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# Lessons in the Use of Probiotics to Inhibit *Listeria monocytogenes* Colonization on Wooden Cheese Aging Surfaces

## ABSTRACT

Boiling effectively decontaminates wooden cheese aging boards but also destroys biofilms on the surface that may inhibit pathogens. We assessed the efficacy of pre-inoculating wooden cheese aging boards with probiotic bacteria to prevent *Listeria monocytogenes* colonization and assessed how prior boiling cycles affected the efficacy of the treatment. On three separate occasions, a commercial lactic acid bacteria (LAB) cocktail was applied to 30/40 wooden boards in three different mediums. The LAB was made into yogurt (n=10), mixed into a commercial autoclaved yogurt (n=10), and mixed in phosphate buffered saline (PBS) (n=10) while the control boards (n=10) were only treated with PBS. All 40 boards were inoculated with 5 log CFU of *L. monocytogenes*. Half of the boards in each treatment group had never been boiled while the other half had previously been boiled three times. Boards were incubated for three weeks at 11°C. The control boards had significantly lower colonization ($P < 0.05$) of *L. monocytogenes* than the probiotic-treated boards; they, however, had the lowest nutrients present. Boiling history did not increase subsequent *L. monocytogenes* colonization. It is crucial to consider growth stage, nutrient presence, and pre-pathogen exposure colonization time to employ probiotics as an anti-*Listeria* control strategy.

## INTRODUCTION

*Listeria monocytogenes* is a foodborne bacterium that is the etiological agent for listeriosis, the leading cause of hospitalization from a foodborne illness in the U.S. with the third highest mortality rate *(27)*. Outbreaks of listeriosis have implicated a wide range of food vehicles, from mushrooms *(24)* to cantaloupes *(18)*, deli meats *(9)* and even unexpected foods such as caramel apples in which dry and acidic conditions should not have ideally favored the growth of *L. monocytogenes (8)*. However, nearly a third (30%) of the 58 outbreaks of listeriosis between 1998 and 2014 in the United States have been associated with soft cheeses, making them a critical target for interventions *(13)*. In aged hard cheeses, the combination of acid, salt and low moisture often means that *L. monocytogenes*, if present, dies during the aging period *(6)*. In contrast, the high moisture content and neutral

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pH of soft and soft ripened cheeses means that the pH only increases during aging due to mold growth or bloom *(6)*. The aging of these soft cheeses therefore has an added risk due to the difficulty of thoroughly cleaning aging boards.

Title 21 of the U.S. Code of Federal Regulations (CFR 110.40(a)), overseen by the FDA, details regulations about the design of food contact surfaces, equipment and utensils. According to this law, the main arguments against wood as a food contact surface are its ability to harbor organic material and adulterants, and more importantly, the difficulty in adequately cleaning and sanitizing wood. Following an *L. monocytogenes* outbreak that was traced back to a cheese facility in New York, the FDA raised these very arguments when they issued what was then interpreted as a ban on the use of wooden boards in cheese aging *(6)*. The resulting backlash from an attempt to enforce existing policy was reinforced by political pressure which ensured the law was never enforced *(6)*. Wood cheese aging boards are currently allowed by the FDA.

Those opposing this policy correctly stressed a lack of evidence that directly implicated wooden cheese aging boards in foodborne disease outbreaks and cited the centuries-old tradition of aging cheese on wooden boards *(1, 6)*. In fact, the rich pore structure and the many hydrophilic groups present in wood makes it the preferred surface for cheese aging *(1)*. Wood absorbs liquid from the cheese during aging which prevents excessive moisture and consequent fouling in the aging rooms and is thus critical for flavor development *(6)*. Although there is limited evidence that wooden food surfaces can transfer pathogenic microorganisms like *L. monocytogenes*, *Salmonella enterica*, *Escherichia coli*, and *Bacillus cereus* to food *(2, 12)*, there are studies that have found that wooden boards can retain or harbor such pathogens, especially when there are blemishes on the board's surface that provide niches for biofilms *(15)*. Wood may also retain residues from chemical disinfectants used to sanitize the wood between use, resulting in chemical residues in the cheese *(16, 29)*. Cheese aging boards are typically cleaned between use by boiling in hot water. However, this indiscriminately eliminates both useful and harmful microbiota and may also affect the material properties and composition of the wood *(16, 21, 23)*.

