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Inhibition of *Listeria monocytogenes* by Broth Cultures of Surface Microbiota of Wooden Boards Used in Cheese Ripening

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Featured Application: Wooden boards are commonly used in the production of artisan cheese. A previous report described the complex bacterial communities present on wooden boards used in cheese ripening. Here, broth cultures of the bacterial communities isolated from these boards were found to inhibit the growth of *L. monocytogenes*. Pure cultures of *Leuconostoc mesenteroides* and *Staphylococcus equorum* isolated from the most inhibitory community also inhibited *L. monocytogenes*. These findings provide new insights into the potential interactions between the microbiota from cheese ripening and the growth of *L. monocytogenes*.

Abstract: *Listeria monocytogenes* is a significant concern in cheese production. It has been assumed by some that wood surfaces pose a greater risk for pathogen contamination during cheese production. The goal of this study was to evaluate the effect of the microbiota obtained from wooden boards used in cheese ripening on the growth of *Listeria monocytogenes*. Bacterial communities from the surface of wooden boards obtained from cheese-ripening facilities were inoculated into tryptic soy broth (TSB) and incubated at 11 °C for 48 h. These communities (10^8 CFU/mL stationary phase cells) were co-incubated with 10^4–10^5 CFU/mL *L. monocytogenes* 2203 at 11 °C for up to eight days. At various times, samples were removed, diluted in sterile saline and plated on modified Oxford agar. Bacterial communities from each of the five boards from three different facilities significantly inhibited growth of *L. monocytogenes* in vitro, compared to growth of *L. monocytogenes* 2203 alone. Using 16S rRNA analysis, we identified sequences belonging to the genera *Carnobacterium*, *Leuconostoc* and *Staphylococcus* as the most abundant in the communities grown in TSB. *Leuconostoc mesenteroides* and *Staphylococcus equorum* isolated from the most inhibitory community significantly inhibited growth of *L. monocytogenes* in TSB at 11 °C, compared to growth of *L. monocytogenes* 2203 alone. These findings suggest that some members of the complex microbial communities on wooden boards in cheese aging facilities might inhibit the growth of *L. monocytogenes*.

Keywords: wood; cheese; bacteria; inhibition; safety; *Listeria monocytogenes*

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1. Introduction

Wood is a natural and sustainable material that has been used in cheese production and ripening for centuries. In the ripening room, wooden boards help moderate hydric balance and serve as a reservoir for microflora important for cheese ripening. Through direct contact between the board and young cheeses, the board microflora contributes a diverse microbial ecosystem that eventually populates the cheeses' rinds. Over the course of aging, this microbial ecosystem is a key contributor to the distinct color, texture, flavor and aroma of the final cheese product [1]. Previous studies have evaluated the effects of

wood in the cheese-processing environment [2,3] and established the ability of wood to provide permeability to air and moisture, which enhance microbial growth.

Listeria monocytogenes contamination is a serious concern in cheese production and has led to costly recalls and rare, but unfortunate, disease outbreaks [4,5]. Wood, because of its porous nature, is presumed to be more difficult to clean than synthetic, smooth materials [1] and, hence, poses a greater risk for harboring pathogenic microflora such as *L. monocytogenes*. However, prior studies suggest that *L. monocytogenes* contamination from wood boards is less likely than commonly assumed. For example, Ak et al. [6,7] demonstrated that with reasonable cleaning efforts, wooden cutting boards are unlikely to create undue risk of pathogen cross-contamination. In another study, Zangerl et al. [8] found that heat treatment was effective in eliminating *L. monocytogenes* from the surface of wooden cheese-ripening boards.

Several previous studies have suggested that the resident indigenous microflora on food-contact surfaces can strongly influence the presence or growth of pathogens such as *L. monocytogenes*. Not surprisingly, the outcomes of these studies varied depending on the unique conditions of each experiment. Mariani et al. [9] analyzed the fate of multiple strains of *L. monocytogenes* in the presence of native microflora from wooden cheese-ripening shelves. The authors concluded that the native microflora present on these boards inhibited the growth of *L. monocytogenes*, an effect that was eliminated when the microflora was inactivated by heat treatment. However, these authors did not further characterize which organisms were responsible for the inhibition of *L. monocytogenes*. In a separate approach, Ref. [10] reported that some *Bacillus* isolates inhibited *L. monocytogenes* biofilm formation, whereas isolates of *Pseudomonas* and *Flavobacterium* allowed for the growth of *L. monocytogenes* on stainless steel surfaces.

