Biofilms in Food Processing Environments

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ABSTRACT

Biofilms are a constant concern in food processing environments. Our overall research focus has been to understand the interaction of factors affecting bacterial attachment and biofilm formation with the ultimate goal of devising strategies to control this problem. This paper briefly describes three areas of biofilm research in which we have been involved. *Listeria monocytogenes*, a foodborne pathogen, survived for prolonged periods on stainless steel and buna-n rubber, materials commonly used in food-processing equipment. Survival was affected by temperature, relative humidity, attachment surface, and soil. Some components in the rubber inhibited growth of the organism on buna-n, which also affected the efficacy of sanitizers on biofilm inactivation. In cheese manufacture, biofilms of *Lactobacillus curvatus* could lead to a defect caused by the formation of calcium lactate crystals in Cheddar cheese. This hardy organism persisted in low numbers on equipment surfaces and was difficult to eradicate. We investigated the relative contributions and interactions of mechanical, thermal, and chemical processes in an air-injected clean-in-place method for milking systems. Overall, it is important to study the interactions between bacteria and the surfaces in a specific food processing environment to provide more effective measures for prevention of biofilm formation and for its removal. (**Key words**: biofilms, food processing)

Abbreviation key: **BN** = buna-n rubber, **CIP** = clean-in-place, **RH** = relative humidity, **SS** = stainless steel.

INTRODUCTION

The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage or transmission of diseases. The surfaces of equipment used for food handling, storage, or processing are recognized as major sources of microbial contamination. Even with acceptable clean-in-place (**CIP**) systems, bacteria can remain on equipment surfaces (3, 6, 7, 14). These organisms may survive for prolonged periods, depending on the amount and nature of residual soil, temperature, and relative humidity (**RH**) (15). Areas that are more prone to biofilm development include dead ends, joints, valves, and gaskets. In addition, equipment surfaces can be corroded with age. Pits and cracks may develop in which soil and bacteria can collect. An understanding of how and why bacteria attach and form biofilms is needed so that strategies can be developed to manage and control this problem.

This paper summarizes three research areas in which we have been involved: biofilm formation by a foodborne pathogen, *Listeria monocytogenes*; contamination of cheese by nonstarter lactic acid bacteria; and cleanability assessment of milking equipment.

ATTACHMENT AND BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES*

Listeria monocytogenes is a foodborne pathogen of significant concern to the food industry. One of the challenges in the control of contamination by this organism is its ubiquitous nature. *Listeria monocytogenes* is commonly found in the environment and has been isolated from both meat and dairy processing plants (1, 5, 8, 18). The highest incidence is associated with wet locations, such as conveyor belts and floor drains. The organism can be found in raw milk and has been associated with outbreaks involving dairy products. Numerous studies (4, 9, 12, 13, 23, 24) have examined the potential for biofilm development by *L. monocytogenes* on various kinds of surfaces and compared the behavior of the biofilm and planktonic bacteria, especially in response to sanitizers and heat. A common conclusion is that biofilm bacteria are more resistant to the effects of adverse environments.

We investigated the attachment and biofilm formation of *L. monocytogenes* on stainless steel (**SS**; type 304, no. 4 finish) and buna-n rubber (**BN**; acrylonitrile butadiene, 70 durometer), two materials com-

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TABLE 1. Effects of milk and milk components on the attachment of *Listeria monocytogenes* to stainless steel (SS) or buna-n rubber (BN).[1]

Attachment menstrua	*L. monocytogenes* attached to					
	SS			BN		
	(log cfu/cm^2)	SD	(% control)	(log cfu/cm^2)	SD	(% control)
PBS	5.04	4.28	100	4.85	4.44	100
Skim milk	2.78	2.78*	0.5	ND[2,*]		0
1% Casein	3.79	3.36*	5.6	3.62	2.63*	5.9
0.3% β-LG	4.10	3.60*	12	3.77	3.77*	8.4
0.07% α-LA	4.67	4.28*	43	4.43	4.23*	19
4% Lactose	5.35	3.03	101	4.60	2.54	79

[1]Adapted from Helke et al. (10); reprinted with permission from *Journal of Food Protection*. Copyright is held by the International Association of Milk, Food and Environmental Sanitarians, Inc.

[2]Nondetectable.

