials, except Buna-N rubber, for which all 3 strains had an increased ability to form biofilms. In the same material, no statistically significant differences were observed among strains, except for Buna-N and EPDM rubber, in which the highly adherent *S. aureus* strain (L1-1171) had greater biofilm formation as compared with other strains. Regular replacement of rubber parts of milking equipment is warranted to reduce the risk of biofilm formation.

Key words: *Staphylococcus aureus*, milking equipment materials, milking equipment, biofilms

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that is frequently found in both humans and animals. It has been described as one of the major pathogens responsible for intramammary infections in cows, which causes considerable losses due to decrease in milk production, wasted milk due to treatments, and early culling (Ruegg, 2017). From a public health perspective, toxin-producing *S. aureus* in milk could be responsible for illness caused by the consumption of contaminated dairy products (Cieza et al., 2024). Cows shedding *S. aureus* when suffering intramammary infections could be a source of *S. aureus* on dairy farms. In addition, transmission of *S. aureus* due to insects, workers' hands, udder cloths, and milking equipment (Zadoks et al., 2011; Latorre et al., 2020) has also been discussed. Kadariya et al. (2014) described in their review several studies that report the presence of *S. aureus* in milk, including enterotoxin-producing strains and methicillin-resistant *S. aureus*. The presence of *S. aureus* from infected udders can cause milk contamination and, subsequently, contamination of the bulk tank milk. Raw dairy products may also become cross-contaminated with *S. aureus* from the environment (Kadariya et al., 2014), including from food handlers and biofilms on surfaces in contact with milk or dairy products.

The ability of *S. aureus* to form biofilms has been well documented, as have the mechanisms underlying the formation of biofilms by this bacterium (Schilcher and Horswill, 2020; Rosenthal et al., 2014). Moreover, the role of *S. aureus* biofilms as the cause of nosocomial

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

infections, as well as diseases in humans such as cystic fibrosis, endocarditis, and infections due to biofilms on medical devices has been described (Parastan et al., 2020; Idrees et al., 2021). An increased resistance to antibiotics, immune evasion mechanisms, and the ability to persist in abiotic surfaces in medical and food industry settings have been also reported as issues caused by *S. aureus* biofilms (Schilcher and Horswill, 2020; Idrees et al., 2021).

Biofilms on milk tanks and on surfaces in direct contact with milk, such as those of milking equipment, are rather frequent on dairy farms (Latorre, 2023). The presence of biofilms on milking equipment on dairy farms can be a source of bulk tank milk contamination (Latorre et al., 2010, 2020), as well as a potential source of intramammary infections for cows (Latorre et al., 2020). Moreover, the presence of biofilms in milking equipment parts, such as hoses used to divert colostrum and waste milk to feed the youngstock, can be a source of pathogens for heifers (Latorre et al., 2022).

The presence of biofilms in milking equipment can be due to failures in the protocols used to clean and sanitize the milking equipment (Latorre et al., 2010), but can also be influenced by the intrinsic ability that certain bacteria might have to attach to surfaces (Latorre et al., 2011; Choi et al., 2023). Thus, the biofilm-forming ability of bacteria, including *S. aureus*, may differ depending on factors such as the roughness of surfaces involved in biofilm attachment (Holah and Gibson, 2000; Oh et al., 2009; Al-Ahmad et al., 2010; Alotaibi and Bukhari, 2021; Choi et al., 2023) or the type of material of the surfaces (Lee et al., 2015). For example, in dairy processing equipment, it has been described that the duration of use influences the wear of Buna-N gaskets, increasing the presence of bacteria, especially in areas of the gaskets that protrude from the lines due to wear, and that are in contact with milk (Czechovski, 1990). Therefore, we investigated the ability of *S. aureus* to form biofilms on 8 different materials that are commonly used for manufacturing pieces of milking equipment in order to assess differences that may have implications for both milk quality and udder health.