In a recent study, our group characterized the indigenous bacterial communities present on wooden boards obtained from cheese-ripening facilities [11]. The purpose of the present study was to determine if broth cultures of these communities can inhibit the growth of *L. monocytogenes* under laboratory conditions and then further characterize these inhibitory communities using a combination of 16S rRNA amplicon sequencing and co-culturing methods.

2. Materials and Methods

2.1. Microflora Sampling of Wooden Cheese-Ripening Boards

Five wooden boards employed for the purpose of cheese aging were obtained from three different Wisconsin cheese-making facilities. We recognize that a wide spectrum of boards with variables such as plant location/geography, wood type, cheese type, board age, board treatment and so forth would have broadened the design and scope of this study. However, manufacturing plants that utilize such boards are relatively rare. Furthermore, plants that do utilize boards for cheese aging are often reluctant to provide these boards for numerous reasons, including the potential disruption to the plant ripening environment and other unintended costs and consequences. While a more comprehensive study design may serve broader goals within this realm of study, this current study design and findings provide unique discoveries relevant for broader application and inference. Boards A and B (cedar and pine, respectively) were sourced from one facility, and board C (spruce) was obtained from a second facility; both facilities produced surface-ripened cheese. Boards D and E (spruce) were obtained from a third facility and were used for cave- and smear-ripened cheeses, respectively. Surface samples were collected from a randomly selected 64 cm^2 area on each board by vigorously scraping the surface with a sterile cell scraper (Biologix Technologies Inc., Monona, WI, USA) and cotton-tipped applicators (Fisher Scientific, Hampton, NH, USA). Stock cultures of the surface sample microbial communities were prepared by inoculating them into 10 mL sterile tryptic soy broth (TSB) and incubating at 11 °C for 24 h (>10^7 CFU/mL). Aliquots (1 mL) of the resulting cultures were stored at −80 °C in TSB with 10% glycerol (*v/v*). For each experiment, an aliquot was thawed, and 1 mL was inoculated into 29 mL of TSB.

2.2. Establishing a Growth Curve for Listeria monocytogenes 2203

L. monocytogenes 2203, used as a challenge inoculum in this study, is a clinical isolate from a foodborne disease outbreak associated with Mexican-style cheese [4]. Cells were stored at −20 °C on Micro bank™ Cryobeads (pro-lab Diagnostics, Richmond Hill, ON, Canada). To establish a growth curve for *L. monocytogenes* 2203, a cryobead was placed into 9 mL of TSB using a sterile loop and incubated overnight at 37 °C with shaking (LabQuake® shaker rotisserie with clips; Model no. 400110; Barnstead/Thermolyne, Dubuque, IA, USA). The bacterial suspension was diluted to the desired concentration (10^4 CFU/mL) in TSB and confirmed by plating on blood agar (BAP) and modified Oxoid (MOX) agar plates. The bacterial suspension was added to a multi-well plate (0.5 mL per well) (Nunc™, cat no: 144530, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 11 °C for seven days in a refrigerated unit (Fisher Scientific, Hampton, NH, USA). Samples were removed every 24 h from each well, serially diluted in sterile phosphate buffered saline (PBS) and plated in duplicate on MOX agar. Plates were incubated at 37 °C for 48 h, the colonies were counted and the results expressed as $\log_{10}$ CFU/mL.

2.3. Growth Curves of the Cheese Board Surface Microflora

To establish growth curves for the microbial communities, 1 mL of stock culture (~10^7 CFU/mL) was thawed and first inoculated into 29 mL of sterile TSB. The resulting microbial suspensions were dispensed (0.5 mL per well) into multi-well plates (Nunc™, cat no: 144530, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 11 °C for 8 days. At 48 h intervals, determined samples were removed, serially diluted in sterile PBS and plated on Trypticase™ Soy Agar (TSA II) with 5% Sheep Blood (BAP; BD® Biosciences, Franklin Lakes, NJ, USA). The plates were incubated at 37 °C for 24 h (boards A, C and D) or at room temperature (RT) for 48 h (boards B and E). Colonies were counted and the results expressed as $\log_{10}$ CFU/mL.