*Values are significantly different from PBS controls ($P < 0.025$).

monly used in food processing equipment (10). When these surfaces were exposed to a suspension of *L. monocytogenes* (ca. 10^7 cfu/ml) in PBS, the organism attached quite readily, resulting in about 10^5 cfu/cm^2 on either surface. Milk and milk proteins (caseins, α-LA, and β-LG) significantly reduced attachment by the organism compared with the levels attained in PBS (Table 1). Pretreatment of the surfaces or the organism with these components had a similar inhibitory effect (Tables 2 and 3). Conversely, the presence of lactose did not affect the attachment by *L. monocytogenes* to either surface. All of the milk proteins that were tested had a pI <6.0 and, therefore, would carry a net negative charge in milk (pH 6.5) or in PBS (pH 7.2). Repulsion between the negatively charged proteins and the negatively charged bacterial cell surface could partially account for the decrease in attachment.

Although results indicated that attachment in the presence of milk or on presoiled surfaces might be reduced, milk soil might harbor surviving bacteria in areas that are not adequately cleaned or are hard to clean, such as gaskets, joints, and crevices. Hence, we examined the interactions of residual soil, surface, temperature (6 and 25°C), and RH (32.5 and 75.5%) on the survival of *L. monocytogenes* (11). In the absence of milk soil, the numbers of attached *L. monocytogenes* (ca. 10^4 cfu/cm^2) decreased over time; the slowest decrease occurred at 6°C and 75.5% RH. A decline in numbers of only about 1 log cfu/cm^2 was observed on both the SS and BN surfaces after 10 d of storage. The other three combinations of temperature and RH were less favorable for survival, but *L. monocytogenes* still survived for 3 to 5 d (Figure 1). The presence of milk soil provided harborage and nutrients for attached *L. monocytogenes*. The organism grew on SS at 25°C and 75.5% RH. Although growth was not observed with any other storage conditions, the numbers remained relatively constant

TABLE 2. Effects of pretreating surfaces of stainless steel (SS) or buna-n rubber (BN) with milk and milk components on the attachment of *Listeria monocytogenes*.[1]

Treatment	*L. monocytogenes* attached to					
	SS			BN		
	(log cfu/cm^2)	SD	(% control)	(log cfu/cm^2)	SD	(% control)
PBS	5.97	5.19	100	5.75	5.23	100
Skim milk	3.08*	3.07	0.1	ND[2,*]		0
1% Casein	3.45*	3.53	0.3	3.03*	3.00	0.001
0.3% β-LG	4.47*	3.78	3.2	3.76*	3.70	1.5
4% Lactose	5.85	5.18	76	5.63	5.25	113

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[2]Nondetectable.

*Values are significantly different from PBS controls ($P < 0.025$).

TABLE 3. Effects of pretreating cells with milk and milk components on the attachment of *Listeria monocytogenes* to stainless steel (SS) or buna-n rubber (BN).[1]

Treatment	*L. monocytogenes* attached to					
	SS			BN		
	(log cfu/cm^2)	SD	(% control)	(log cfu/cm^2)	SD	(% control)
PBS	5.70	5.19	100	5.16	3.20	100
Skim milk	2.95*	2.48	0.2	2.78*	2.78	0.4
1% Casein	2.78*	2.78	0.1	ND[2],*		0
0.3% β-LG	4.77*	4.34	12	4.33*	4.21	15
4% Lactose	5.76	5.02	115	5.11	4.62	89

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[2]Nondetectable.

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over 10 d (Figure 2). When SS and BN surfaces were artificially soiled with raw milk and held at 6 and 25°C and 75.5% RH, *L. monocytogenes* persisted in the presence of raw milk natural flora and was recovered under all conditions through 10 d of storage. In addition, the organism grew on SS at 25°C despite high numbers of competing raw milk flora (Figure 3).