MATERIALS AND METHODS

We analyzed 3 *S. aureus* strains isolated from biofilms in milking equipment (Pachá et al., 2021) and previously characterized for high (strain L1-1171), medium (strain L1-030), or low (strain L1-256) adherence ability by microtiter plate assay (**MPA**) according to Stepanović et al. (2001), with the modifications described by Latorre et al. (2011), in order to assess their ability to form biofilms. In vitro biofilms for each of the 3 strains were developed using a CDC Biofilm Reactor (**CBR**; Biosurface Technologies, Bozeman, MT) as described by Gambino et al. (2022), Vidal et al. (2020), and Goeres et al. (2005), with modifications. Briefly, *S. aureus* strains were removed from −80°C storage and cultured in brain heart infusion agar (Difco, BD Diagnostics, Sparks, MD) at 37°C for 24 h. After incubation, one single isolated colony was transferred to 100 mL of 0.01X trypticase soy broth (**TSB**; Difco, BD Diagnostics, Sparks, MD) and incubated at 37°C for 24 h. Next, 1 mL of this culture was transferred under sterile conditions to 0.01X TSB in the CBR and incubated at 125 rpm at 30°C for 24 h to achieve initial cell adhesion to the coupons. Biofilms grew in the CBR on removable coupons (Biosurface Technologies, Bozeman, MT), each with a diameter of 1.27 cm and thickness of 0.3 cm. Coupons were made of stainless steel (304 and 316 electropolished), nitrile (Buna-N) rubber, ethylene propylene diene monomer (**EPDM**) rubber, silicone rubber, borosilicate glass, polycarbonate, and polyvinyl chloride (**PVC**; 3 coupons of each of the 8 materials, a total of 24 coupons per assay, in duplicate experiments). Following initial adhesion, the system was switched to a continuous flow (12.1–12.7 mL/min) of 0.003X TSB at 125 rpm at 30°C for 96 h to promote biofilm formation and minimize planktonic growth.

After biofilm growth, the coupons with the biofilms were removed from the CBR and aseptically transferred to a sterile conical tube containing 10 mL of sterile 1X PBS. Biofilms were removed from the coupons by vortexing and sonication (81 W, 40 kHz; Webber et al., 2015) for at least 5 min, until the biofilms were completely dislodged from the coupons. Bacterial counts of the biofilm suspended in 1X PBS were assessed in Reasoner's 2A agar (Merck, Darmstadt, Germany) in triplicate for each coupon at 30°C for 24 h.

Scanning electron microscopy was performed on one coupon of each material for the 2 experiments (i.e., 2 replicates for each of the 3 *S. aureus* strains), following the procedures described by Latorre et al. (2020) with minor modifications. Briefly, coupons were rinsed with 1X PBS to remove nonadherent cells and then were placed in 2.5% glutaraldehyde solution. Next, the coupons were washed and gradually dehydrated using a graded series of ethanol. The samples were then critical point dried, attached to a specimen support, and coated with gold. Biofilms were confirmed when both bacteria attached to a surface and an exopolymeric matrix were visualized by scanning electron microscopy.

Statistical analyses of the bacterial counts ($\log_{10}$) were performed in SAS 9.4 (SAS Institute Inc., Cary, NC). Descriptive statistics were calculated by strain and material using PROC MEANS. Differences in $\log_{10}$ cfu/mL among strains and among materials were assessed using ANOVA (PROC MIXED). Statistical significance was determined at $P < 0.05$.

