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► **To cite this version:**

Chloé Desmousseaux, Morgan Guilbaud, Gwenaëlle Jard, Hélène Tormo, Nadia Oulahal, et al.. Biofilms in the milking machine, from laboratory scale to on-farm results. *Journal of Dairy Science*, 2025, 108 (8), pp.8120-8140. <10.3168/jds.2024-25352>. <hal-05015640>

HAL Id: hal-05015640

<https://hal.science/hal-05015640v1>

Submitted on 22 Oct 2025

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Biofilm Formation in Dairy: A Food Safety Concern—Biofilms in the milking machine, from laboratory scale to on-farm results

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ABSTRACT

Raw milk is known to harbor a complex microbial community, including microorganisms of technological and human health interest. However, it can also be a source of pathogenic and spoilage bacteria, such as spore-forming bacteria and *Pseudomonas* spp. Despite cleaning and disinfection procedures, biofilms in milking machines are difficult to remove and represent a major source of milk contamination. This work aims to describe biofilms in milking machines at both the laboratory and farm scales. Encouraging studies on the microbiota of milking machine biofilms, the parameters influencing changes in biofilm composition, and the methods used to characterize them are essential for managing the formation and composition of these biofilms. Enhancing such knowledge will help improve the understanding of milking machine biofilms and their impact on the quality of milk and dairy products.

Key words: biofilms, milking machine, materials, dairy farm, microbiota

INTRODUCTION

Raw milk contains a complex microbial community, including microorganisms of nutritional and technological importance, such as lactic acid bacteria (LAB; Ayivi et al., 2020). The microbiota of milk can be altered, potentially influencing milk production, processing, spoilage, and consumer health (Quigley et al., 2011). Biofilms are structured bacterial communities embedded in a self-produced polymeric matrix, which provides specific advantages to the species and makes them resistant to cleaning and disinfection methods (Flemming et al.,

2016). Biofilms also contribute to pitting and corrosion of equipment and pipes in the dairy industry (Lv et al., 2024). However, the main concern is that dairy biofilms, through both inter- and intraspecies interactions, including LAB, pathogenic bacteria, and spore-forming bacteria (Alonso et al., 2022), serve as sources of milk and product contamination. Indeed, dairy biofilms can contain human pathogens such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus* (Galié et al., 2018). Therefore, understanding the composition of the microbial community in the milking machine, as the starting point of dairy production, is essential to ensuring milk quality. The milking machine consists of different materials, including stainless steel, polymers, and glass, which can influence the adhesion properties of microorganisms. The presence of milk residues in the milking machine can lead to increased microbial cell adhesion to surfaces and consequently to the formation of biofilms. This type of contamination is not limited to cow milk farms, but also occurs in farms with other milking animals, such as goats, buffalo, camels, and sheep. The traditional sanitation and disinfection procedures currently applied are ineffective at completely removing established biofilms in dairy farms (Weber et al., 2019; Wilson et al., 2024). Some alternatives to reducing dairy biofilms have been studied but are not widely used in farms. These methods include enzymatic disruption of biofilms (Kumar et al., 2021), ozonation (Varga and Szigeti, 2016), and UV light treatment (Prestes et al., 2024).

Over the past decade, interest has grown in the role of milking machine biofilms in milk quality at both the laboratory and farm scales. For example, studies have shown that biofilms in milking machines harbor a highly diverse microbiota (Weber et al., 2019), exhibit some resistance to antibiotics (Weber et al., 2023), and may contain *Staph. aureus* (Latorre et al., 2020), which can pose health risks to both animals and humans. In this review,

Received June 28, 2024.

Accepted February 4, 2025.

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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.

we provide an overview of (1) the characteristics of milk and the milking machine as a favorable environment for biofilm formation, (2) the formation and maturation of biofilms in milking machines, and (3) methods for sampling and characterizing biofilms.

SPECIFICITIES OF DAIRY ENVIRONMENT

Milk

Due to its high nutritional value, high water content, and nearly neutral pH (Quigley et al., 2011), milk is an ideal medium for hosting a diverse microbial community (Guo et al., 2024; Irlinger et al., 2024; Quigley et al., 2011). This community includes bacteria and yeasts of technological interest for dairy products (Laithier et al., 2005; Ayivi et al., 2020; Martin et al., 2023), such as LAB, *Corynebacteriaceae*, *Geotrichum candidum*, and *Kluyveromyces lactis*. Contamination during the milk production process and inadequate hygiene practices can lead to the presence of spoilage (Yuan et al., 2020) and pathogenic microorganisms (Fusco et al., 2020), including psychrotrophic bacteria (*Pseudomonas* spp.), spore-forming and thermophilic bacteria (*Clostridium* spp. and *Bacillus* spp.), and pathogenic species (*Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp. and mycotoxin-producing fungi). In addition, cows with bacterial infections, such as mastitis, contribute to milk contamination by pathogenic species such as *Staphylococcus* spp., *Pseudomonas* spp. *Streptococcus* spp., and *Klebsiella* spp. (Fusco et al., 2020)

Lactic acid bacteria play a crucial role in traditional cheesemaking, either as starter cultures that rapidly acidify milk or as nonstarter cultures that contribute substantially to cheese ripening. Many LAB exhibit antimicrobial activity, possess probiotic properties, and have health-promoting effects through the production of bioactive compounds (Coelho et al., 2022). In a recent study, Irlinger et al. (2024) analyzed 370 milk samples from 3 dairy species (cow, goat, and sheep). The mesophilic LAB *Lactococcus lactis* was one of the 4 species in the core microbiota of the 370 analyzed milk samples and was found in most cheeses. *Lactococcus lactis* colonizes a wide range of ecological niches and is one of the most extensively used microorganisms in dairy products (Laroute et al., 2017). It converts sugars into lactic acid, leading to milk acidification and coagulation, which prolongs food shelf life and provides biocontrol against foodborne pathogens. Additionally, *Lactococcus lactis* produces various metabolites and enzymes that contribute to the aroma and flavor of cheese. Some studies have demonstrated its ability to decarboxylate glutamate into γ -aminobutyric acid, a bioactive compound known as a

nutritional supplement that can promote relaxation and improve mood (Laroute et al., 2016).

Spoilage bacteria in raw milk are often psychrotrophic and are of particular concern to the dairy industry due to their ability to produce heat-stable enzymes (Machado et al., 2017). Additionally, the microbiota of raw milk and its associated biofilms are influenced by storage temperature. For example, *Pseudomonas* predominates at 4°C, whereas *Lactobacillus* is more prevalent at 25°C (Ban et al., 2023). Among these, *Pseudomonas* spp., particularly *Pseudomonas fluorescens*, is one of the most significant bacterial species responsible for dairy product spoilage (Marchand et al., 2012). The extracellular heat-resistant enzymes produced by these bacteria can lead to milk bitterness, sedimentation, and gelation during the storage of heated milk (Stoeckel et al., 2016; Vithanage et al., 2016). Furthermore, *P. fluorescens* can cause significant defects in cheese, including yellow to brown discoloration of the rind due to the development of undesirable ripening flora, as well as taste defects (Kumar et al., 2019). The impact of spoilage is further exacerbated when strains possess biofilm-forming ability, which is frequently the case (Yan et al., 2023).

Spores are commonly found in soil and animal bedding, and in the absence of proper handling, good hygienic practices, and adequate cleaning and sanitation during milking, they can contaminate milk (Ostrov et al., 2016). The sporulated form of microorganisms makes them resistant to heat, pressure, and cleaning and disinfection methods. Sporulated bacteria, such as *Bacillus* and *Geobacillus*, pose significant challenges to the dairy industry due to their ability to withstand heat treatment and form both mono- and multispecies biofilms (Ostrov et al., 2016). Because of their heat resistance, spores can survive milking machine cleaning, pasteurization, and milk sterilization (Alonso et al., 2021). Later, they germinate into their vegetative form and cause milk spoilage. They can also form biofilms, serving as a continuous source of contamination from the milking machine to the final product (Oliveira et al., 2018; Alonso et al., 2021). In raw milk, the level of spore-forming bacteria is initially low but increases slightly during storage in the milk tank and transport, rising from an average of 0.58 to 1.68 log cfu/mL. To produce high-quality powdered milk for international trade, spore contamination should not exceed 1.70 log cfu/mL in raw milk, a threshold that is frequently surpassed in the industry. After pasteurization and packaging, the average spore level continues to rise, reaching 2.34 and then 2.60 log cfu/mL. As a result, one-third of dairy production is lost each year in the United States, largely due to spore contamination (Ortuzar et al., 2018). To mitigate this issue, raw milk collected on farms must