2.4. Growth of Listeria monocytogenes 2203 in the Presence of the Microbial Communities

To assess the growth of *L. monocytogenes* 2203 when co-cultured with the microbial communities, the latter were incubated at 11 °C in a multi-well plate for two days to reach stationary phase (10^8–10^9 CFU/mL). *L. monocytogenes* 2203 (10^4–10^5 CFU/mL) was then added to each well and incubated at 11 °C for eight days. Samples were removed (0.1 mL) from each well every 48 h, serially diluted in sterile PBS and plated on MOX (to quantify *L. monocytogenes* 2203) and BAP (to quantify the microbial community). BAP plates (boards A, C and E) and MOX plates (all boards) were incubated at 37 °C for 48 h. BAP plates for boards B and E were incubated at 25 °C for 48 h. Colonies were counted and the results expressed as mean ± SEM $\log_{10}$ CFU/mL.

Representative bacterial colonies isolated on BAP plates from surface samples of board E were sent to the School of Veterinary Medicine diagnostic microbiology service for identification by a MALDI-TOF MS Biotyper (Bruker, Billerica, MA, USA), using the direct transfer (DT) technique according to the manufacturer's recommendations. A score > 1.7 is consistent with a species-level identification of Gram-positive species. One isolate from community E was identified by MALDI-TOF MS (Wisconsin Veterinary Diagnostic Laboratory) as a probable *Leuconostoc mesenteroides* (score of 1.860) and a second as *Staphylococcus equorum* (score of 1.840). These two bacterial species were separately incubated in BHI at 11 °C in a multi-well plate for two days to reach stationary phase (10^8–10^9 CFU/mL). *L. monocytogenes* 2203 (10^4–10^5 CFU/mL) was then added to each well and incubated at 11 °C for eight days. Samples were removed from each well every 48 h, serially diluted in sterile PBS and plated on MOX (to quantify *L. monocytogenes* 2203) and BAP (to quantify the competing bacteria). BAP plates and MOX plates were incubated at 37 °C for 48 h. BAP plates for boards B and E were incubated at 25 °C for 48 h. Colonies were counted and the results expressed as mean ± SEM $\log_{10}$ CFU/mL.

2.5. 16S rRNA Analysis of the Cheese Board Inhibitory Communities

Additionally, 16S rRNA analysis of broth of the cheese board communities was performed as described previously [11]. For each of the microbial communities, aliquots were inoculated into tubes of sterile TSB. *L. monocytogenes* 2203 (10^4 CFU/mL) were added to three of these tubes, while the remaining three tubes were left uninoculated to serve as controls. Samples were removed at 0, 4 and 8 days of co-incubation and centrifuged (4500× *g*) for 15 min at 4 °C (Eppendorf®, Centrifuge 5804R, and rotor: A-4-44, Hauppauge, NY, USA). The resulting cell pellets were washed twice with sterile PBS, suspended in sterile PBS and stored at −80 °C until total DNA extraction was performed [12–14]. Briefly, 1 mL of each suspension was transferred to a 2 mL screw-cap tube with 0.5 g of 0.1 mm zirconium beads (Thermo Fisher Scientific, Waltham, MA, USA). A total of 50 µL of 20% sodium dodecyl sulfate (SDS; Promega, Madison, WI, USA) and 700 µL cold equilibrated phenol (Thermo Fisher Scientific, Waltham, MA, USA) were added to each tube and subjected to bead beating for 2 min on a tabletop bead beater (Mini Bead Beater, Biospec Products, Bartlesville, OK, USA). The mixture was then heated in a 60 °C water bath for 10 min before a second 2 min round of bead beating. Tubes were then centrifuged for 10 min at 4 °C on a tabletop centrifuge (11,500× *g*, Microfuge 20R, Beckman Coulter, Brea, CA, USA). The aqueous layer was washed 2–4 times with 500 µL of cold equilibrated Phenol:Chloroform:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, Waltham, MA, USA) to remove the lipid layer and then transferred to a new tube. A total of 50 µL of 2M sodium acetate (Thermo Fisher Scientific, Waltham, MA, USA) and 300 µL of isopropanol (Thermo Fisher Scientific, Waltham, MA, USA) were added to the washed aqueous layer, and the DNA was allowed to precipitate overnight at −20 °C. The DNA was then pelleted in the same centrifuge (11,500× *g* for 20 min at 4 °C), washed twice with 70% ethanol (Decon Labs, Inc., King of Prussia, PA, USA) and then dried overnight in a fume hood. DNA Pellets were re-suspended in 20 µL of elution buffer (Thermo Fisher Scientific, Waltham, MA, USA), quantified using an individual Qubit® 2.0 Fluorometer (Invitrogen, San Diego, CA, USA) and stored at 4 °C. As a negative control, water was used instead of the DNA template for each DNA extraction and PCR reaction and was processed in a similar manner to the surface samples.