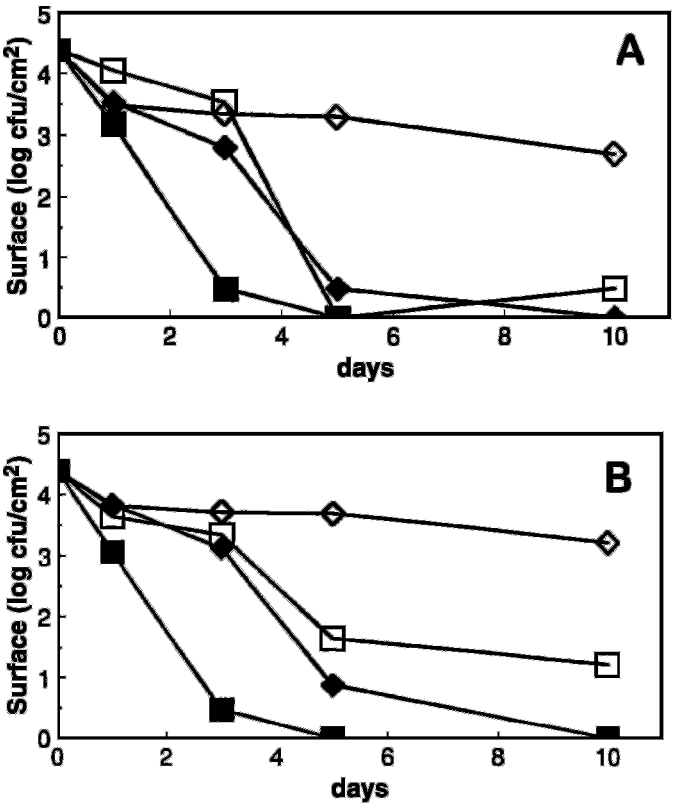

Figure 1. Survival of *Listeria monocytogenes* in PBS on stainless steel (SS; A) and on buna-n rubber (BN; B). Legend: ■ = 25°C and 32.5% relative humidity (RH), ♦ = 25°C and 75.5% RH, □ = 6°C and 32.5% RH, and ◊ = 6°C and 75.5% RH. (Adapted from Helke and Wong (11); reprinted with permission from *Journal of Food Protection*. Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc.).

Of interest was that some as yet unidentified components in BN inhibited the growth of *L. monocytogenes* and several other foodborne pathogens (11, 21). This inhibitory effect was enhanced when the nutrient level in the growth medium was suboptimal. With a high nutrient medium such as tryptose-phosphate broth, an increased lag phase was observed in the presence of BN. In a medium containing one-fifth the amount of nutrients found in tryptose-phosphate broth, biofilm formation on BN was either totally inhibited or was reduced in four of seven *L. monocytogenes* strains tested (21). The bacteriostatic effect of BN was stable to cleaning procedures and was not inactivated or removed by 20 cycles of a simulated CIP process (11).

The effectiveness of four types of commonly used sanitizers containing chlorine, iodine, quaternary ammonium compounds, and anionic acid were tested on planktonic and biofilm cells of *L. monocytogenes* (21). In agreement with results of other researchers (9, 12, 13), we found that biofilm bacteria were more resistant to the action of sanitizers. Planktonic cells were reduced by 7 to 8 log cfu/ml by the sanitizers tested. Resistance of biofilm bacteria to sanitizers was strongly influenced by the type of surface. Bacterial biofilm populations on SS were reduced 3 to 5 log cfu/cm^2, and those on BN were reduced <1 to 2 log cfu/cm^2. It is not known whether the inhibitory effects of BN on biofilm formation as described earlier and sanitizer efficacy are caused by the same factors. Slow growth might enhance the resistance of cells to antimicrobial agents (2), which could partially explain why the biofilm cells that formed on BN were more resistant to sanitizers.

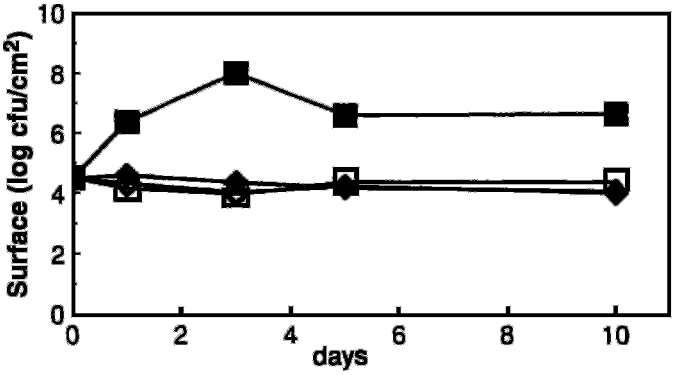

Figure 2. Survival of *Listeria monocytogenes* in pasteurized whole milk on stainless steel (SS) and buna-n rubber (BN) at 75% relative humidity (RH). Legend: ■ = 25°C, SS; ♦ = 25°C, BN; □ = 6°C, SS; and ◊ = 6°C, BN. (Adapted from Helke and Wong (11); reprinted with permission from *Journal of Food Protection.* Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc.)