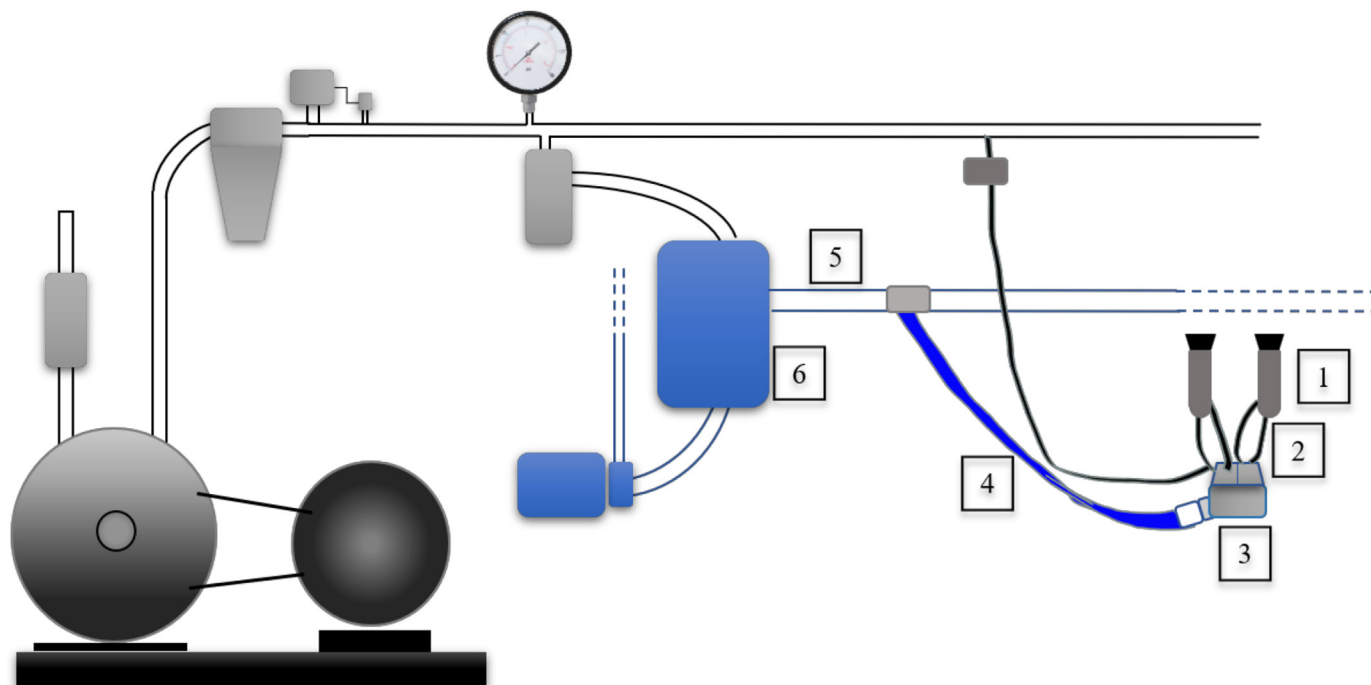


Figure 1. Materials of milking machine components in contact with milk (adapted from Poulet and De Cremoux, 2015). (1) Milk liners (nitrile or silicone rubber); (2) short milk tubes (nitrile rubber); (3) milk claw (stainless steel, polycarbonate, polycarbonate/polysulfone, and polymethylmethacrylate); (4) long milk tube (nitrile or silicone rubber); (5) milkline (stainless steel); and (6) receiver (glass and stainless steel).

have the lowest possible spore concentration. During growth, many spore-forming bacteria produce proteolytic enzymes that cause spoilage (Trmčić et al., 2015; Alles et al., 2018). For example, the psychrotolerant spore-forming bacterium *Bacillus weihenstephanensis* is responsible for sweet curdling, a coagulation defect in fluid milk (Martin et al., 2019). Additionally, raw milk can serve as a source of pathogens or mycotoxin-producing fungi (Zastempowska et al., 2016). The main pathogens found in milk and dairy products include Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter* spp., *Salmonella* spp., and coagulase-positive *Staphylococcus* spp. (Fusco et al., 2020; Williams et al., 2023). Some of these pathogens can form multispecies biofilms with other microorganisms, such as *Staph. aureus* (Emiliano et al., 2024). Archaea, fungi, and viruses may also interact with bacteria, potentially influencing the milk microbiota (Guo et al., 2024).

Microorganisms in milk originate from various sources, including external sources such as drinking and rinsing water, milking utensils, bedding, skin, feces, the barn environment, and mammary resident microorganisms (Doyle et al., 2015; Martin et al., 2023; Irlinger et al., 2024). Recent studies also emphasize endogenous transfer pathways, including entero-mammary and rumen-mammary routes (Guo et al., 2024). Additionally,

milk and water circulating in the milking machine play a critical role in contamination and biofilm formation.

Milking Machine Specificities

The milking machine consists of several components (Figure 1), each subject to challenges related to its use and environment. As a mechanized system, it includes functional parts such as vacuum, milk, and cleaning circuits. Stainless steel, polymers, and glass are commonly used materials in these devices. Examples of materials used in milking machines are provided in Table 1.

In general, the materials used in this equipment must meet specific requirements, including the following:

- (1) Vacuum resistance, with a minimum vacuum level of 90 kPa without deformation. Glass, in particular, must be designed with a safety factor 5 times the external pressure (5×90 kPa; standard 5707:2007, ISO, 2007).
- (2) High-temperature resistance, as cleaning and disinfection steps involve temperatures ranging from $<50^{\circ}\text{C}$ (prewash) to 75°C (wash step; Marchand et al., 2012; Lianou et al., 2023).
- (3) Chemical resistance to cleaning and sanitizing agents as well as milk components (e.g., milk fat).
- (4) An intact surface, free from scratches or cracks.

Table 1. Properties of materials used for devices of the milking machines

Material ¹	Devices	Regulated properties			Properties correlated with bioreceptivity			References
		Mechanical resistance ² (MPa)	Thermal resistance ³ (°C)	Chemical resistance ⁴	Roughness ⁵ (µm)	Water wettability ⁶ (°)		
SS	Milk lines, claws	620	870 (for SS 304) 925 (for SS 316)	Corrosion resistant Chlorine sensitivity: SS 304 > SS 316 Good resistance to acids	0.01; 0.16 ± 0.04; 1.17; 0.22 ± 0.02; 0.3 ± 0.1 ⁷	62 ± 7; 70 ± 2; 83 ± 1; 81.6 ± 2.6	Hilbert et al. (2003); Bruzard et al. (2017); Boulange-Petermann (1993); Santos et al. (2004); Teixeira et al. (2005); Guilbaud et al. (2017); personal data ⁷ (milkline)	
NiRB	Liners, tubes	1–20	120	Good resistance to fat products and diluted acids/bases	1 ± 0.4 ⁷	96.4 ± 3; 99.2 ± 2.1 ⁷	Teixeira et al. (2005); Katan (1996); personal data ⁷ (nitrile rubber liner)	
PSU	Claws	70	150	Good resistance to fat products, acids and bases, and detergents, but sensitive to organic polar solvents	2.5	80; 65	Trotignon et al. (1996); Kessler et al. (2013); Watanabe et al. (2020)	
PPSU		70	200	Very good chemical resistance (except concentrated acids)			Trotignon et al. (1996); Pakizeh et al. (2022)	
PC		67	135	Resistance to diluted acid but sensitive to strong bases	1	89.9 ± 1.8; 60	Trotignon et al. (1996); Park et al. (2017); Watanabe et al. (2020)	
PPSU/PC				Excellent chemical stability	0.2 ⁷	73.4 ± 4.4 ⁷	personal data ⁷ (claw)	
PMMA		60–70	80	Good resistance to diluted acids and bases until 60°C	0.22 ± 0.07	67.6 ± 1.5; 66 ± 4.7; 79.4 ± 3.2	Trotignon et al. (1996); Teixeira et al. (2005); Al-Dwairi et al. (2019); Park et al. (2017)	
Glass	Claw, receiver	50	>300	Not recommended for basic solution storage. Borosilicate glass offers superior resistance to chemical products	0.02; 0.03 ± 0.004	18 ± 4; 49.7 ± 2.0	Faille et al. (2002); Teixeira et al. (2005); Guilbaud et al. (2017); Zemanova et al. (2018)	
SiRB	Liners, tubes	4–10	225	Remarkable chemical inertness	1.4; 0.23 ± 0.06 ⁷	102.2 ± 2; 83.1 ± 4.2 ⁷	Nguyen et al. (2016); Trotignon et al. (1996); personal data ⁷ (long milk tube)	
PTFE	Seals	16	260	Exceptional chemical stability, even when exposed to strong concentrated acids	0.44	105	Trotignon et al. (1996); Faille et al. (2002); Prime (2020)	

¹SS = stainless steel; NiRB = nitrile rubber; PSU = polysulfone; PPSU = polyphenylsulfone; PC = polycarbonate; PMMA = polymethylmethacrylate; SiRB = silicone rubber; PTFE = polytetrafluoroethylene.

²Tensile strength: Maximum load that a material can support without fracturing when being stretched.

³Thermal resistance, expressed in deformation temperature for SS and maximum temperature of use for glass and polymers.

⁴Chemical resistance properties to products used in milking machines.

⁵Ra (arithmetic roughness); for SS, roughness is regulated (ISO, 2007) to be <2.5 µm.

⁶Water contact angles on the surfaces.

⁷Personal data: Roughness and water wettability measured on the milking machine devices by the authors and not yet published.

Therefore, the choice of materials for contact surfaces must comply with current regulations, including Regulation (EC) no. 1935/2004 for the European Union, Code of Federal Regulations Title 21 for the United States, and the Canada Consumer Product Safety Act for Canada (Sharma et al., 2023b).