Several studies have investigated the use of probiotics, predominantly lactic acid bacteria (LAB), to control colonization of *L. monocytogenes* and other key pathogens in food matrices *(3, 25, 28)*. Probiotic bacteria used have included *Leuconostoc* spp., *Lactococcus* spp., *Lactobacillus rhamnosus*, *Pediococcus* spp., and *Streptococcus* spp. *(7, 14, 17, 19, 25, 26, 30)*. The authors of these studies determined that biofilms of probiotic bacteria formed on the surface of wood aging boards prevented the colonization of *S. enterica*, *L. monocytogenes*, *E. coli*, and *Staphylococcus aureus (12, 25)*. The primary mechanisms of action for lactic acid bacteria against *L. monocytogenes* are hypothesized to include competition for energy and nutrients *(14)* and the secretion of biocins, a group of peptides that inhibits growth or disrupts the quorum sensing of *L. monocytogenes (14, 19)*.

The effect of probiotics on *L. monocytogenes* have also been studied on other food contact surfaces including polystyrene, stainless steel, rubber and silicon *(10, 11)*. Wood, however, has been the focus of more investigations due to its ability to retain or transfer pathogens once contaminated *(1, 2, 12)*. Even more studies have been done on the microbiome of wooden cheese aging boards and storage shelves and their potential to confer protection against pathogens like *L. monocytogenes (1, 3, 17, 22, 28)*.

This study was designed in response to a local cheesemaker who sanitized their cheese aging boards by boiling and wanted to investigate the efficacy in replacing the destroyed protective LAB biofilms with a commercial probiotic which also comprised predominantly of LAB. We therefore investigated: (i) Whether the application of the probiotic through a food vs. non-food matrix influenced the biocidal or inhibitory properties of the probiotic against *L. monocytogenes* contamination, and (ii) how the repeated practice of boiling wood as a means of sanitization affected wood board colonization by *L. monocytogenes*. Our methods were therefore largely based on Standard Operating Procedures at the cheese maker's facility.

## MATERIALS AND METHODS

### Board Preparation

New wooden boards never previously used in cheese aging were cut into 5 inch × 5 inch × 1.5 inch squares and branded with sample IDs. Board pieces in Groups A–H were washed with hot soapy water in a board washer (JNJ, model mp2n82). The boards were then rinsed in hot water before being submerged in 88–130 ppm of peroxyacetic solution (IBA Inc, Super-San LpH, Sutton, MA) for 30 seconds. Finally, they were dried on a rack for a minimum of 48 hours. Board pieces in groups B, D, F, and H were boiled 3× in water at 165°C for 20 minutes and allowed to air dry for 24 hours between cycles in a room with dehumidifiers. All boards were then wrapped in aluminum foil and sterilized in an autoclave at 122°C for 15 minutes with 30 minutes drying time. The experimental design is illustrated in *Figure 1*.

### Preparation of Board Inoculum

*Groups A&B*: Raw milk was heated to 70°C for 10 minutes and subsequently cooled to 35°C before 0.05 grams of a probiotic powder, SuperSile Dry Hay Inoculant (Strong Microbials, Milwaukee, WI) containing *Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, and *Lactobacillus casei*, was added per liter of milk. Yogurt was made by holding the mixture at 37°C in an incubator until the consistency thickened after 48 hours. The yogurt was stored at 4°C until use. The yogurt was diluted in 250ml sterile PBS to $10^8$ CFU/ml of probiotic bacteria (determined