2.6. Amplification and Sequencing of Bacterial 16S rRNA

The hypervariable 4 (V4) region of the bacterial 16S rRNA coding region was PCR amplified using barcoded, 1-step sequencing primers for each sample [15]. PCR reactions were performed using varying concentrations (5 ng/µL–10 ng/µL) of total sample DNA, along with 0.5 µL of each primer (forward and reverse), 12.5 µL of 2X Hot Start Ready Mix (KAPA Biosystems, Wilmington, MA, USA) and nuclease-free water (Integrated DNA Technologies (IDT), Coralville, IA, USA) to a total volume of 25 µL. PCR reactions were conducted using the following cycling conditions: initial denaturation of 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min (Thermocycler; C1000 Touch Thermal Cycler, BioRad, Hercules, CA, USA). PCR products were then assessed by gel electrophoresis in a 1.0% low-melt agarose gel (National Diagnostics, Atlanta, GA, USA). Bright bands at ~380 bp were cut out of the gel, placed on a 96-well collection plate and purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Purified DNA was quantified using a Qubit® 2.0 fluorometer, equimolarly pooled and sequenced on an Illumina MiSeq using a v2 kit (2 × 250 bp) (Illumina, San Diego, CA, USA) with custom sequencing primers as described by Kozich et al. and Li et al. [13,15]. A total of 34 samples were sequenced, including 15 experimental (with *L. monocytogenes*), 15 controls (without *L. monocytogenes*) and 4 negative controls (for both DNA extraction and PCR amplification).

2.7. Sequence Processing and Analysis

Sequences were demultiplexed on the Illumina MiSeq system. Further sequence processing was performed using mothur v.1.46.1 [16], following a protocol adapted from

Kozich et al. [15]. Briefly, paired end sequences were assembled into continuous segments and poor quality sequences were removed. Sequences were aligned to the SILVA 16S rRNA gene reference database v138, and those not aligning to the v4 region were removed. Pre-clustering was performed (diffs = 2) to reduce error and computational load, and chimeric sequences were detected (UCHIME; [17], http://drive5.com/uchime, accessed on 1 March 2023) and removed. Sequences were taxonomically classified using the SILVA 16S rRNA gene reference database (v138) [18], with a bootstrap value cutoff of 80. Sequences classified as *Cyanobacteria*, mitochondria, Eukarya or Archaea were removed, and singleton sequences were also removed. The remaining bacterial sequences were grouped into operational taxonomic units (OTUs) at 97% sequence similarity using the opticlust method. Good's coverage was calculated for each sample, and those with coverage < 95% were removed. Sequences were normalized to 10,753 sequences per sample. Sequences for this study are available in the NCBI's Sequence Read Archive under BioProject accession PRJNA884361 [asteinberger9 (2020). Asteinberger9/seq_scripts v1.1. URL https://doi.org/10.5281/zenodo.4270481 (accessed on 1 March 2023)].