Each device in contact with milk has specific characteristics depending on its function (Figure 1). Milking liners (Figure 1, step 1) are among the most technically advanced components of the milking machine. They must be both resistant and flexible to facilitate milk extraction from the animal's teats through alternating massage and pulsation. They are made of nitrile rubber (NiRB) or silicone rubber (SiRB). The former deteriorates more quickly and requires replacement every 2,500 milkings, whereas the latter is more durable and needs to be changed every 5,000 milkings. Milking tubes must be lightweight, portable, and flexible to transport milk efficiently. The short milk tube (Figure 1, step 2) conducts milk from the teat cup to the claw, and the long milk tube (Figure 1, step 3) carries milk from the claw to the milking pipeline. These tubes are primarily made of NiRB, SiRB, or a blend of polyvinyl chloride, silicone, and urethane. Milk claws (Figure 1, step 4), which are part of the clusters like short milking tubes, must be lightweight and possess strong mechanical properties to withstand vacuum pressure and potential animal kicks. They are made of stainless steel (SS), polycarbonate (PC), polymethyl methacrylate (PMMA), or a blend of PC with polysulfone (PSU) or polyphenyl sulfone (PPSU). Milk lines (Figure 1, step 5) are fixed components that transport milk to the milk receiver. They are several meters long (between 10 and 50 m, depending on the milking parlor) and made of SS. Receivers (Figure 1, step 6) collect the milk before it is transferred to the milk tank. They are designed with SS or glass. The former is more expensive, whereas the latter is more prone to breaking. Seals used in the milking machine are made of SiRB, NiRB, or polytetrafluoroethylene. The properties of the materials used in the milking machine are detailed in Table 1. Chemical, temperature, and mechanical resistance are regulated for each material. We also included data on physicochemical properties correlated with the bioreceptivity of the materials, which refers to their ability to be colonized by living organisms, such as roughness and wettability. Tensile strength is commonly used to assess the mechanical resistance of a material. It represents the maximum load a material can withstand without fracturing when stretched. Materials used in the milking machine exhibit tensile strengths ranging from 1 MPa for rubbers to 620 MPa for SS. Most rigid plastics (PSU, PPSU, PC, PMMA) have a similar tensile strength of around 70 MPa (Trotignon et al., 1996). In terms of temperature resistance, all materials except PMMA can

withstand temperatures exceeding 120°C, allowing for more effective high-temperature cleaning procedures. However, PMMA has a maximum operating temperature of 80°C.

The integrity of material surfaces in contact with milk must be maintained in the milking machine. Surface degradation can have adverse effects, such as reduced cleaning efficiency, which promotes the accumulation of organic and mineral residues, or, in extreme cases, the release of material debris or particles into the milk (Atapour et al., 2019). This degradation can result from exposure to cleaning products as well as from the milk itself, which consists of water and total solids, including proteins, fat globules, lactose, minerals, and vitamins. If the materials are not resistant to fat, contact with milk can lead to the migration of undesirable compounds (Arvanitoyannis and Bosnea, 2004). For this reason, all materials used in milking machines are selected for their high resistance to fats and chemical agents, such as detergents, acids and bases. Chloride solutions can corrode SS (Wang et al., 1988) and must be used strictly according to the manufacturer's recommendations.

Roughness and wettability are properties known to influence biological fouling and microbial bioadhesion (Dürr, 2007). In the milking machine, metal surfaces in contact with milk must have a surface roughness (**Ra**) of less than 2.5 µm, whereas the Ra of welded connections must be below 16 µm (standard 5707:2007; ISO, 2007). There are no specific regulations for other materials, which may explain why this information is not readily provided by suppliers. The roughness values presented in Table 1 correspond to bibliographic data on materials used in milking machines, as well as our own measurements from milking machine components. Even for the same material, such as SS and certain plastics, different roughness levels exist. It is important to prioritize smooth surfaces, as recommended by the European Hygienic Engineering and Design Group (Ra <0.8 µm) for food contact surfaces (Curiel et al., 1993). Our findings show that the claw made of a PPSU/PC blend exhibited low roughness (Ra = 0.2 µm), similar to the long milk tube (Ra = 0.2 µm), whereas nitrile rubber liners had a slightly rougher surface (Ra = 1 µm). Repeated use of these devices may increase their surface roughness due to chemical exposure and flow abrasion. Excessively rough surfaces can act as traps for microorganisms, shielding them from cleaning chemicals and fluid flow, thereby reducing cleaning efficiency (Frank and Chmielewski, 2001; Waldhans et al., 2023). Figure 2 illustrates examples of SS surface defects that can harbor organic matter, minerals, or microorganisms, such as pitting corrosion and scratches. For plastic materials, rubber seals with low mechanical strength are particularly concerning and should be replaced regularly, or at the very least, as soon as cracks become visible.

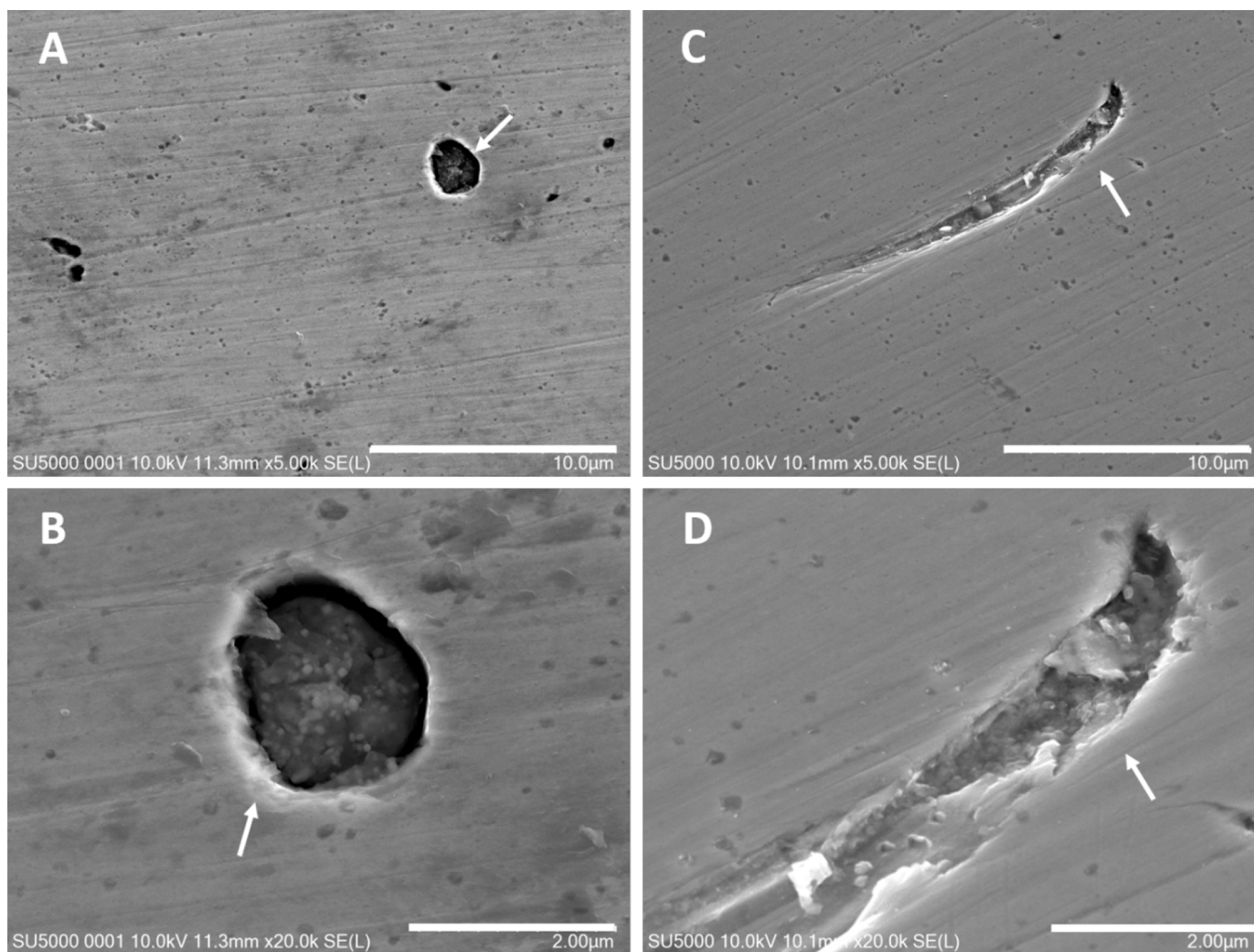


Figure 2. Scanning electron microscopy observations of a 316 SS surface (authors' unpublished data) from the PiloTraite milking machine (Laithier et al., 2024). White arrows show pitting corrosion (A, B) and scratches (C, D). Sample preparation and scanning electron microscope analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRAE, Jouy-en-Josas, France).

Most materials used in a milking machine setup exhibit moderately hydrophobic properties ($>70^\circ$), except for glass, which is hydrophilic. Wettability can vary for the same material across different studies. For example, glass surfaces have been reported to exhibit contact angles ranging from 18° to 49.7° (Faille et al., 2002; Teixeira et al., 2005). The wettability of a surface depends on factors such as surface fouling, cleaning and disinfection procedures, and rinsing water hardness (Sinde and Carballo, 2000). Wettability plays a crucial role in the initial colonization of microorganisms; however, it is difficult to control, as it is substantially altered by contact with products such as milk.

