The Journal of Undergraduate Research

Volume 18 Article 3

2023

Comparing Biofilm Forming Potential of *Listeria* Species on Non-food and Food-contact Surfaces of Importance to the Dairy Industry

Hannah Cooper
South Dakota State University

Comparing biofilm forming potential of *Listeria* species on non-food and food-contact surfaces of importance to the dairy industry

Author: Hannah Cooper

Advisor: Dr. Sanjeev Anand

Graduate Student Mentor: Bhaswati Chowdhury

Department: Department of Dairy and Food Science

Abstract

Listeria monocytogenes (*Lm*) is a foodborne pathogen of major safety concerns. This bacterium can resist/adapt to environmental stressors and persist through forming robust biofilms despite dairy plants following stringent surface cleaning protocols. Surface roughness is considered a major factor controlling bacterial attachment and biofilm formation. Food contact surfaces like stainless steel (SS) have low surface roughness for easy cleaning. On the other hand, non-contact surfaces like floors need a rougher surface to ensure employee safety. Hence, we hypothesized that *Listeria* biofilm formation would be lower on stainless steel compared to clay brick (CB; common flooring material in dairy plants). This study used *L. innocua* (*Li*; non-pathogenic) as the test strain instead of *Lm* because of its similar biofilm-forming capacity and isolation from similar environmental settings. Biofilms of *Li* were developed on SS and CB tiles (2.54x2.54 cm^2) using 7-log_{10}CFU/mL *Li* spiked reconstituted non-fat dry milk (11%w/v; R-NFDM) for 24h at 37°C. The R-NFDM was analyzed for fat, protein, lactose, total solid, solid-not-fat content, and pH. Simultaneously, as a control, biofilm on the tiles was formed using brain heart infusion broth. Biofilm counts were obtained through swabbing. Dilutions were plated on brain heart infusion

agar and incubated at 37°C for 24-48h, and counts were expressed as $\log_{10}$CFU/coupon. Three trials in duplicate were completed, with 6 tiles each. Means were compared using a one-way analysis of variance (ANOVA). The biofilm counts for SS were 5.33±0.12, and for CB tiles were 5.54±0.08 (P-value>0.05). The findings demonstrated that both surfaces equally supported biofilm development by *Li*. Therefore, establishing effective interventions to eliminate *Lm* biofilms and lower the risk of product cross-contamination is critical.

1. Introduction

Listeria monocytogenes (*Lm*) is a gram-positive, rod-shaped bacteria responsible for human listeriosis from consuming contaminated food (Colagiorgi, et. al., 2017). Although rare, listeriosis is severe with a higher mortality rate of 20-30% compared to other foodborne illnesses (FDA, 2020). People at high risk from this disease include pregnant women and newborns, adults 65 or older, and people with compromised immune systems (CDC, 2023). In the U.S., the Centers for Disease Control and Prevention (CDC) estimated that about 1,600 people develop listeriosis each year, and about 260 die (FDA, 2020). The hospitalization rate of reported cases is 94%, meaning that out of 1,600 infected people, about 1,500 are hospitalized each year (FDA, 2020). The CDC website also states that about 1,600 people develop listeriosis each year (CDC, 2023).

This pathogen can be found everywhere in the environment and has been identified in water, soil, dust, plants, animal feeds, feces, and sewage (ADEC, 2011). Since *Lm* is ubiquitous, these organisms can easily be introduced through different routes to food-processing environments and may become persistent (Møretrø & Langsrud, 2004). In dairy plants, *L. monocytogenes* can be introduced through dairy cows carrying the bacteria without clinical signs of the disease and can contaminate dairy products (ADEC, 2011). Researchers have also found that processing equipment like holding tanks, storage coolers, table tops, and conveyor systems are all vulnerable to contamination as well as other sites such as drains, floors, and storage areas (ADEC, 2011). Cross-contamination can occur from spreading *L. monocytogenes* from processing equipment and table tops to food products through the ventilation system, from dripping and splashing when cleaning with high-powered hoses (aerosol formation), and by workers themselves (ADEC, 2011).