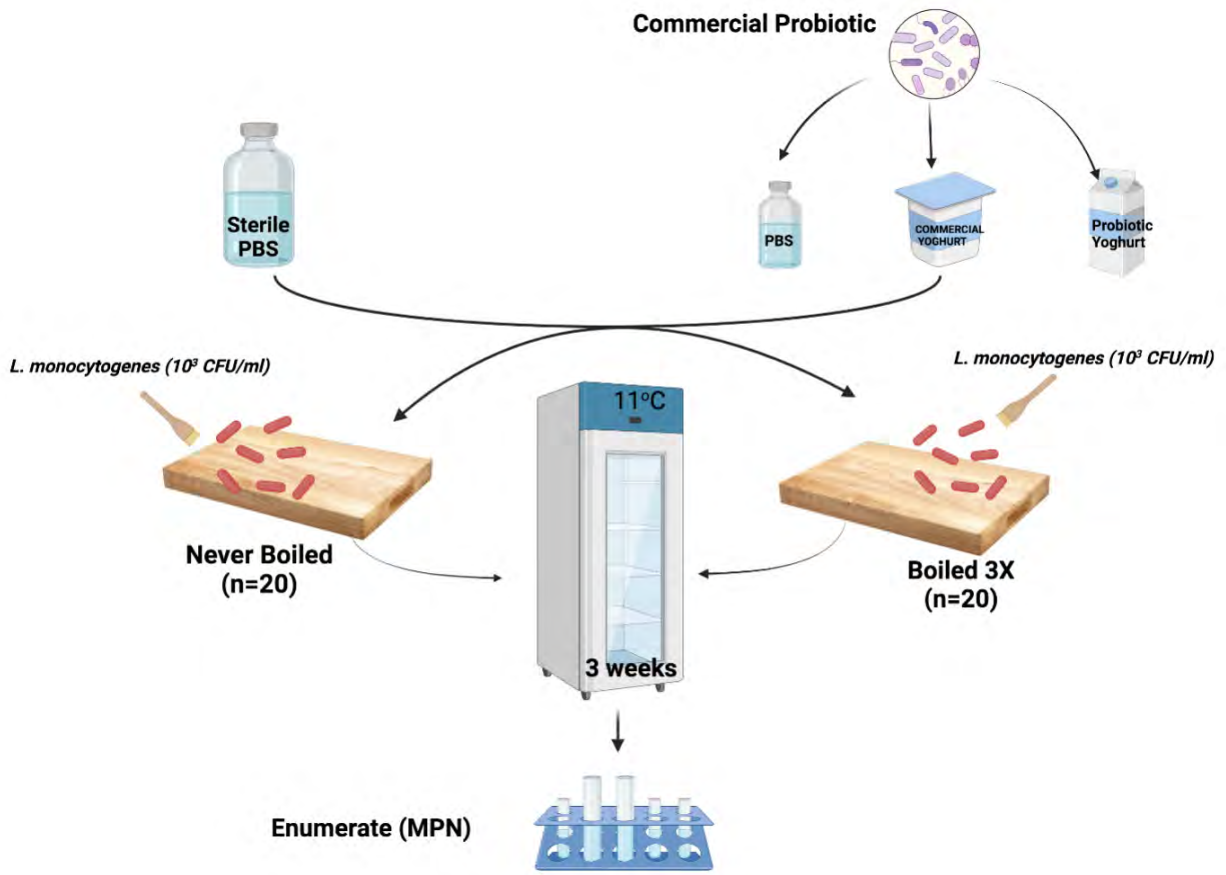


**Figure 1. Schematic representation of experimental design and microbiological testing methods. Created in BioRender. Aboagye, E. (2024) https://BioRender.com/m56z297.**

by serial dilution in PBS and plating on 3M Lactic Acid Bacteria Petrifilm)(Reference # 6461/6462). *Groups C&D*: the commercial probiotic was diluted in 250ml sterile PBS to achieve a probiotic cell concentration of $10^8$ CFU/ml. Calculations were based on manufacturer provided specifications of total CFU/gram in purchased packet. *Groups E&F*: the commercial probiotic was diluted in 250ml autoclaved commercial brand yogurt to achieve a probiotic cell concentration of $10^8$ CFU/ml; calculations were based on manufacturer provided specifications of total CFU/gram in purchased packet. *Groups G&H*: these were the control boards; they were soaked in 250ml of sterile PBS.

### *L. monocytogenes* Inoculum Preparation

Two strains of *L. monocytogenes* (Lm 008 and Lm 010 from Lineage II and Lineage I, respectively) previously isolated from raw milk cheese processing environments *(4, 5)* were streaked onto Brain Heart Infusion (BHI) agar (Becton, Dickinson, and Company, Franklin Lakes, NJ) from frozen stock and then sub-cultured in BHI Broth overnight at 37°C. The cultures were then further incubated for 12 hours at 11°C. The cells were subsequently spun down (16,700 xg for one minute) and resuspended in sterile PBS at $10^5$ CFU/ml.

### Board Treatment and Inoculation

Each treatment (250ml) was poured into sterilized stainless steel square pans. Boards were aseptically transferred from their foil wrapping and immersed in the appropriate inoculum for one minute. They were then handed to the second researcher, who applied one milliliter of *L. monocytogenes* culture on the branded side of each board and spread over the entire surface of the board with a sterile cotton applicator. The boards were then returned to their original wrapping and placed in a wine cooler at 11°C for 3 weeks. An open 100ml volume beaker of water maintained at the 60ml mark was placed inside the cooler to create a humid environment during the 3-week period.