The milking machine is a complex system composed of numerous pipelines and components with varying geometries and materials depending on their function. Clean-

ing this system is a challenging task but is facilitated by existing hygienic design recommendations, such as (1) sloping the milk line (0.5% to 2%) to promote milk flow during milking, (2) installing 2 valves in the reception chamber to reverse the flow direction of cleaning solutions, (3) incorporating air injectors in large-diameter milk lines to create air plugs, and (4) fitting a removable milk line to facilitate cleanliness inspections.

Hygienic design could still be improved by eliminating right angles and horizontal surfaces where water may stagnate (Bénézech and Faille, 2020). Current standards for milking machine construction provide limited guidance on this aspect, giving manufacturers substantial design flexibility, which can lead to challenges in cleaning efficiency. Therefore, it is essential to carefully consider the design of milking installations and adhere to mainte-

nance and cleaning recommendations to prevent hygiene issues resulting from inadequate cleaning.

BIOFILMS IN MILKING MACHINES

Composition and Structure of Biofilms

Biofilms are complex microbial communities that can adhere to various surfaces, including biotic and abiotic environments, as well as liquid or solid interfaces (Jiang et al., 2021; Palková and Váchová, 2021). These communities produce an extracellular matrix, primarily composed of extracellular polymeric substances (EPS), along with proteins, carbohydrates, extracellular DNA, lipids, and signaling molecules. This extracellular matrix acts as a protective physical barrier, restricting the diffusion of antimicrobial agents and facilitating nutrient and waste exchange (Marchand et al., 2012; Whitehead and Verran, 2015; Karaca et al., 2019; Rumbaugh and Sauer, 2020; Palková and Váchová, 2021). Proteins such as adhesins play a critical role in enhancing biofilm cohesion, adhesion, and defense mechanisms (Paharik and Horswill, 2016; Lipke and Ragonis-Bachar, 2023). The ratio of polysaccharides to proteins within the extracellular matrix varies depending on bacterial species and environmental factors (Campanac et al., 2002; Whitehead and Verran, 2015; Chagas et al., 2017). This variability influences the physical properties and resilience of the biofilm matrix.

Biofilm Formation and Development

The development of biofilm results from the successful attachment and subsequent growth of microorganisms on a surface. The transition from the planktonic to biofilm mode is a dynamic, multistage process influenced by environmental and physiological factors, including nutrient availability, cellular stress, and intercellular communication mediated by quorum sensing (Sharma et al., 2023a). These environmental factors can influence microbial interactions, leading to changes in quorum sensing, a process by which bacteria communicate and synchronize gene expression in response to variations in cell density and species composition, thereby optimizing biofilm development. Additionally, microbial metabolic activity and gene expression are crucial, as they regulate the production of EPS that form the biofilm matrix and enhance microbial adhesion to surfaces (Marchand et al., 2012).

Biofilms may consist of a single species or multiple species and can form either single layers or complex 3-dimensional (3D) structures. These structures may contain water channels, which facilitate nutrient distribution,

metabolite exchange, and waste removal, contributing to biofilm adaptability (Quan et al., 2022; Alonso et al., 2022). Interactions among species can be cooperative, competitive, or neutral. In cooperative interactions, bacteria enhance biofilm formation by improving adhesion, promoting growth, and increasing resistance to biocides (Elias and Banin, 2012; Alonso et al., 2020; Yuan et al., 2020). Competitive interactions, on the other hand, may involve competition for space, nutrients, and energy sources (exploitative competition) or the production of secondary metabolites such as bacteriocins, enzymes, hydrogen peroxide, and organic acids (interference competition), ultimately leading to competitive exclusion (Alonso et al., 2020; Yuan et al., 2020).

Factors influencing bacterial attachment and biofilm formation include growth phase, viability, contact time with the surface, conditioning film, temperature, flow rate, surface roughness, hydrophobicity, and charge (Bayles, 2007; Marchand et al., 2012; Karaca et al., 2019; Alonso et al., 2021). Biofilms can form within several hours or over several weeks, depending on environmental conditions. Dynamic biofilm structures adapt to their environment, with their formation significantly influenced by hydrodynamics. Fluid shear, in particular, plays a critical role in shaping the physical characteristics of biofilms. Shear stress caused by fluid flow impacts both the structural integrity and density of biofilms, with higher shear conditions often leading to denser biofilms. Additionally, biofilms formed in zones exposed to shear forces exhibit substantially greater resistance to mechanical stress. Conversely, under static conditions at the air-liquid-material (ALM) interface, one-way transfers occur between the planktonic population and the biofilm. This is driven by the ecological opportunity created by the simultaneous availability of high levels of both oxygen and nutrients (Jha et al., 2020). Once integrated, planktonic cells do not mix with sessile cells, but instead localize in specific clusters within the biofilm pellicle. A ring-like structure often forms at the ALM interface, creating a favorable area for bacterial adhesion and biofilm formation. This phenomenon illustrates the large-scale migration of planktonic cells into specific regions of the biofilm, contributing to its spatial heterogeneity (El-Khoury et al., 2021). Studies have shown that strains of *Salmonella* Enteritidis and *Bacillus cereus* produce 100 times more biofilm on a semi-immersed stainless steel coupon than on a fully immersed coupon (Bénézech and Faille, 2020). Other pathogens, such as *Escherichia coli*, *Salmonella* Typhimurium, and *Pseudomonas* spp., have also been shown to produce ALM biofilms. These ALM interface biofilms exhibit high resistance to rinsing and cleaning procedures (Jha et al., 2020). This understanding of biofilm formation and resilience provides a foundation

for exploring their impact in specific environments, such as milking machines.

Milking Machine Case

Milking machines provide an environment conducive to biofilm formation, with microbial colonization affecting both equipment functionality and milk quality. This is evidenced by the frequent detection of *Pseudomonas* spp., *Lactococcus lactis*, *Klebsiella*, *Staphylococcus* spp., and *Enterococcus faecalis* (Teixeira et al., 2005). The presence of *Listeria monocytogenes*, the causative agent of listeriosis, has also been documented on stainless steel, rubber, and plastic components of milking machines (Latorre et al., 2010). This pathogen has been isolated from critical areas such as milk meters, teat cup liners, and bulk tank outlets (Latorre et al., 2020). *Pseudomonas* spp. are particularly adept at forming robust biofilms with high metabolic activity, even at low temperatures, making them resistant to disinfection and persistent within milking machine pipes (Khalilzadeh, 2009; Pécastaings, 2010; Aswathanarayan and Vittal, 2014; Rossi et al., 2016). Milking equipment biofilms have also been identified as a source of *Staph. aureus* contamination during milking, compromising the microbiological quality of milk used in dairy manufacturing (Latorre et al., 2020).

Although most studies on milking machine biofilms have focused on cow dairy farms, research has also investigated biofilm composition in goat farming systems. Biofilms in goat farming milking machines, studied by Laithier et al. (2012), predominantly contained beneficial acidifying bacteria alongside spoilage organisms. Weber et al. (2019) examined the composition of bacterial biofilms in milking machines from 2 dairy farms using both culture-dependent and culture-independent approaches. In total, 135 species were identified, with 8 species detected on both farms. Most bacteria isolated in this study belonged to genera typically associated with the dairy environment. The study highlights that the microbial profile at the genus level varied between farms, potentially influenced by seasonal factors. Bacteria from the phylum *Actinobacteria* were identified as initial colonizers of surfaces but were not found in large quantities in mature biofilms. Various bacterial genera detected on both farms included *Acinetobacter*, *Bacillus*, *Chryseobacterium*, *Kocuria*, *Microbacterium*, *Pseudomonas*, and *Staphylococcus*. Other bacteria frequently found in milking machines included *Lactobacillus* species (LAB), *Listeria monocytogenes*, and coliforms.

These findings emphasize the critical need for effective biofilm control strategies in milking machines to prevent contamination, maintain milk quality, and ensure the safety of dairy products.

Factors Influencing Biofilm Persistence in Milking Machines

Milking machines provide an ideal environment for biofilm formation due to nutrient availability, oxygen presence, and hydrodynamics forces during milk flow. Milk composition plays a critical role, as residues form conditioning films composed of proteins, lipids, minerals, and carbohydrates that promote bacterial adhesion and biofilm development. These films are often incompletely removed during cleaning, resulting in persistent contamination (Silva et al., 2018; Jiménez-Pichardo et al., 2021). In addition, the circulation of milk at 38°C in milking machines raises the system's temperature, promoting the growth of mesophilic bacteria such as LAB and pathogens such as *Escherichia coli*. Moist areas that remain after milk passage or cleaning further encourage bacterial and mold growth. Additionally, the continuous flow of milk provides nutrients to bacteria, facilitating their distribution throughout the system.

Different components of milking machines, such as teat cup liners, pipelines, and bulk milk storage chambers, are prone to ALM biofilm formation due to undrained liquid. Stagnant liquid (water or milk) in these areas creates conditions that promote microbial growth. Poor structural design of milking machines, particularly inadequate hygienic aspects, creates areas susceptible to bacterial adhesion and biofilm persistence and complicates cleaning efforts.