L. monocytogenes has been observed to adhere to materials commonly used in the food industry by forming biofilms (Møretrø & Langsrud, 2004). Biofilms are a survival mechanism for microorganisms and can occur on any given surface if enough nutrients are present (Ossiform, 2022). The resistance to environmental stresses, like sanitizers, is directly related to *L. monocytogene's* ability to form biofilms (Mazaheri et al., 2021). *L. monocytogenes* biofilms were significantly more resistant to disinfection than its single cells. Thick, complex biofilms are more difficult to remove than adherent single cells (Møretrø & Langsrud, 2004).

Moreover, the roughness of a surface influences biofilm formation. Surface roughness increases the surface area available for bacterial attachment and provides a scaffold for adhesion (Zheng, Bawazir, & Dhall, 2021). Food contact surfaces like stainless steel (SS) have low surface roughness for easy cleaning. Stainless steel is made of chromium which provides durability and resistance properties, and SS is a nonporous material that will prevent the build-up of bacteria (Chemseal Inc, 2020). In contrast, non-contact surfaces like floors have a rougher surface to improve employee safety. Different floor surface finishes require different levels of surface coarseness for different types of environmental conditions to effectively manage slippery walking environments (Kim, 2018).

In this experiment, *Listeria innocua*, a non-pathogenic strain of *Listeria spp.*, was used instead of *L. monocytogenes* because of its similar biofilm-forming abilities, resistance to disinfectants, and ability to be isolated from environments similar to *L. monocytogenes* (Costa et al., 2018). The objective was to elucidate the difference in the biofilm-forming ability of *L. innocua* in 24-h on stainless steel, a food-contact surface, and clay brick, a common flooring material used in dairy industries (non-food contact surface). This research will test how fast *Listeria* can form biofilms on floors and stainless-steel surfaces.

5. Materials and Methods

5.1. Sourcing and propagation of L. innocua

The bacterial isolate used for this research was a previously isolated strain of *Listeria innocua* from a commercial dairy plant located in the United States. The isolate was stored in cryovials in glycerin solution at -80℃ until further use. For activating the culture, frozen stock culture with the cryovials was thawed and 100 µL of stock culture with two cryobeads was transferred into 10 mL of Brain Heart Infusion (BHI) broth (Oxoid, Thermo Scientific, UK) and inoculated for 24-h at 37℃. After 24-h, the activated culture was subcultured into 9 mL of fresh BHI broth to maintain the logarithmic growth of listerial cells.

5.2. Sourcing of stainless-steel and clay brick coupons, and preparation of reconstituted Non-fat dry milk (NFDM)

Stainless steel and clay brick coupons (**Figure 1**) of 2.54x2.54 cm^2 area were sourced from a commercial dairy facility. The stainless-steel coupons were selected to mimic food contact surfaces like bulk milk storage tanks, pipelines, and milk pasteurizers. The clay brick floor coupons were selected to represent the floor or drain areas in dairy processing facilities. Before initiating the biofilm study, the coupons were cleaned by submersion in liquid soap for 30 minutes, followed by scrubbing thoroughly. The clean coupons were then sterilized by autoclaving at 121℃ for 15-20 minutes.

Reconstituted NFDM was used because NFDM resembles milk. In a dairy plant, accumulated milk products are often left behind because of improper equipment design or inefficient cleaning, providing *L. monocytogenes* the opportunity to grow. Product spillage on the plant floor may also allow *Listeria* to grow; therefore, drains could have *Listeria* buildup. To prepare an 11% solution of NFDM, 110 g of NFDM was mixed with 1000 mL at 350℃. After

homogenization, NFDM solution was autoclaved at 121℃ for 15-20 minutes. The chemical composition of NFDM was determined by Fourier-Transform Infrared Spectrometer (FTIR; Dairy Spec FT, Bentley Instruments Incorporated®, Chaska, EUA). This was done by taking 50 mL of NFDM solution and placing the sample in the sampling port of the machine to determine the fat, protein, lactose, total solids, and solid not fat content. The pH was also taken for the NFDM solution using a pH probe (Fisher Science Education, United States).