2.8. Statistical Analyses

Figures 1, 2 and 6 were created using Python v.3.8.5 [19], whereas Figures 3–6 were generated in R v3.53 [20], using RStudio v1.4.1106 [21] and packages including tidyverse, phyloseq v1.26.1 [22,23]), vegan [24], dplyr ([25]) and ggplot2 [23]. To calculate differences among experimental and control groups, days and boards, the Kruskal–Wallis test was used for alpha diversity and PERMANOVA for beta diversity. Pairwise Wilcoxon tests were performed for pairwise comparisons with FDR-correction applied to resultant *p*-values. The Kruskal–Wallis test was also used to calculate the difference between the growth of *L. monocytogenes* with and without the microbial communities (Figures 2 and 6).

3. Results

3.1. Growth Curves for the Wooden Board Microbial Communities and L. monocytogenes 2203

We first determined the growth curves for both the microbial communities recovered from the surface of the wooden ripening boards and for *L. monocytogenes* 2203 when each was individually inoculated into TSB and incubated at 11 °C for eight days. For all five communities, CFUs increased during the first two days of incubation and then remained stable through the later eight days of incubation (Figure 1A). The growth curve for *L. monocytogenes* 2203 demonstrated that maximal numbers (>10^9 CFU/mL) were achieved at four days and remained stable through seven days (Figure 1B).

3.2. Growth of L. monocytogenes 2203 in the Presence of Microbial Communities

The goal of our study was to evaluate the ability of *L. monocytogenes* 2203 to grow in the presence of the microbial communities obtained from our five cheese boards. To test this, we inoculated *L. monocytogenes* 2203 into broth cultures of each cheese board's surface microbiota, as shown in Figure 2. We found that growth of *L. monocytogenes* 2203 was significantly inhibited ($p < 0.05$) by each of the five microbial communities. The greatest inhibition was seen with surface microbial communities from boards A and E and the least with the communities from boards C and D.

3.3. Sequencing Summary

16S rRNA amplicon sequencing yielded 1,222,505 raw sequences, of which 915,465 quality-filtered sequences remained from the thirty samples and four negative controls. Negative controls were removed during normalization, as their sequence counts were less than the normalization cutoff. After normalization, board communities yielded an average of 10,751 ± 2.4 SD quality-filtered sequences per sample, resulting in 101 unique OTUs. Sequencing depths for all samples were satisfactory before and after normalization (Good's coverage: 99.8 ± 0.0002 SD %; range: 99.88–99.97%) [26], indicating sufficient capture of the species diversity in all samples.

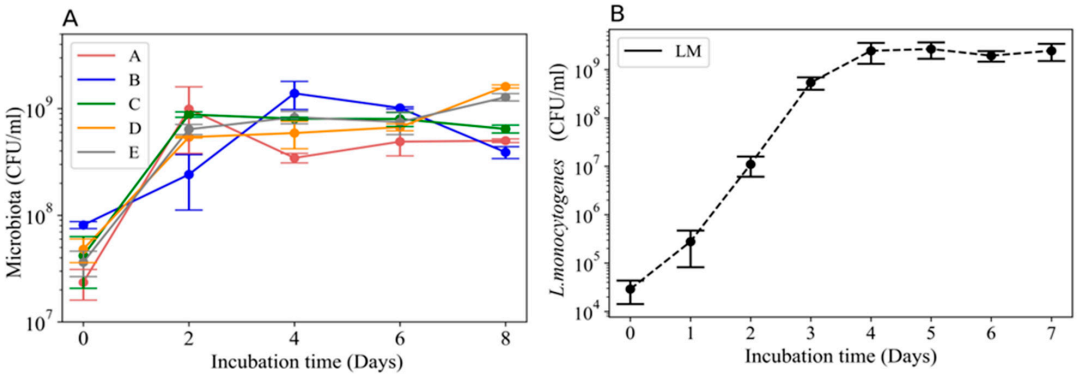

Figure 1. Growth curves for broth cultures of wooden cheese-ripening board microbial communities and *L. monocytogenes* 2203. (**A**) Microbial communities from five wooden cheese-ripening boards (A–E) were inoculated into TSB (initial inocula 7.2–7.8 Log_{10} CFU/mL) and incubated at 11°C. (**B**) Growth curve for *L. monocytogenes* 2203 inoculated into TSB (initial inocula 4.1–4.6 Log_{10} CFU/mL) and incubated at 11 °C. Error bars depict standard error of the mean (n = 4).