### Enumeration of *L. monocytogenes* on Boards

The branded side of the boards were held down with C-clamps and gouged with a chisel that was sterilized by immersing in 70% ethyl alcohol and flaming between use.

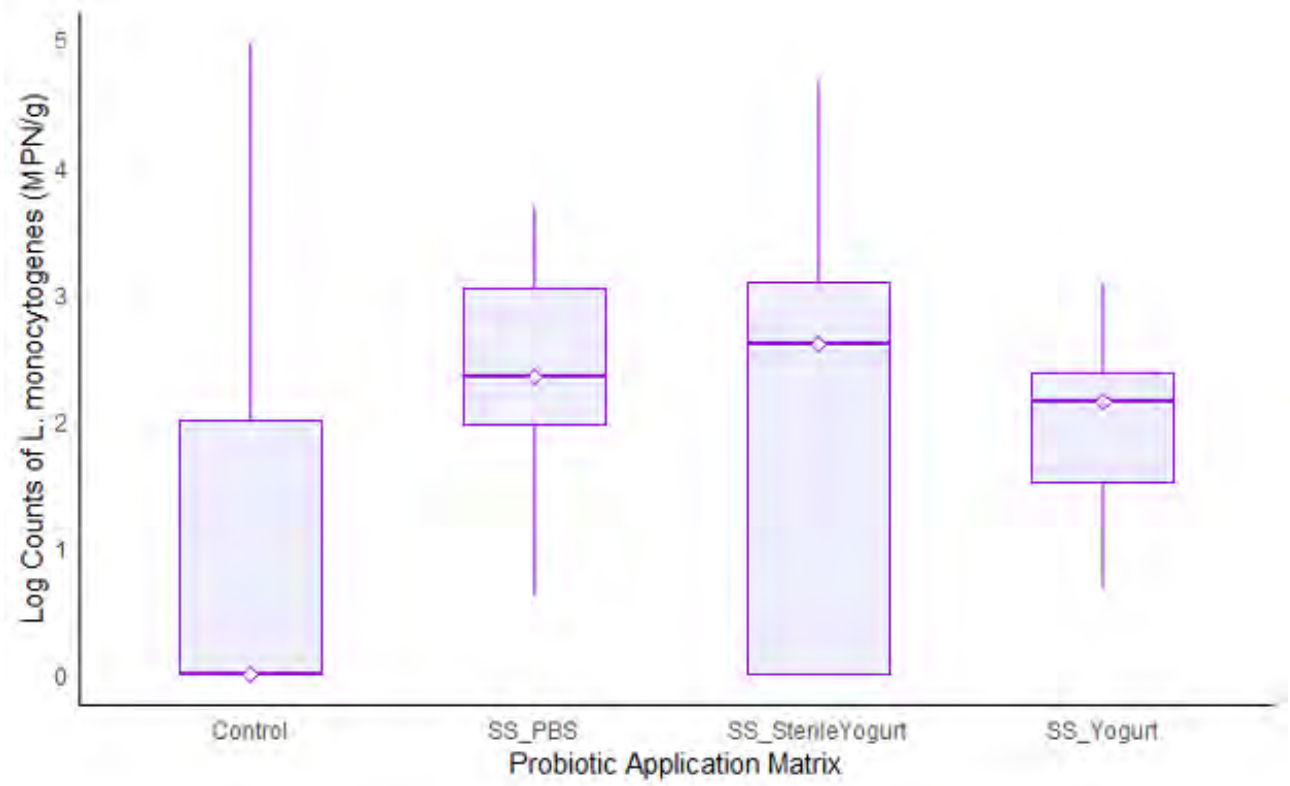


**Figure 2. Colonization of probiotic treated wooden cheese aging boards by *L. monocytogenes* after three weeks incubation at 11°C. Control was treated with sterile PBS prior to *L. monocytogenes* inoculation, while SS_PBS was treated with a commercial probiotic in PBS, the SS_SterileYogurt group was treated with a commercial probiotic in autoclaved commercial yogurt, and the SS_Yogurt group was treated with yogurt made from a commercial probiotic.**

Five grams total of wood chips (1–2 millimeters thick) were chiseled per board and submerged in 45ml of Buffered *Listeria* Enrichment Broth (BLEB; Becton, Dickinson, and Company, Franklin Lakes, NJ) and stomached for 1 minute at 260 rpm to dislodge all adhering or embedded bacteria. The homogenate was then incubated at 30°C for 4 hours.