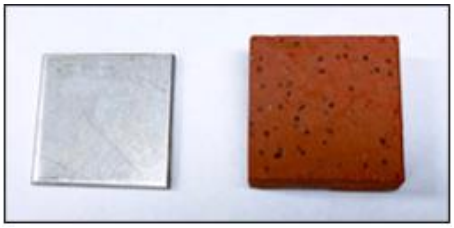

Figure 1 Image of stainless steel (left) and clay brick floor (right) coupon.

5.3. Gram staining, shape, and colony morphology of L. innocua

Gram staining of *L. innocua* was completed to observe the morphology. Preparation for gram staining included smearing a loop full of *L. innocua* culture onto a glass slide surface after dipping the inoculation loop in *L. innocua* sub-cultured BHI broth. After applying immersion oil, prepared slides were observed under a light microscope at 100X magnification. To observe colony morphology of *L. innocua,* plating techniques were used. For preparing serial dilutions, 1 mL of sub-cultured *L. innocua* was transferred into a test tube containing 9 mL of phosphate buffer saline (PBS) solution to make the 1^{st} dilution. The test tube was then vortexed, and 1 mL of solution was transferred to a second test tube with 9 mL of PBS (2^{nd} dilution). The same steps were repeated through the 7^{th} dilution. Once completing the dilution series, 100 µL from the 5^{th}, 6^{th}, and 7^{th} dilution tubes were plated onto BHI and Modified Oxford (MOX) agar using the spread plate technique. Plates were incubated for 24-h at 37℃. After incubation, colony

morphology for *L. innocua* was observed on BHI agar plates with the number of colonies ranging between 25-250.

5.4. Biofilm study on stainless steel and clay brick surfaces

The sterile coupons (stainless steel and clay brick) were kept side by side, without any overlaps, inside an instrument sterilizing tray (16.83cm. x 10.90cm. x 5.72cm.) with a cover. The coupons were submerged in 200 mL reconstituted NFDM and inoculated with 7 log10 CFU/mL *L. innocua* inoculum. Thereafter, incubation was carried out in a shaking incubator at 37°C for 24-h for the development of *L. innocua* biofilms. For control, coupons were simultaneously incubated using BHI broth only.

The swab technique was utilized to obtain viable *L. innocua* counts from coupons. Each coupon used one quick swab (#7000030171, 3M, LLC, St. Paul, MN) containing 1 mL of letheen broth. Biofilm swabbing was done in three directions: vertically (10 times), horizontally (10 times), and diagonally (10 times). After swabbing, the swab was returned to the container containing letheen broth. Thereafter, 10^{-1} dilution (first dilution) was prepared by transferring 1 mL letheen broth in the swab container to 9 mL PBS and vortexed well. Further dilutions were prepared using PBS as previously described. Thereafter the tubes were vortexed for 60 s and plated on BHI agar using the spread plate technique. These plates were incubated for 24 to 48-h at 37°C and the counts were reported as $\log_{10}$ colony-forming units per coupon squared ($\log_{10}$ CFU/coupon^2).