Mechanical action and pressure within milking machines can cause abrasions and surface imperfections, creating prime sites for bacterial adhesion. These micro-environments protect bacteria from cleaning agents and physical removal (Qiu et al., 2022). Regular maintenance is crucial to limiting biofilm development. Replacing worn parts, such as teat cup liners and milk hoses, helps prevent fouling and microbial adhesion.

Experiments conducted on a pilot milking machine demonstrated that even stringent cleaning and disinfection procedures cannot eliminate all microorganisms. This is due to the complex structural design of milking machines, which hinders thorough cleaning, along with intrinsic biofilm factors such as multiple resistance mechanisms to disinfectants (Jang et al., 2006; Laithier et al., 2024). Therefore, the impact of chemical agents currently used for cleaning or with potential to eliminate biofilms is of significant interest in this field of study. Numerous studies have examined the effects of various chemical compounds on biofilm elimination. Campanac et al. (2002) compared the effect of cationic surfactants, specifically quaternary ammonium compounds (QAC), on planktonic cells and mature biofilms of *Pseudomonas aeruginosa* and *Staph. aureus*. Their study demonstrated that QAC exhibit significant bactericidal activity against

planktonic cells but are much less effective on biofilms. Strong oxidizing agents, such as chlorine (Cl_2) and peracetic acid, are the most commonly used disinfectants for biofilm control. However, chlorination can lead to the formation of byproducts, such as trihalomethanes and haloacetic acids, which pose risks to consumer health. Additionally, biofilms exhibit significant resistance to common chemical compounds such as QAC and chlorine, primarily due to the protective EPS matrix and phenotypic adaptations of bacterial cells (Campanac et al., 2002; Jang et al., 2006). One promising alternative is chlorine dioxide (ClO_2); however, biofilm thickness remains a limiting factor in its effectiveness (Jang et al., 2006). Jiménez-Pichardo et al. (2021) tested a procedure using electrolyzed water, showing promising results, provided that the sequential order of the procedure was followed.

Santos et al. (2013) studied the effect of cleaning treatments on the adhesion of *Streptococcus agalactiae* to milking machine surfaces. From a thermodynamic perspective, cleaning the liners reduces the likelihood of microbial adhesion. However, the surface wear induced by these cleaning procedures must be considered, as it may hinder the removal of adherent cells. The regular replacement of rubber and silicone components in milking equipment is crucial and can help prevent contamination. Laithier et al. (2005) conducted experiments on cleaning methods aimed at selectively decontaminating undesirable microorganisms while preserving those of technological interest. They tested alkaline detergents in which chlorine was replaced by sodium sulfate (Na_2SO_4). Although the procedure yielded promising results under laboratory conditions, these results were not replicated in field trials. The differences between the results obtained in the laboratory and those obtained on the farm are probably because the biofilms studied have different characteristics in the 2 cases. The microbial communities of milking machine biofilms are complex, unlike those of laboratory biofilms. In addition, the surfaces of milking machine components are more conducive to biofilm formation than the glass slides used in the laboratory, and milking machine biofilms are older. All these factors can make milking machine biofilms more resistant to cleaning and disinfection procedures. The interactions among species play a crucial role in the formation, structure, and function of bacterial communities (Liu et al., 2017). Interspecies competition and biocontrol strategies involving beneficial biofilms are gaining attention in various industries (Simões et al., 2010; Elias and Banin, 2012; Giaouris et al., 2014). These approaches could provide innovative methods for managing microbial ecosystems in the dairy industry. Most previous studies have focused on biofilm competi-

tion against *Listeria monocytogenes* using LAB such as *Lactobacillus* spp. (Jara et al., 2020) or nisin-producing *Lactococcus lactis* (Leriche et al., 1999; Guerrieri et al., 2009). The interaction between the spoilage bacterium *Pseudomonas fluorescens* and *Lactococcus lactis* was investigated by Quintieri et al. (2021). In the biofilm phase, both proto-cooperation and commensalism were observed. The type and intensity of these interactions depended on the *Lactococcus* strain and the cell density of each species. Attachment was enhanced up to 100-fold for *P. fluorescens* and up to 20,000-fold for one *Lactococcus lactis* strain. In an unpublished study, Jard et al. (2018) evaluated the interactions between *Lactococcus lactis* ssp. and *P. fluorescens* isolated from raw goat milk. A minimal culture medium supplemented with milk was used to grow the strains and assess biofilm formation. Both *Lactococcus lactis* and *P. fluorescens* were able to form stable monospecies biofilms after 24 h. For dual-species biofilm assays, 2 types of experiments were conducted: (1) co-inoculation of both *Lactococcus lactis* and *Pseudomonas fluorescens*, and (2) delayed inoculation of *P. fluorescens* onto a preformed *Lactococcus lactis* biofilm. In both conditions, the 2 species successfully coexisted within dual-species biofilms. Confocal microscopy observations revealed that *Lactococcus lactis* formed a uniform cell layer, while *P. fluorescens* formed aggregates. Therefore, food-spoilage and technologically relevant microorganisms can coexist in a dairy context. These findings may serve as a foundation for future studies aimed at selecting microorganisms that inhibit spoilage bacteria, thereby optimizing the milking machines microbiota.

Seasonal variations in raw milk microbiota can occur, with mesophilic and thermophilic bacteria predominating in summer and psychrotrophs more prevalent in winter. This variation may therefore significantly affect biofilm composition. Environmental and herd factors such as geographical location, climatic conditions, type of forage, bedding, soil, animal genetics, herd size, type of housing, lactation stage, and udder health play a crucial role in this variability (Vacheyrou et al., 2011; Chemidlin Prévost-Bouré et al., 2021; Ouamba et al., 2022; Irlinger et al., 2024). Additionally, spores from the soil can contaminate forage plants, persist in silage, pass through the cows' gastrointestinal tract, and ultimately end up in the milk (Queiroz et al., 2018). Air quality, bedding, and feces are also key factors influencing the milk microbiota (Ouamba et al., 2022). These findings emphasize the importance of adopting specific management practices to ensure the microbiological quality of raw milk.

This underscores the need for detailed studies that closely replicate real-world conditions, considering both process and environmental factors, to better understand

fouling and cleaning in milking devices. These studies should investigate biofilm complexity, including resistance mechanisms and variations across different machine designs and materials. Additionally, emphasis should be placed on novel cleaning strategies, ranging from physical to biological approaches, to enhance the hygienic profile of milking machines. Such strategies would not only improve milk safety for consumers but also promote livestock health by minimizing microbial contamination. Furthermore, integrating a hygienic design approach can improve cleaning efficiency, reduce waste, and lower environmental impact by conserving water, energy, and chemicals.

BIOFILM IMPACTS ON MILK AND DAIRY PRODUCT QUALITY

Impact on Food Safety

Biofilms in milking machines on dairy farms are a potential source of raw milk contamination (Weber et al., 2019; Teixeira et al., 2005). These biofilms can harbor pathogenic bacteria that contaminate raw milk and persist throughout the dairy chain, posing a potential risk to human health, particularly when the milk is not subjected to heat treatments such as pasteurization or sterilization. Among the potentially pathogenic species detected in milking machine biofilms, *Listeria monocytogenes*, *Staph. aureus*, and *Escherichia coli* have occasionally been identified (Latorre et al., 2020; Weber et al., 2019). Coliforms, commonly found in biofilms, are considered hygiene indicators, and their high presence reflects ineffective cleaning (Weber et al., 2019). *Bacillus cereus*, a pathogen capable of forming biofilms on solid surfaces like those of a milking machine, produces heat-resistant endospores that can survive pasteurization. These endospores generate enterotoxins, which can cause food poisoning in humans (Ortuzar et al., 2018; Silva et al., 2018; Weber et al., 2019).

Impact on Technological and Sensory Quality of Dairy Products

Certain bacteria can impact the quality of dairy products at various levels. Contamination may render the product unsellable, reduce its shelf life, and cause defects in texture, taste, color, and odor. These bacteria can originate from the milking machine, as demonstrated by Weber et al. (2019) with *Acinetobacter* and *Pseudomonas*. Species from these genera are considered psychrotolerant spoilage enzyme producers in the dairy environment, meaning they can degrade product quality even under refrigeration, making them difficult to control

(Marchand et al., 2012; Ouamba et al., 2022). Similarly, some *Bacillus* species produce spoilage enzymes in addition to the previously mentioned enterotoxins and endospores. These bacteria are prolific biofilm producers and can induce lipolysis and proteolysis in dairy products. Furthermore, biofilms produced by *Pseudomonas* can enhance the biofilm-forming abilities of other pathogens. For instance, Simões et al. (2010) demonstrated that dual biofilms of *P. fluorescens* and *B. cereus* were ~5 times more metabolically active than *P. fluorescens* monospecies biofilms.

Although biofilms formed by pathogenic and spoilage microorganisms in milking machines are a concern and undesirable, not all biofilms are detrimental. Some contribute positively by inoculating milk with beneficial bacteria, particularly in the raw milk sector. These microorganisms play a key role in the acidification and maturation of cheeses. Laithier et al. (2012, 2004) demonstrated that these microorganisms, consisting of mesophilic acidifying bacteria, micrococci, and corynebacteria, were dominant in the milking machines of a goat farm. Additionally, a significant proportion of LAB possess technological properties that facilitate milk transformation and offer health benefits (Ouamba et al., 2022).