Table 1. Descriptive statistics of the adherence ability (log_{10} cfu/mL) of 3 *Staphylococcus aureus* strains to polyvinyl chloride (PVC), ethylene propylene diene monomer (EPDM) rubber, stainless steel (SS) 316 electropolished, borosilicate glass (BG), silicone rubber, SS 304, polycarbonate, and nitrile (Buna-N) rubber coupons[1]

Material	Mean	SD	Median	Minimum/maximum
S. aureus L1-1171 (high adherence in MPA)				
PVC	3.16	0.21	3.12	2.95/3.43
EPDM	4.21	0.03	4.21	4.17/4.26
SS 316	3.38	0.66	3.41	2.73/3.96
BG	3.21	0.69	3.27	2.31/4.00
Silicone	3.89	0.15	3.95	3.67/4.00
SS 304	3.70	0.31	3.75	3.29/4.03
Polycarbonate	2.99	0.82	3.01	2.07/3.86
Buna-N	4.54	0.30	4.44	4.32/4.98
S. aureus L1-030 (medium adherence in MPA)				
PVC	3.25	0.75	3.34	2.29/4.04
EPDM	1.97	0.21	1.89	1.81/2.27
SS 316	3.11	0.95	3.20	2.06/3.99
BG	3.00	0.74	3.3	1.90/3.51
Silicone	3.64	1.14	3.73	2.42/4.67
SS 304	3.33	1.03	3.34	2.38/4.26
Polycarbonate	3.62	0.53	3.73	2.94/4.06
Buna-N	4.18	0.36	4.20	3.81/4.50
S. aureus L1-256 (low adherence in MPA)				
PVC	2.84	0.69	2.96	2.04/3.43
EPDM	1.87	0.84	1.54	1.30/3.11
SS 316	3.20	0.88	3.26	2.26/4.04
BG	3.31	0.73	3.57	2.48/3.89
Silicone	3.01	1.74	4.02	1.00/4.03
SS 304	3.23	0.74	3.26	2.43/3.99
Polycarbonate	3.05	0.94	3.27	1.84/3.82
Buna-N	3.71	0.75	3.49	3.10/4.77

[1]MPA = microtiter plate assay; mean = arithmetic mean of bacterial counts (log_{10} cfu/mL) across experiments.

RESULTS

Descriptive statistics of bacterial counts (log_{10} cfu/mL) of biofilms removed from coupons of each of the analyzed materials are provided in Table 1. The highest bacterial counts were observed in Buna-N surfaces for high-adherence (L1-1171), medium-adherence (L1-030), and low-adherence (L1-256) *S. aureus* (Table 1). Overall, the biofilm-forming ability for the same *S. aureus* strain, either with high (L1-1171), medium (L1-030), or low (L1-256) adherence abilities, was not significantly different among the tested surface materials, with the exception of Buna-N rubber, for which all 3 strains had an increased ability to form biofilms in comparison with several other materials (Table 2). Analysis using scanning electron microscopy showed that the 3 *S. aureus* strains had the ability to notably attach to Buna-N coupons, although the coverage of the biofilm was not necessarily consistent with the putative adherence ability in MPA (Figure 1). Furthermore, high-adherence *S. aureus* L1-1171, showed an increased biofilm formation to rubber materials (Buna-N, EPDM, and silicone) and to stainless steel 304, as compared with the other materials (Table 2).

In addition, a decreased adherence to EPDM, as compared with other materials, was observed for *S. aureus* L1-030 (medium adherence ability) and L1-256 (low adherence ability) (Table 2).

In a same material, no statistically significant differences were observed among strains, except for Buna-N in which the highly adherent *S. aureus* strain (L1-1171) had a greater biofilm formation as compared with the low-adherence strain (L1-256; $P = 0.0488$). In addition, the highly adherent *S. aureus* strain (L1-1171) had a significantly greater biofilm formation on EPDM rubber as compared with the medium (L1-030) and low (L1-256) adherence strains ($P < 0.0001$; Figure 2).

Overall, scanning electron microscopy analysis showed that the high-adherence *S. aureus* strain was able to successfully form biofilms on all 8 of the materials that were tested (Figure 3).