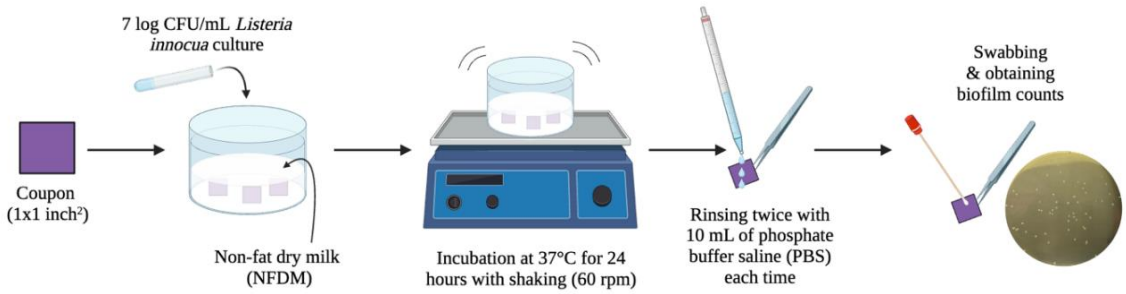

Figure 2 Experimental steps for the biofilm formation study on stainless steel and clay brick floor coupons

5.5 Statistical Analysis

Counts were collected in duplicates three times. Mean and standard deviation was calculated for each treatment. Means between treatments were compared using Microsoft Excel (Microsoft Corp., Redmond, WA.). Differences in all experiments were considered statistically significant at P-value < 0.05.

6. Results and Discussion

6.1. Fourier-Transform Infrared Spectroscopy of NFDM

Upon analyzing the NFDM using FTIR (DairySpec FT, Bentley, USA), the NFDM chemical composition can be found in **Table 1**, and it was compared to the chemical composition of bovine milk. The composition of bovine milk is 4.2% fat, 3.4% protein, 4.6% lactose, 13.0 % Total Solids (TS) 13.0%, 9% Solid Not Fat (SNF), and a pH 6.7-6.9 (Månsson, 2008). *Listeria monocytogenes* can survive and grow in a pH range of 4.0 – 9.6 (Food Standards Australia New Zealand, 2013). Since *Listeria* can grow in a large pH range, the neutral pH of bovine milk that is used in dairy plants is a convenient source for bacteria growth.

Table 1 Chemical composition of the Non-Fat Dry Milk (NFDM)

Fat (%)	Protein (%)	Lactose (%)	Total Solids (TS, %)	Solid Not Fat (SNF, %)	pH
0.01 ± 0.00	3.81± 0.00	5.31± 0.01	9.94± 0.02	9.92± 0.01	6.27± 0.00

6.2 Gram staining, shape, and colony morphology of L. innocua

The gram staining results (**Figure 3**) confirmed the presence of short-rod-shaped, Gram-positive bacteria. *Listeria innocua* has been confirmed to be Gram-positive (Liu et al., 2003). All the species within the genus *Listeria* are Gram-positive bacteria (Gasanov et al., 2005). Upon observing colony morphology of *L. innocua* on BHI agar, colonies of *L. innocua* are tiny, round, cream-color, opaque, and shiny, as reported previously (**Figure 4A**) (Jones & D'Orazio, 2013). On MOX agar, *L. innocua* appeared as tiny, round, darker colonies with black zones, also as previously reported (**Figure 4B**) (Remel, 2008). Both agars used to observe colony morphology produced different morphologies based on the properties of these agars. The BHI agar is a non-selective agar that is suitable for cultivation of a wide variety of organism types (Becton, Dickinson & Company, 2013). In contrast, MOX agar produced a different colony morphology because this agar is a selective media used for isolation and detection of *Listeria monocytogenes* (ThermoFisher Scientific, 2022). However, MOX agar cannot differentiate among *Listeria* species or strains. The differentiation of *Listeria* species is based on a limited number of biochemical markers (Allerberger, 2003).