After incubation, filter sterilized selective agents, concentrations of: 10mg/L acriflavine (Thermo Scientific, Waltham, MA), 50mg/L cycloheximide (Acros Organics Fisher Scientific, Pittsburgh, PA) and 40mg/L sodium nalidixic acid (Janssen Pharmaceuticals, Fairlawn, NJ) were added to each bag of the BLEB pre-enriched homogenates. One milliliter of the homogenate was then directly plated by spreading onto *Listeria* Chromogenic Plating Medium (RF Products, Downers Grove, IL) and incubated at 35°C for 48 hours, after which blue-green convex colonies were directly enumerated. The most probable number (MPN) of *L. monocytogenes* was also determined using the supplemented pre-enriched homogenate according to methods outlined in the FDA Bacterial Analytical Manual (2022). Positive tubes were confirmed by streaking onto *Listeria* Chromogenic Plating Medium.

**Data Analysis**

Colonization of boards was determined by MPN and confirmed by direct plating. One-way ANOVA was then used to compare colonization between treatments while a student's *t*-test was used to determine the effect of boiling. All analyses were carried out using RStudio 2024.04.2+764 "Chocolate Cosmos" Release for windows.

## RESULTS AND DISCUSSION

Overall, *L. monocytogenes* colonization of the wooden aging boards was comparatively weak. Three weeks after inoculation with 5 log CFU of *L. monocytogenes*, we detected a mean of 0.98 log MPN/g on the control boards, which was significantly lower than the 2.13 log MPN/g on the probiotic treated boards ($P$= 0.0006; *Figure 2*). There were no significant differences found among the different matrices used for probiotic application on boards *(Figure 2)*. Previously published studies had demonstrated inhibitory effects of LAB on *L. monocytogenes* colonization *(3, 12, 17, 19, 28, 30)*; however, the commercial probiotic we used contained a sucrose base, which would provide nutrients for any bacteria present, increasing their growth compared to the control. Additionally, we added the dried probiotic mixture