DISCUSSION

Our results show increased biofilm formation on Buna-N rubber across all tested *S. aureus* strains, as compared with other materials, regardless of their adherence ability shown in screening tests. This suggests that

Table 2. Materials[1] where high, medium, and low biofilm production *Staphylococcus aureus* strains had a significantly increased or decreased biofilm formation, compared with other materials commonly found in milking equipment

Material with significant biofilm formation[2]	Strains	*P*-value
Buna-N rubber (↑)	*S. aureus* L1-1171 (high adherence)	
	SS 304	0.0213
	SS 316 electropolished	0.0023
	PVC	0.0005
	Polycarbonate	0.0001
	BG	0.0007
SS 304 (↑)	Polycarbonate	0.0048
EPDM (↑)	SS 316 electropolished	0.0218
	PVC	0.0005
	Polycarbonate	0.0015
	BG	0.0073
Silicone (↑)	PVC	0.0413
	Polycarbonate	0.0142
	S. aureus L1-030 (medium adherence)	
Buna-N rubber (↑)	EPDM	0.0005
	BG	0.0442
EPDM (↓)	SS 304	0.0214
	SS 316 electropolished	0.0498
	PVC	0.0290
	Polycarbonate	0.0065
	Silicone	0.0059
	S. aureus L1-256 (low adherence)	
Buna-N rubber (↑)	EPDM	0.0108

[1]SS = stainless steel; PVC: polyvinyl chloride; BG = borosilicate glass; EPDM: ethylene propylene diene monomer.
[2]Significantly increased biofilm formation indicated by ↑; significantly decreased biofilm formation indicated by ↓.

S. aureus may have preference for certain materials and subsequently may behave differently in terms of biofilm formation based on the material, per se, rather than their putative intrinsic ability to attach to surfaces.

Other studies have also reported the ability of *S. aureus* to form biofilms on several materials, such stainless steel, polystyrene, polypropylene, glass, cobalt-chromium alloy, titanium, and zirconium (Lee et al., 2015; Avila-Novoa et al., 2018; Piechota et al., 2018; Hiltunen et al., 2019; Minkiewicz-Zochniak et al., 2021; Vargová et al., 2023). When comparing differences in adherence and growth of biofilms to different materials, Minkiewicz-Zochniak et al. (2021) found increased biofilm formation on cobalt-chromium alloy surfaces as compared with titanium or zirconia surfaces used for dental implants. Moreover, Lee et al. (2015) report a higher biofilm formation on certain materials such as polystyrene, glass, and polypropylene, but with a significant variation among strains. Hiltunen et al. (2019) reported that *S. aureus* ATCC 25923 had an increased biofilm formation on polystyrene surfaces as compared with other clinical materials. Vargová et al. (2023) showed in their study

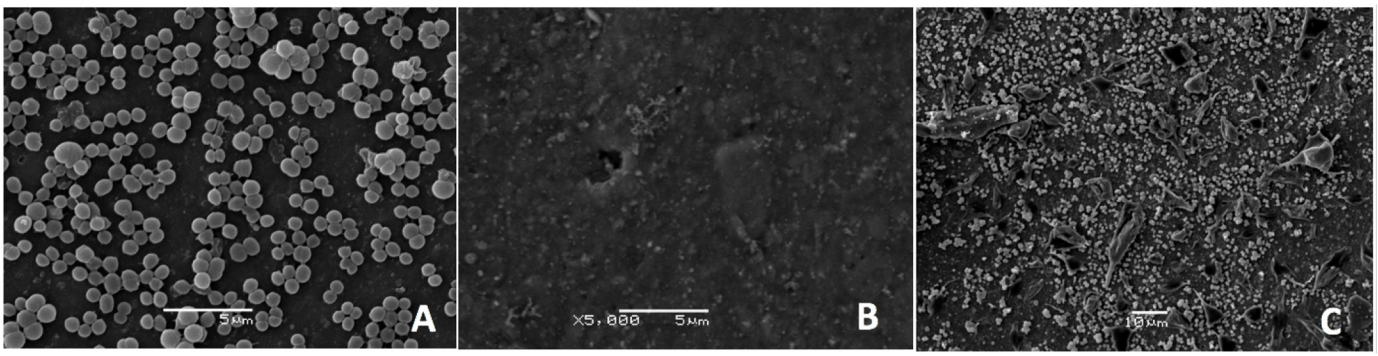

Figure 1. Scanning electron microscopy images of *Staphylococcus aureus* biofilms created in vitro on nitrile (Buna-N) rubber coupons using (A) high, (B) medium, and (C) low adherence ability strains.