Figure 3 Gram's staining of *L. innocua*

(a) (b)

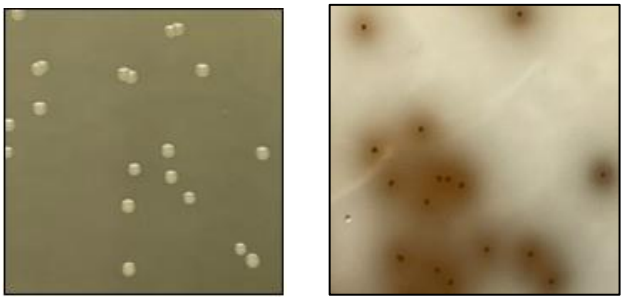

Figure 4 Colony morphology of *L. innocua* observed on Brain Heart Infusion (BHI) agar (a) and Modified Oxford (MOX) agar (b) plates.

6.2. Biofilm-formation by L. innocua on stainless-steel and clay brick surfaces

After incubation for 24-h at 37℃ in NFDM, both surfaces had comparable biofilm densities (P-value > 0.05), indicating that regardless of surface type, *Listeria* species can attach and develop biofilm on these industrially important surfaces (**Table 2**). Hence, despite using a more hygienic design for dairy processing equipment, the presence of *Listeria monocytogenes* in the dairy environment may cause biofilms to grow on equipment surfaces. Biofilms allow *Listeria* to be resistant to environmental stressors, like sanitizers, and these biofilms can lower sanitizer efficacy (Mazaheri et al., 2021). Inadequate cleaning of food-processing equipment constitutes a potential source of *Listeria* resulting in the contamination of foods that are exposed

to such equipment (Poimenidou et al., 2009). For example, in 2022 the CDC and FDA investigated a multistate outbreak of *Listeria monocytogenes* infections from cheese made by Old Europe Cheese, Inc. (CDC, 2022). Since *L. innocua* could form substantial amounts of biofilms (5.54 $Log_{10}CFU/coupon^2$) within an incubation of just 24-h on floor surfaces, *Listeria* species cells can easily be transferred to neighboring equipment. People, air, and cleaning systems can serve as vectors for the microorganism's transmission to food (Colagiorgi et al., 2017). Once a biofilm of *Listeria* matures to an irreversible stage, it becomes even harder to eliminate (Ossiform, 2022). These results emphasize the importance of developing effective interventions for control of this pathogen.

Table 3 Biofilm counts of *L. innocua* obtained on stainless steel and clay brick surfaces after 24h

Surface type	Biofilm counts ($Log_{10}CFU/coupon$)
Stainless-steel	5.33 ± 0.12^a
Clay brick	5.54 ± 0.08^a

*[a] Means within a column with the same superscripts were not statistically different (P-value > 0.05)

7. Conclusions

These findings demonstrate that *Listeria* species, specifically *L. innocua*, formed biofilms on food-contact (stainless steel) and non-food-contact (clay brick) surfaces. The dairy industry is continually attempting to improve equipment cleanability (through hygienic design using stainless steel) and lower *Listeria* amounts; however, *L. innocua* still formed biofilms on stainless steel in substantial numbers within a short contact time. Further, comparable biofilm formation by *L. innocua* on both stainless-steel and clay brick floors, despite the former having lower surface roughness points to the pathogen's ability to form resilient biofilms regardless of

surface characteristics. Lastly, future studies will focus on comparing *L. innocua* biofilm-formation on stainless steel and clay brick at 48-h and 72-h. This experiment will allow us to better understand the pathogen's biofilm-forming capacity and establish effective interventions to eliminate this pathogen from within dairy processing facilities.

Acknowledgments

The Midwest Dairy Association is acknowledged for providing the funding to conduct this study. The Dairy and Food Science Department and the AES, SDSU are also acknowledged for their infrastructural support.

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Author Biography

Hannah Cooper is a senior at South Dakota State University where she is majoring in Microbiology with minors in Biology and Chemistry. Her interest in microbiology began while working for a food testing lab in high school. She learned about what goes into food testing and how it impacts both the clients and consumers. Inspired by her job, she joined a research lab dealing with food safety. Hannah aspires to pursue a career in food microbiology and be a part of keeping consumers safe. She enjoys spending time with family or picking up a book when she is not busy working with bacteria.