BIOFILM FORMATION BY NONSTARTER LACTIC ACID BACTERIA

Nonstarter lactic acid bacteria are common causes of cheese defects, such as undesirable flavors, gas formation, and the white haze from calcium lactate crystals. The source of these nonstarter lactic acid bacteria is thought to be primarily from postpasteurization contamination in the dairy plant environment (19). A series of studies was conducted to determine the potential for biofilm formation and contamination of Cheddar cheese by a *Lactobacillus curvatus* isolate capable of forming D-(–)-lactic acid, the isomer responsible for the formation of calcium lactate crystals (22). Presterilized SS chips (2.5 cm^2) were glued to the inside of a cheese vat. Cheddar cheese was made with pasteurized milk that had been inoculated with *Lb. curvatus* at 10^4 cfu/ml, and the chips were retrieved at various intervals during cheese manufacture, cleaning, and sanitizing to determine the presence of attached organisms. The *Lb. curvatus* was present in the curd (ca. 10^5 cfu/g) and also on the surface of the vat (ca. 10^2 to 10^3 cfu/cm^2) after it had been cleaned and sanitized. During ripening of the cheese at 7.2°C, *Lb. curvatus* grew to about 10^7 cfu/g in 2 wk; D-(–)-lactic acid was 48% of the total lactic acid content.

To determine whether residual *Lb. curvatus* could contaminate subsequent cheese making, a second batch of cheese was made in the same vat after it had been cleaned and sanitized but was made without the addition of the *Lb. curvatus* culture. Cross-contamination from the first batch of cheese occurred, as evidenced by the recovery of *Lb. curvatus* from the second batch of cheese (ca. 10^2 cfu/g). The organism persisted on the surface of the vat (ca. 10 cfu/cm^2) after cleaning and sanitizing.

A second approach was designed to mimic possible contamination of the product by contact with biofilms that developed on equipment surfaces. Biofilms of *Lb. curvatus* were developed on SS chips and were added to the cheese vat along with milk at the beginning of the cheese-making process. Approximately 10^6 cfu were present in the biofilm, giving a potential inoculum of 10 to 30 cfu/ml of milk. The *Lb. curvatus* could not be detected in the milk at the time of addition of the starter culture or in the curd at the hooping stage. However, after 1 wk of storage at 7.2°C, *Lb. curvatus* was recovered from the cheese (<10^2 cfu/g), and, by 10 wk, the number had increased to about 10^4 cfu/g; 15% of the total lactic acid in the cheese was present as the D-isomer.

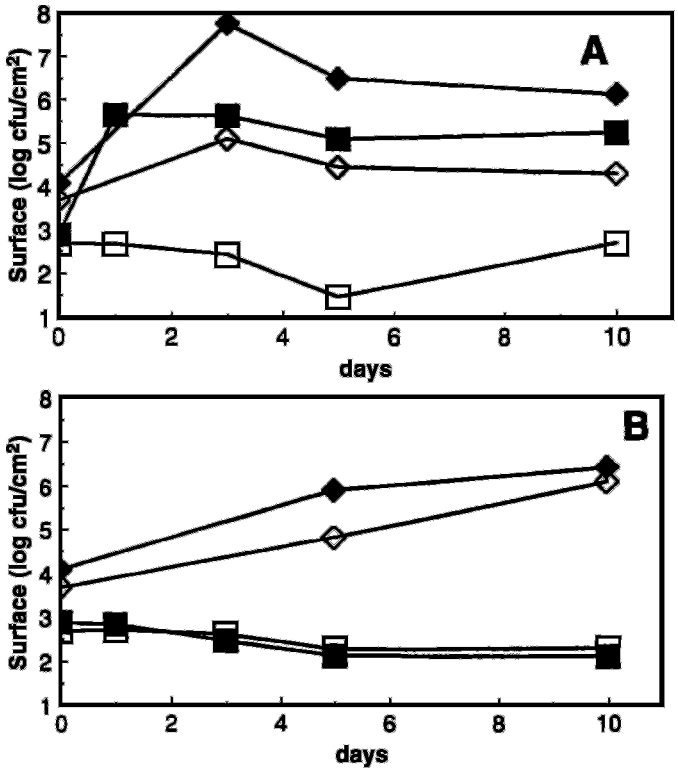

Figure 3. Survival of *Listeria monocytogenes* in raw milk on stainless steel (SS) and buna-n rubber (BN). *Listeria monocytogenes* and raw milk flora at 75.5% relative humidity (RH) at (A) 25°C and (B) 6°C. Legend: ■ = *Listeria monocytogenes*, SS; ♦ = raw milk flora, SS; □ = *L. monocytogenes*, BN; and ◊ = raw milk flora, BN. (Adapted from Helke and Wong (11); reprinted with permission from *Journal of Food Protection.* Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc.).