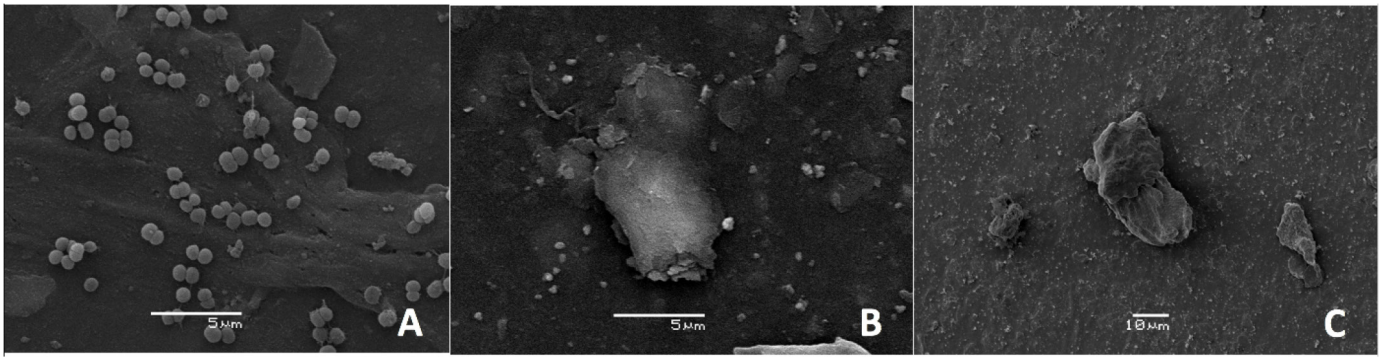

Figure 2. Scanning electron microscopy images of *Staphylococcus aureus* biofilms created in vitro on EPDM rubber coupons using (A) high, (B) medium, and (C) low adherence ability strains.

that *S. aureus* isolated from surfaces in a milking parlor had a higher ability to form biofilms as compared with those strains isolated from milk from mastitis cows. Variations in the results may be explained by the *S. aureus* strains that were used; the origin of the strains (clinical vs. surfaces); the topography, roughness, and other characteristics of the materials that were analyzed; as well as the methods that were used to grow biofilms (culture medium, time, and temperature of incubation). It is important to note that in all these studies, biofilms were grown under static conditions and for shorter times, unlike our study, which used continuous flow of media and a total time of incubation of 120 h (i.e., 5 d).

In our study, highly adherent *S. aureus* L1-1171 showed a significantly increased biofilm formation on Buna-N rubber as compared with all other materials. Nitrile rubber, or Buna-N rubber, is frequently found in milking equipment in pieces such as rubber liners, O-rings and gaskets, collector valves, and milk hoses, among others. A previous study (Czechovski, 1990) showed an association between the time in which Buna-N gaskets stay in milk processing lines and the wear of these gaskets. The longer the Buna-N gaskets stayed in the lines, the more wear occurred, and bacteria were more frequently found, particularly in the area of the gasket that was in contact with milk during processing.