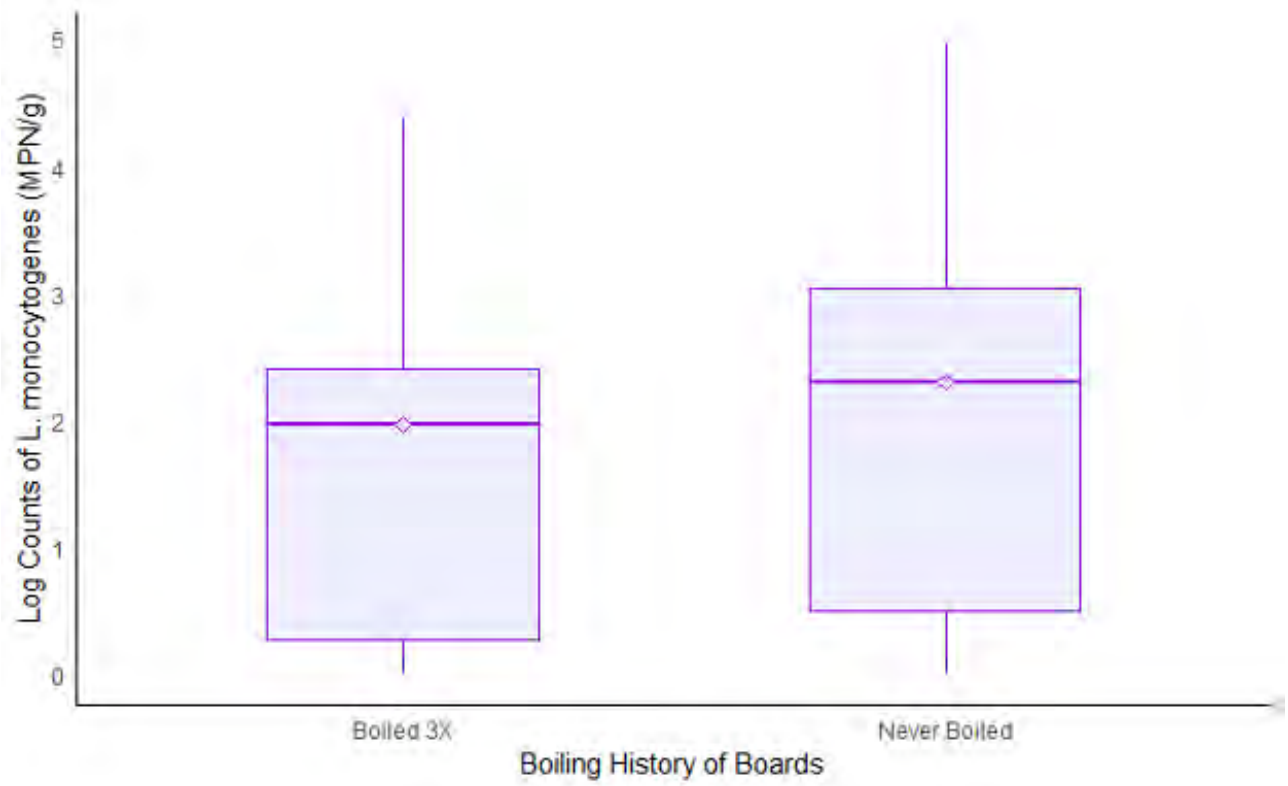


**Figure 3. Colonization of *L. monocytogenes* on previously boiled and never boiled wooden cheese aging boards after 3-week incubation at 11°C.**

to the PBS and autoclaved yogurt matrices immediately before application onto wood boards and the application of *L. monocytogenes*. There was, therefore, limited time for the dried LAB in the probiotic product to recover prior to *L. monocytogenes* inoculation. This does not fully explain why the non-autoclaved probiotic yogurt-based treatment (SSYogurt) did not inhibit *L. monocytogenes* colonization; however, the LAB had not yet colonized the wood boards and may have been outcompeted by the *L. monocytogenes*.

We had, however, hoped that the probiotic bacteria would be established as the dominant species by virtue of their higher inoculum numbers (the "Jameson Effect" for colonization *(20)*). Instead, the high initial concentrations of *L. monocytogenes* present on the boards, and the lack of a recovery period for the LAB may have allowed the *L. monocytogenes* to outcompete the probiotic population. Previous studies observed that the dominance of probiotic bacteria over pathogenic bacteria like *L. monocytogenes* allowed the probiotic bacteria time to form biofilms on the surface before introducing the pathogenic species *(3, 12, 17, 19, 28, 30)*. Our decision to apply the probiotic and the *L. monocytogenes* inoculum simultaneously was informed by our collaborator, who sought a time-saving solution to replace LAB biofilms that were lost to boiling. Ultimately, it is crucial to consider factors such as growth stage of the probiotic bacteria, nutrient presence, and colonization time for the probiotic prior to attempting to use probiotics as an anti-*Listeria* control strategy.

It has been suggested that exposure to high temperatures may alter the physical structure of the wood or increase the loss of volatile organic compounds that have antimicrobial properties *(16, 23)*. One study even reported reduced antimicrobial activity against *S. aureus* in oak wood after it had been sterilized by autoclaving *(21)*. In contrast, we found *(Figure 3)* that there was no significant difference between *L. monocytogenes* colonization on previously boiled boards compared to the un-boiled boards ($P$=0.064). Boards that have a history of repeated boiling cycles may therefore not carry any additional risk of *L. monocytogenes* colonization compared to newer boards and may be safely used in cheese aging if appropriately sanitized. However, it is important to note that the boards' surface was still visually intact and undamaged after three boiling cycles. Boiling and reuse of boards beyond three cycles may cause surface deterioration and increase the risk of *L. monocytogenes* colonization, which is beyond the scope of this study.

## CONCLUSION

While the use of probiotics as a preventive treatment against *L. monocytogenes* on wooden food contact surfaces shows promise, our study highlights the complexity of implementing such strategies effectively. Cheesemakers must

be aware that pre-application of LAB probiotics on boards immediately before using them for cheese aging may not confer equal protection compared to LAB biofilms developed over the duration of aging in the event of *L. monocytogenes* contamination. Future research should consider factors such as probiotic recovery time, nutrient availability, and LAB colonization periods when developing biocontrol measures for *L. monocytogenes* using probiotics. Additionally, while limited boiling cycles appear to be a safe sanitization practice for wooden boards, further investigation into the long-term effects of repeated boiling on wood structure and microbial colonization is needed.

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# FUTURE ANNUAL MEETINGS

**IAFP 2026**
**July 26–29**
New Orleans, Louisiana

**IAFP 2027**
**July 18–21**
Kansas City, Missouri

**IAFP 2028**
**July 23–26**
Charlotte, North Carolina