Impact on Mammary Infections

Biofilms on milking machines can serve as a major source of contamination for dairy animals, leading to bacterial mammary infections such as mastitis. This disease is primarily caused by *Staph. aureus* (Lee et al., 2014; Almeida et al., 2017; Latorre et al., 2020; Lianou et al., 2023), *Staphylococcus epidermidis*, and *Escherichia coli* (Chagas et al., 2017), although *Streptococcus agalactiae* is also recognized as a significant pathogen (Fröhling et al., 2010). Once a herd is infected, eliminating the pathogen becomes challenging, leading to declining herd health, reduced milk production, and compromised milk quality. These factors collectively result in substantial economic losses for farmers.

In summary, milking machine biofilms play a crucial role in microbial introduction, whether by harboring spoilage bacteria that compromise milk quality or by contributing beneficial bacteria that support raw milk fermentation. Although pathogenic biofilms pose risks to food safety, spoilage, and mammary infections, certain biofilms can enhance dairy product transformation. Future research should focus on developing biofilm management strategies that not only eliminate harmful bacteria but also shape microbial composition to support dairy-friendly microorganisms and improve milk-related product quality.

ANALYTICAL METHODS TO STUDY MILKING MACHINE BIOFILMS

Sample Collections

The presence of bacterial biofilms in raw milk processing lines poses a critical challenge to milk quality and dairy product safety, leading to significant economic consequences. To address this issue, reliable, robust, and time-efficient biofilm analysis methods are essential. The first step in these methods is accurate sample collection, as it directly affects the reliability of downstream analysis. Given the complexity of biofilms, representative and reliable sampling is crucial to ensure both surface and internal biofilm components are captured for analysis. Sampling can be performed using direct agar contact, swabs, sterile cloths, or sponges, following the ISO 18593 standard (ISO, 2018).

Direct agar contact is a rapid method for quantifying biofilm bacteria on surfaces. However, it is limited to a small surface area and only detects bacteria present on the biofilm surface. For larger surface sampling, sponges, swabs, or sterile cloths are recommended, particularly in industries such as raw milk manufacturing (Chagas et al., 2017; Almeida et al., 2017). These methods offer the advantage of being applicable to irregular surfaces, recovering significant quantities of bacteria from both the biofilm surface and interior (Niboucha et al., 2022). Additionally, they allow for targeted investigation of contamination in specific milking machine components that may be difficult to clean.

Alternative sampling methods include ultrasonication, which is feasible in the laboratory but not directly applicable in industrial settings. A spatula can also be used to dislodge biofilm; however, swabs have been shown to achieve better biofilm detachment and more repeatable results (Laithier et al., 2024). Removable stainless steel coupons can be placed in the milking machine to serve as reference sampling sites (Weber et al., 2019; Laithier et al., 2024). Although manual sampling methods are effective for localized biofilm assessment, they may not provide a comprehensive view of contamination across the entire milking system.

As an alternative to manual sampling, the rinsing method is preferred for assessing contamination across the entire milking machine. This process involves circulating a fluid through the system to collect microorganisms that may contaminate milk during milking. The fluids used include UHT milk, sterile water, or farm water (RMT Fromages de Terroirs, 2011). Among these, sterile water is the official standardized method. However, UHT milk is often preferred for small installations and narrow pipelines as it minimizes deposits within the milking machine, requiring only a single rinse before milking.

This technique can be implemented using 2 different approaches. The first approach involves sampling the sleeves located at the end of the milking machine circuit, with 2 sleeves if the milk pipeline is linear and 4 if it forms a “Y” configuration. The second approach selects 2 stations on one side and 2 on the other, alternating between the left and right milk pipes for each station. Milk is collected at the reception cannula in a sterile bucket after ~2 L have circulated through the machine, with the remainder drained by gravity. The collected milk is then homogenized with a disinfected ladle and stored in sterile bottles. Although this method is simple to implement and operates within the existing milk circuit, it requires careful adjustment of the milk volume passing through the system. Ultimately, the biofilm sampling method must be adapted to the surface being tested and the type of subsequent analysis to ensure optimal sample characterization.

Once samples are collected, various analytical approaches can be employed to study biofilms in detail. These include microbiological, molecular, and physicochemical techniques, as discussed in the following section.

Analytical Approach

Biofilm formation by monospecies microorganisms is often evaluated at a laboratory scale under static conditions using microplates. This method involves counting adherent cells in biofilms and observing them with fluorescence microscopy (details provided later in this section; Makovcova et al., 2017). The use of a minimal medium instead of a rich medium is beneficial as it promotes the growth of adhered bacteria rather than planktonic cells (Khalilzadeh, 2009). However, static conditions do not replicate the environmental forces that biofilms encounter in industrial systems. To better understand biofilm behavior under real-world conditions, dynamic models are used to simulate shear forces and flow characteristics. These models mimic the physical stresses biofilms experience in industrial settings, such as fluid flow and shear forces, enabling researchers to study both biofilm formation and detachment under shear flow (Mercier-Bonin et al., 2012). To ensure reproducible and reliable *in vitro* results when studying biofilm formation on industrial surfaces, standardized devices such as biofilm reactors are recommended (Gomes et al., 2019). For specific applications, custom experimental setups can be developed, provided that critical parameters such as shear forces and flow characteristics are carefully considered (André et al., 2015).

Several approaches have been developed to detect, quantify, and characterize biofilms in industrial settings. These methods include microbiological, molecular, physicochemical, and microscopic techniques. The most com-

monly used methods are listed in Table 2 and discussed in this section. One of the most basic techniques for biofilm measurement is crystal violet staining in microtiter plates (Almeida et al., 2017; Chagas et al., 2017; Weber et al., 2019). This widely used method quantifies total biofilm biomass, including bacterial cells and exopolysaccharides, by measuring optical density. However, it cannot distinguish between live and dead bacterial cells, and signal saturation may occur in thick biofilms. Extracellular matrix production, such as exopolysaccharides, can also be assessed using the Congo red agar test, a low-cost method that is as simple to perform as the crystal violet assay (Almeida et al., 2017; Chagas et al., 2017; Lianou et al., 2023). The main advantage of microtiter plate assays is that optical density can be measured in real time, allowing researchers to monitor biofilm formation dynamics over several hours. Although these microbiological techniques are rapid and provide quantitative data on biofilm characteristics, they may not be suitable in all cases, as not all bacteria can be cultivated under laboratory conditions.

To overcome the limitations of the biofilm quantification methods mentioned above, it is recommended to combine them with molecular techniques. These methods allow for an in-depth study of bacteria within biofilms by analyzing their genetic potential, identifying species with greater precision, exploring microbial interactions, and detecting nonculturable bacterial species. Among these, pulsed-field gel electrophoresis (PFGE) is widely used for generating DNA fingerprints, enabling the comparison of genetic profiles. This technique is particularly useful for bacteria of interest such as *Listeria monocytogenes* and *Staph. aureus*, which are detected within biofilms after selective subculture and isolation (Lee et al., 2014). Pulsed-field gel electrophoresis enables strain-level bacterial comparison, helping to track cross-contamination and bacterial colonization within environments to identify contamination sources. Using this approach, a predominant and persistent *Listeria monocytogenes* strain was detected in biofilms collected from milk meters, bulk milk, and the bulk tank outlet of a dairy farm (Latorre et al., 2010). However, PFGE is a subtyping method that requires the isolation of target microorganisms from the biofilm and does not provide quantitative data on bacterial load within the biofilm.

As an alternative to conventional microbiological enumeration assays, culture-independent quantification methods can be employed for detecting and estimating target bacteria. Polymerase chain reaction is a widely used, sensitive, and specific technique that enables genetic analysis of biofilm communities through DNA extraction and amplification. The 16S rRNA gene serves as a common marker for studying bacterial community composition, ranging from the phylum to species level.

Weber et al. (2019) amplified the 16S rRNA gene from DNA extracted from biofilms on milking equipment retainers and bulk tank outlets. Sequencing revealed dominant bacterial genera, including *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Serratia*. In combination with culture-dependent methods, PCR can confirm bacterial genus and species by amplifying specific genes of interest or detecting genes linked to biofilm formation, virulence, and antimicrobial resistance. For example, *Staph. aureus* was detected in biofilms collected from milk and milking machines on a dairy farm in Brazil using culture methods (Almeida et al., 2017). Polymerase chain reaction confirmed its presence by amplifying the sa442 gene, which is specific to *Staph. aureus*, and identified additional genes associated with biofilm formation and virulence. Similarly, Latorre et al. (2010) detected the pathogenic species *Listeria monocytogenes* in milk meters and rubber liners from a dairy farm using PCR amplification of the species-specific hly gene.