These studies showed that the crystal-forming strain of *Lb. curvatus* is a hardy organism and can survive and grow during the cheese-making and ripening processes. Even with diligent cleaning and sanitizing, low numbers may remain on equipment surfaces. In addition, biofilms formed by the organism can be a source of contamination in the dairy environment.

AIR-INJECTED CIP FOR MILKING SYSTEMS

Most modern milking systems are cleaned using air-injected CIP systems. Cleaning and sanitizing are accomplished by a combination of mechanical, thermal, and chemical processes. Air-injected CIP systems make use of the milking vacuum pump to supply the force required to circulate cleaning solutions. Air is injected to increase the circulating velocity and mechanical cleaning action of the wash solution (20). The wall shear stress that develops can be 10 to 20 times higher than those in flooded CIP systems (16). Some studies have shown the importance of shear effects on the removal of milk soil and bacteria. However, only a few studies have evaluated the cleaning effectiveness of these systems, and limited information exists on the relative contributions and interactions of the mechanical, chemical, and thermal actions in these cleaning systems.

To assess the cleaning effectiveness of an air-injected CIP system, a series of studies was performed at the Milking Research and Instruction Laboratory at the University of Wisconsin-Madison. This facility has a complete, air-injected CIP system with 72-mm (i.d.) pipelines. An experimental SS pipe section with six removable SS chips (3.8 cm^2) was constructed. A spraying apparatus was designed to apply a standardized milk soil to the SS chips (17). Three types of milk soil were tested: type 1 contained pasteurized whole milk with added albumin; type 2 contained pasteurized whole milk plus albumin and was inoculated with a culture of *Lactobacillus fermentum*; and type 3 had the same composition as type 2, but the soiled chips were incubated at 5°C for 24 h prior to cleaning. Milk soil was estimated by measurement of total ATP and bacterial counts.

Milk soil types 2 and 3 were more resistant to cleaning than was type 1; type 3 was the most tenacious. After a detergent wash cycle at 48.9°C and a shear force of 84 N/m^2, 84.6, 78.8, and 55.4% of the residues in milk soil types 1, 2, and 3, respectively, were removed. The results suggested that milk soils containing high numbers of bacteria are more tenacious and that soils developed during prolonged intervals between milking and cleaning may be harder to clean. More effective cleaning was achieved by applying a high shear level (114 N/m^2) and temperature (60°C).

The cleaning effectiveness of each cycle of a typical complete CIP system, namely, water rinse, detergent wash, acid rinse, and sanitizer rinse was evaluated. The water rinse removed about 45% of the total milk soil residue. A subsequent detergent wash removed an additional 27%, and an acid rinse decreased the amount of ATP by another 22%. After the final sanitizer rinse, the amount of ATP was reduced to <1% of the initial level. The milk soil residue was removed mainly by water rinse and detergent wash, indicating the importance of the mechanical and chemical effects. The sanitizer was important to reduce bacterial cell numbers further at the end of a complete cleaning cycle.

CONCLUSIONS

The model systems we studied indicate that the bacteria encountered in food processing environments can be very hardy and difficult to eliminate. Bacterial attachment and subsequent survival involve interactions between a bacterial cell, a surface, and the surrounding microenvironment. *Listeria monocytogenes* can survive for prolonged periods on SS and BN and, under favorable conditions, even multiply on SS. Temperature, RH, soil, and surface affected the behavior of surface-associated *L. monocytogenes*. In addition, the nature of the attachment surface affected the efficacy of sanitizers. Similarly, the nature of the soil affected cleaning of a milking system. *Lb. curvatus*, even at low levels on equipment surfaces, can potentially cause quality defects in the final cheese product. It is important to understand the interactions between bacteria and the surfaces in specific food processing and food service environments and the impacts of surface-associated bacteria on cleaning and sanitizing to provide more effective measures for prevention of biofilm formation and for biofilm removal.

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