Screening tests to assess adherence ability of bacteria (i.e., MPA) are conducted on PVC or polystyrene plates, which may not necessarily reflect the adhesion ability of *S. aureus* strains to other surface materials. Moreover, other factors on milking equipment (such as wear of the materials, presence of milk residues or milk stones, and scratches), as well as the dairy environment per se, might also influence biofilm formation. For example, a study

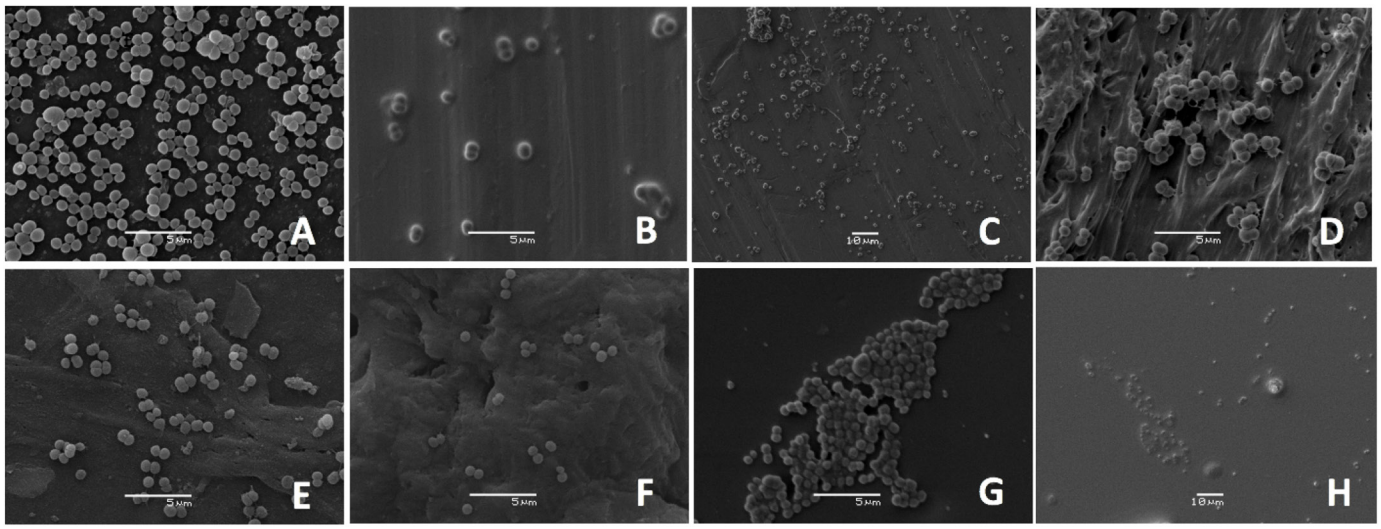

Figure 3. Scanning electron microscopy images of *Staphylococcus aureus* biofilms created in vitro using the high adherence ability strain L1-1171 on (A) nitrile (Buna-N) rubber, (B) stainless steel 316 electropolished, (C) stainless steel 304, (D) PVC, (E) EPDM rubber, (F) polycarbonate, (G) silicone, and (H) borosilicate glass coupons.

conducted with bacteria from the oral cavity showed differences in adhesion related to the surface properties, and increased adhesion to rough surfaces than smooth ones from the same material (Choi et al., 2023). Similarly, a study conducted by Oh et al. (2009) showed that the roughness of the surface was an important factor for biofilm formation, and less biofilm was formed in those surfaces that were less rough. Moreover, another study described differences in adhesion of *Vibrio harveyi* to different materials, which seemed to prefer plastic surfaces as compared with concrete slabs and steel (Karunasagar et al., 1996). On another hand, Sharan et al. (2022) reported in their review of biofilms in the food industry that there were variable results on biofilm formation on polystyrene, glass, Teflon, or metals. In addition to surface substrates, other factors may also play a role in biofilm formation, such as the presence of a conditioning layer (in this case, milk residues), temperature, pH, nutrient availability, and time (Holah and Gibson, 2000), and can be relevant for milking equipment on dairy farms. The presence of other microorganisms that are known to have an increased biofilm-forming ability, such as *Pseudomonas aeruginosa*, may also provide aid to certain bacterial strains that have a reduced adherence in MPA (Latorre, Pachá, Gonzalez-Cordova, and Vidal; unpublished data). The presence of other bacterial strains within a biofilm is also relevant because biofilms in the environment (including the dairy farm environment and milking equipment) naturally comprise very diverse microbial communities (Latorre et al., 2022) and may contribute to the formation of biofilms by less adherent strains (Moons et al., 2009). Therefore, several factors that are relevant to biofilm formation must be considered in addition to the materials that act as a contact surface and site of biofilm adhesion. These factors are not reflected by the laboratory conditions used for in vitro biofilm development in our experiments, which are controlled and differ from real-life settings