Building upon conventional PCR, quantitative PCR enhances sensitivity, specificity, and speed through real-time detection of the amplified gene. It also enables precise quantification of target genes in a sample using fluorescent dyes and specific probes. Reverse-transcription PCR (RT-PCR) further expands molecular analysis by reverse-transcribing extracted RNA into complementary DNA, allowing gene expression studies. This technique has been particularly useful for examining variations in *Staph. aureus* gene expression at different stages of biofilm development (Atshan et al., 2013). Although molecular techniques such as RT-PCR provide insights into the genetic and functional characteristics of biofilms, they are complemented by physicochemical techniques, which analyze the structural and chemical composition of biofilms.

Among advanced biofilm detection techniques, multiplex fluorescence in situ hybridization (M-FISH) is particularly powerful, as it combines molecular identification with fluorescence imaging to visualize bacterial species within biofilms (Karygianni et al., 2014; Dutta et al., 2021). This method allows multiple bacterial species to be simultaneously labeled with distinct fluorescent probes, enabling the generation of 3D images that reveal the spatial distribution of microbial communities. However, M-FISH is rarely used today due to its complexity and high implementation costs. Additionally, probe sequence optimization and validation are required to ensure accurate detection, and the penetration of probes can be limited in dense or structurally complex biofilms.

Spectrum-based methods also provide valuable insights into biofilm composition by analyzing protein content and chemical structures. One such method is MALDI-TOF, which examines the protein profile of biofilms, enabling the detection of specific protein markers and

Table 2. List of microbiological, molecular, and physicochemical methods most commonly used to study and characterize biofilms¹

Method	Description	Advantages	Disadvantages and limits	References
Microbiological Crystal violet assay	<ul style="list-style-type: none"> Positively charged dye binds to negatively charged components (polysaccharides, proteins, and nucleic acids) Optical density measurement correlates with biofilm biomass 	<ul style="list-style-type: none"> Quantitative Simple and rapid Low cost 	<ul style="list-style-type: none"> Does not differentiate living and dead cells Saturation problems for thick biofilms 	Chagas et al., 2017; Almeida et al., 2017; Weber et al., 2019
Congo red agar test	<ul style="list-style-type: none"> Detects the production of exopolysaccharides (EPS) by bacteria Cultured on agar plates with the dye Congo red that changes color upon contact with EPS 	<ul style="list-style-type: none"> Simple Low cost 	<ul style="list-style-type: none"> Qualitative rather than quantitative Not suitable for all bacterial species 	Almeida et al., 2017; Chagas et al., 2017; Lianou et al., 2023
Microtiter assay	<ul style="list-style-type: none"> Bacteria are cultured in the wells of a microtiter plate. After incubation, biofilm production is evaluated by measuring optical density 	<ul style="list-style-type: none"> Enables the screening of many conditions at the same time. Cost-effective Easily adaptable for different types of biofilm and bacterial species 	<ul style="list-style-type: none"> Variable results Nonspecific: total biofilm biomass is measured Biofilm heterogeneity: inaccurate measurements 	Almeida et al., 2017; Chagas et al., 2017; Weber et al., 2019; Lianou et al., 2023
Molecular PFGE	<ul style="list-style-type: none"> Molecular technique used to generate DNA fingerprints to analyze genetic profiles of bacteria within the biofilm 	<ul style="list-style-type: none"> High-resolution and genetic analysis Reproducible results Standardized protocol 	<ul style="list-style-type: none"> Time-consuming Complex and expensive equipment Limited throughput 	Latorre et al., 2010; Lee et al., 2014
PCR	<ul style="list-style-type: none"> Identifies specific bacterial species, detect genes associated with biofilm formation, and analyze genetic diversity in biofilm community by DNA extraction and amplification 	<ul style="list-style-type: none"> High sensitivity Specificity Rapid and efficient High throughput 	<ul style="list-style-type: none"> Contamination risk Need for specific primers Conventional PCR: qualitative results; additional techniques like qPCR needed for quantification Limited to known sequences 	Latorre et al., 2010; Almeida et al., 2017; Weber et al., 2019
M-FISH	<ul style="list-style-type: none"> Visualize and identify specific nucleic acid sequences in biofilms. Simultaneously detect multiple target sequences, such as bacterial species or functional genes, within the biofilm matrix 	<ul style="list-style-type: none"> Specific and simultaneous detection Quantitative analysis Visualization of spatial organization 	<ul style="list-style-type: none"> Requires careful optimization and validation of probe sequences to ensure accurate and reliable detection Limited throughput Penetration of FISH probes into dense or complex biofilm matrices can be limited 	Karygianni et al., 2014; Dutta et al., 2021
MALDI-TOF	<ul style="list-style-type: none"> Analyzes protein composition Detects specific bio-molecular markers Identifies bacterial species within biofilms 	<ul style="list-style-type: none"> Rapid identification and high sensitivity Comprehensive proteomic analysis 	<ul style="list-style-type: none"> Limited quantitative capability Matrix interference and sample complexity Limited to ionizable molecules and dependent on database quality 	Caputo et al., 2018; Yan et al., 2023
Physicochemical				

Continued

Table 2 (Continued). List of microbiological, molecular, and physicochemical methods most commonly used to study and characterize biofilms¹

Method	Description	Advantages	Disadvantages and limits	References
SPR	<ul style="list-style-type: none"> Monitors the formation and growth of biofilms Study binding interactions between biofilm components (such as proteins, polysaccharides, and nucleic acids) and various substances (like antimicrobial agents) by detecting changes in the refractive index near a sensor surface, which occur when biomolecules bind to the surface Characterizes the biochemical composition of biofilms by detecting the vibrational modes of chemical bonds within the biofilm matrix (analyze of infrared spectra) 	<ul style="list-style-type: none"> Real-time monitoring Label-free detection (no fluorescent or radioactive labels) Quantitative analysis and sensitive 	<ul style="list-style-type: none"> Surface-dependency: may not fully represent the complexity of biofilm interactions occurring in the 3-dimensional (3D) structures High cost and specialized expertise needed Limited throughput 	<p>Abadian et al., 2014; Bajaj et al., 2023</p>
FTIR	<ul style="list-style-type: none"> Characterizes the biochemical composition of biofilms by detecting the vibrational modes of chemical bonds within the biofilm matrix (analyze of infrared spectra) 	<ul style="list-style-type: none"> Nondestructive analysis Rapid and efficient analysis of the biofilm composition Minimal sample preparation Detailed molecular information Nondestructive and quantitative analysis Possible to analyze biofilms in their native hydrated state 	<ul style="list-style-type: none"> FTIR primarily analyzes the surface layers of biofilms Complex spectra interpretation and overlap of absorption bands Environmental sensitivity High cost and complex Large amounts of sample and high concentrations of analytes required Long acquisition time Sensitivity to magnetic field inhomogeneities Complex data interpretation 	<p>Gieroba et al., 2020; Kamnev et al., 2023</p>
NMR	<ul style="list-style-type: none"> Studying the structure of biofilm components such as polysaccharides, proteins, and metabolites by observing the interaction of molecule nuclear spins when placed in a magnetic field 	<ul style="list-style-type: none"> Detailed molecular information Nondestructive and quantitative analysis Possible to analyze biofilms in their native hydrated state 	<ul style="list-style-type: none"> High cost and complex Large amounts of sample and high concentrations of analytes required Long acquisition time Sensitivity to magnetic field inhomogeneities Complex data interpretation 	<p>Zhang and Powers, 2012; Reichhardt and Cegelski, 2014</p>
Scanning electron microscopy	<ul style="list-style-type: none"> Powerful imaging technique used to examine the surface structure and morphology of biofilms at high magnifications 	<ul style="list-style-type: none"> High-resolution imaging Three-dimensional visualization Wide range of sample types (dry and wet coated samples) 	<ul style="list-style-type: none"> Preparation process: time-consuming, complex, and may introduce artifacts or alter the natural state of the biofilm 	<p>Latorre et al., 2010; Santos et al., 2013; Latorre et al., 2020</p>
Fluorescence microscopy	<ul style="list-style-type: none"> Visualizes biofilm structure, cell morphology, and EPS content using fluorescent staining Enables real-time monitoring of biofilm formation and detachment 	<ul style="list-style-type: none"> Differentiates live and dead cells Provides both qualitative and quantitative analysis Captures biofilm dynamics CLSM allows for detailed 3D structure analysis Quick and easy to operate 	<ul style="list-style-type: none"> High cost Limited in distinguishing VBNC cells from dead cells Conventional fluorescence microscopy may have out-of-focus glare issues Requires complementary molecular methods CLSM imaging quality can be affected by biofilm thickness 	<p>Jha et al., 2019, 2020, 2022</p>

¹PGFE = pulsed-field gel electrophoresis; M-FISH = multiplex fluorescence in situ hybridization; SPR = surface plasmon resonance; SPH = surface plasmon resonance; NMR = nuclear magnetic resonance; qPCR = quantitative PCR.

the identification of bacteria after subculturing. Notably, MALDI-TOF distinguishes biofilm-producing from non-producing strains by comparing protein profiles against reference databases. This approach has been successfully applied to clinical strains of *Staphylococcus epidermidis* and *Pseudomonas fluorescens*, which contribute to raw milk spoilage (Caputo et al., 2018; Yan et al., 2023).

Similarly, Fourier transform infrared and nuclear magnetic resonance spectroscopy are effective for characterizing the chemical content of biofilm samples without prior preparation, under both dry and wet conditions, to identify the main components of the extracellular matrix (Zhang and Powers, 2012; Reichhardt and Cegelski, 2014; Gieroba et al., 2020; Kamnev et al., 2023). These methods are also used to evaluate the impact of antimicrobial agents on biofilm deterioration.

Although spectrum-based methods provide valuable biochemical insights into biofilms, additional physicochemical techniques are necessary to study molecular interactions and biofilm dynamics in real time. These include surface plasmon resonance, a label-free optical measurement technique that detects molecular interactions on a surface and monitors biofilm removal in real time (Abadian et al., 2014; Bajaj et al., 2023). Surface plasmon resonance is particularly useful for assessing the effectiveness of antimicrobial compounds in reducing biofilms on a specific surface.

Where molecular and physicochemical techniques provide molecular-level insights into biofilms, scanning electron microscopy produces high-resolution images in the nanometer range, offering detailed observation of the 3D structure of biofilms adhering to a surface, including bacterial morphology and the extracellular matrix. This is achieved by using an electron beam, rather than visible light, to scan the surface of the sample (Latorre et al., 2020; Santos et al., 2013). These different approaches are complementary, providing a better understanding of biofilm structure and resistance and offering valuable opportunities for developing new strategies to prevent biofilm formation in the industrial environment.

Fluorescence microscopy, including confocal laser scanning microscopy (CLSM), is widely used for studying biofilm structure and estimating key characteristics such as biofilm quantity, cell morphology, and EPS content (Jha et al., 2020). This technique enables an in-depth understanding of cell physiology through staining processes that differentiate between live and dead cells, allowing both qualitative and quantitative analyses of biofilms (Jha et al., 2022). Additionally, fluorescence microscopy provides real-time data on biofilm formation and detachment, elucidating biofilm development mechanisms under both static and dynamic conditions. Notably, CLSM allows for detailed 3D visualization of biofilm structures, offering insights into their spatial organiza-

tion and layering. Although fluorescence microscopy offers significant advantages, it has certain limitations. For example, it may struggle to distinguish between dead cells and those in a viable but nonculturable (VBNC) state (Jha et al., 2020). This limitation highlights the need to integrate molecular methods as complementary tools for biofilm analysis. In the dairy industry, VBNC cells are particularly concerning, as they can evade regular screening protocols, potentially leading to foodborne outbreaks. This underscores the necessity of robust and multifaceted approaches for biofilm assessment, especially in milk processing and safety monitoring.

Recently, the multi-omics approach, combined with advanced bioinformatics tools, has been explored to gain deeper insights into microbial communities, gene and protein expression, and metabolite production in mixed-species biofilms (Yuan et al., 2023). This method contributes to the development of effective biofilm control strategies.

Metagenomics facilitates high-throughput and detailed identification of microbial compositions, including unculturable species, within mixed-species biofilms in dairy environments.

Whole-genome sequencing is a reliable tool for identifying genes involved in biofilm formation by various dairy isolates. Additionally, a pan-GWAS has identified genetic variants of *L. monocytogenes* associated with biofilm phenotypes, highlighting its persistence in low-temperature, nutrient-deprived dairy environments (Lee et al., 2019).

Transcriptomics, proteomics, and metabolomics can be applied to study interactions within mixed-species biofilms. Using these approaches, numerous studies have confirmed social interactions in biofilms formed by dairy strains (Yuan et al., 2023). These methods provide insights spanning cellular to molecular levels, each offering distinct advantages and limitations. Often, they are used in combination to achieve a more comprehensive analysis.

Although laboratory-based methods offer valuable information on biofilm behavior, they frequently fail to replicate the complexity of real-world conditions. Bridging this gap requires tools such as PiloTraite (Laithier et al., 2024), which simulate operational environments while allowing controlled studies to optimize biofilm management strategies in the dairy sector, as discussed in the following section.

Approach Closer to Real Conditions

The hygienic profile of milking machines is assessed differently in laboratory settings compared with real-field environments, often yielding varying results due to differences in environmental conditions and operational

parameters. Laboratory experiments, although designed to mimic milking machine conditions, cannot fully replicate the complexities of real-world dairy environments. Conducting reproducible and reliable experiments in field settings presents challenges, as factors such as temperature, humidity, and milk production are difficult to control. This has led to the development of intermediate tools that simulate milking machine conditions and enable controlled and reliable studies.

A laboratory tool was developed in the United States to study clean-in-place systems for milking parlors under conditions closer to real-world settings (Reinemann et al., 1997; Reinemann and Ruegg, 2000). However, it does not include a specific apparatus for studying biofilms. The PiloTraite tool (Laithier et al., 2024) was therefore created to meet this need. This is a laboratory-installed milking machine located at the Derval experimental farm (Derval, France), designed to operate without animals by simulating milking through artificial udders at 4 stations. The main components include claws with teat cups, the milk line, pulsator, receiving chamber, and cleaning automaton.

Various stakeholders in the dairy sector were consulted before the development of the tool to assess needs and expectations regarding the milking machine. The survey identified key objectives for the PiloTraite tool, including (1) improving knowledge and control of biofilms, (2) optimizing cleaning and disinfection procedures, and (3) improving understanding of the mechanics of milking and the relationship between the milking machine and milk quality. Following its installation, initial experiments focused on biofilms in the milking machine, helping to establish protocols for sampling, implantation, and removal. It was demonstrated that eliminating established biofilms from the milking machine was not feasible, even with rigorous cleaning procedures (Laithier et al., 2024). As a result, a resident biofilm persists in the tool, composed of both beneficial and spoilage microflora, with a microbial diversity closely resembling that found in real farming environments. The composition of this biofilm can be modified by introducing specific microflora from the circulating milk (Laithier et al., 2024). These initial results show that achieving sterility in the milking machine is impractical, making it necessary to work with the existing biofilms. However, these biofilms must not harbor pathogens that could contaminate the milk and be difficult to eliminate. Therefore, a rigorous and efficient cleaning procedure must be maintained to control biofilms and ensure milk quality.

CONCLUSIONS

Biofilms have been extensively studied since the 1990s, leading to substantial advancements in microbiology and

a deeper understanding of microbial behavior. Although their structure and formation are well understood, biofilm detachment remains relatively unexplored, highlighting the need for further research.

Milking machines create ideal conditions for biofilm formation and persistence, as food-spoilage and pathogenic bacteria present in raw milk or on machine surfaces can form biofilms. Once these biofilms detach, they can contaminate bulk milk, posing significant food safety concerns. Controlling biofilms is therefore critical to maintaining milk quality and safety. However, their dynamic nature and the continuous evolution of microbial communities make them difficult to analyze and control. Effective biofilm management requires a comprehensive approach that integrates machine design, optimized cleaning and disinfection protocols, and proper farm management practices. The interaction between machine components, milk residues, and microbial communities complicates biofilm control, reinforcing the need for precise and effective cleaning procedures. Cleaning the milking machine remains fundamental in biofilm management, preventing the establishment of spoilage and pathogenic flora. To achieve this, hygienic equipment design and adherence to cleaning recommendations from dairy organizations and product suppliers are essential.

Despite these efforts, laboratory-based studies often fail to replicate real-world farm conditions, making field validation crucial. Pilot-scale tools such as PiloTraite help bridge this gap by simulating milking machine conditions, allowing for better evaluation of biofilm behavior, cleaning efficiency, and microbial persistence. Additionally, improving biofilm detection and quantification methods will enhance contamination assessments, enabling more targeted mitigation strategies. Standardizing biofilm assessment techniques will further aid in developing effective prevention and removal protocols.

In addition to technological solutions, farm-level interventions remain equally important. Good farming practices, from feeding to barn hygiene, play a crucial role in promoting beneficial microorganisms while limiting undesirable ones. Maintaining a balanced microbial ecosystem is essential not only for minimizing biofilm-related contamination risks, but also for preserving beneficial bacteria that contribute to milk processing and quality.

Ultimately, effective biofilm management in milking machines is crucial for ensuring milk safety and sustainable dairy processing. Achieving this requires balancing the removal of harmful biofilms and preserving beneficial microbial communities. A multidisciplinary approach integrating microbiology, engineering, and hygiene management is essential to optimizing cleaning strategies. Future research should focus on understanding the biofilm life cycle, particularly detachment, under conditions that closely mimic real-world dairy settings. Ad-

ditionally, improving detection methods and real-world validation tools will enhance biofilm control, ensuring hygienic milking systems and safe dairy production.

NOTES

This study received no external funding. No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: 3D = 3-dimensional; ALM = air-liquid-material; CLSM = confocal laser scanning microscopy; EPS = extracellular polymeric substance; LAB = lactic acid bacteria; M-FISH = multiplex fluorescence in situ hybridization; NiRB = nitrile rubber; NMR = nuclear magnetic resonance; PC = polycarbonate; PEFE = polytetrafluoroethylene; PGFE = pulsed-field gel electrophoresis; PMMA = polymethyl methacrylate; PPSU = polyphenyl sulfone; PSU = polysulfone; QAC = quaternary ammonium compound; qPCR = quantitative PCR; Ra = surface roughness; RT-PCR = reverse-transcription PCR; SiRB = silicone rubber; SPR = surface plasmon resonance; SS = stainless steel; VBNC = viable but nonculturable.

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