Staphylococcus aureus is a bacterium frequently found in biofilms in the food industry, and its adherence ability on these environments is similar to that of *S. aureus* found in clinical settings (Møretrø and Langsrud, 2017). Bacteria in biofilms have shown to have an increased resistance to sanitizers, antimicrobial compounds, and harsh environments (Sharan et al., 2022). For example, Sadekuzzaman et al. (2015) report that *S. aureus* was resistant to several methods commonly used for biofilm control, highlighting the need of novel approaches in biofilm removal. In addition, Ueda and Kuwabara (2007) described that *S. aureus* was more tolerant to several sanitizers as compared with *Escherichia coli* O157 and *Salmonella* Enteritidis. The same study showed that even at high concentrations of chlorine, a sanitizer commonly used in dairy operations, biofilms were not completely removed, and *S. aureus* was more resistant to chlorine than *E. coli* and *Salmonella* Enteritidis. Our study did not assess the resistance of *S. aureus* contained in biofilms to sanitizers, which would be relevant, as bacteria in biofilms have shown an increased resistance to sanitizing compounds, thus posing a risk of persistence of *S. aureus* on different milking equipment surface materials.

Moreover, surface topography, such as rough surfaces, scratches, abrasions, and crevices may impede effective cleaning and sanitation of milking equipment and may provide protection to bacteria during these procedures (Wirtanen et al., 1995; Palmer et al., 2015), thus creating sites of persistence for bacteria, such as *S. aureus*, in the form of biofilms on dairy farms. Milking equipment can act as a reservoir of pathogenic bacteria (Latorre et al., 2010, 2020), particularly in pieces such as rubber liners that are in close contact with teat ends during milking and are also shared among cows. Moreover, bacteria detached from biofilms on the surfaces of milking equipment in contact with milk, such as milk hoses and gaskets, can cause bulk tank milk contamination, thus compromising the microbiological quality of the milk.

Increased biofilm formation by *S. aureus* strains was observed on Buna-N rubber, silicone rubber, and EPDM rubber, which are materials commonly used in milking equipment, particularly in rubber liners, O-rings, and milk hoses. Regular replacement of these and other parts of milking equipment is warranted to reduce the risk of biofilm formation, which may pose a risk for udder health and can also compromise the microbiological quality of the milk.

CONCLUSIONS

Staphylococcus aureus successfully forms biofilms on different materials that are commonly used for manufacturing milking equipment parts and pieces. In our study, the analyzed *S. aureus strains* seemed to prefer rubber materials such as Buna-N, EPDM, and silicone rubber. These materials are widely used for the manufacturing of rubber liners, O-rings, and milk hoses, which are surfaces in contact with teat ends or milk during milking, posing thus a risk for both bulk tank milk and udder health. Further research is necessary to account for factors that may also influence biofilm formation, such as topography and roughness of materials used in milking equipment, as well as the influence of sanitizers and other chemicals on both the formation and resistance of biofilms on these materials.

NOTES

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Nonstandard abbreviations used: BG = borosilicate glass; CBR = CDC Biofilm Reactor; EPDM = ethylene propylene diene monomer; MPA = microtiter plate assay; PVC = polyvinyl chloride; SS = stainless steel; TSB = trypticase soy broth.

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