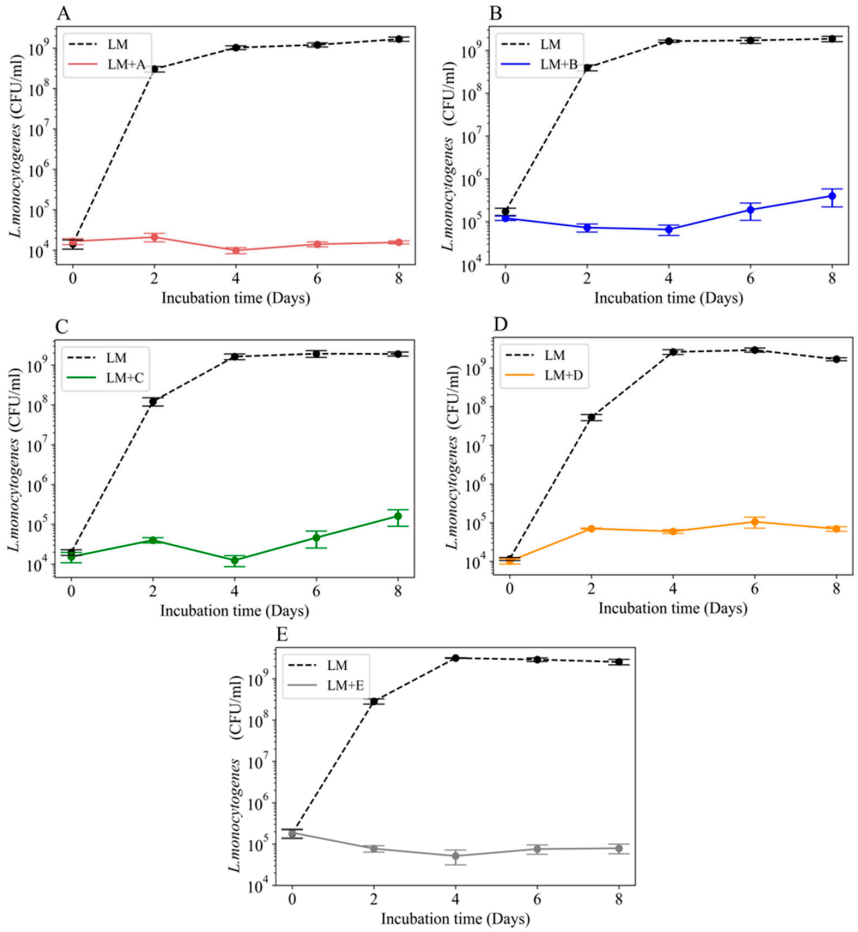

Figure 2. Inhibition of *L. monocytogenes* 2203 by broth cultures of the microbial communities from five different wooden boards (**A**–**E**). Board communities were incubated in TSB at 11 °C for 48 h until they reached stationary growth phase (10^8 CFU/mL), at which point 10^5 CFU/mL *L. monocytogenes* 2203 (LM) was added. The cultures were then incubated at 11 °C for 8 days. Samples were removed at 2-day intervals and plated on MOX agar to quantify LM 2203 (mean ± SEM CFU/mL). Dashed lines correspond to LM 2203 alone (positive control) and solid lines to LM 2203 in the presence of each microbial community (n = 6).

3.4. Diversity and Composition of Cultured Cheese Board Bacterial Communities

To evaluate and compare the bacterial community from each board, alpha (within sample; richness and evenness) and beta (between sample) diversity were first compared among boards. For alpha diversity, no differences were found between board communities for either Chao's richness or Shannon's diversity metrics ($p > 0.05$, Figure 3, Table A1). Beta diversity differed across boards for both the Bray–Curtis dissimilarity and the Jaccard index ($p < 0.05$, Figure 4, Table A1). Pairwise comparisons found cultured communities from boards A and B, A and D, A and E, B and C, B and D, B and E, C and E and D and E to be distinct for both metrics ($p < 0.05$) (Table A2), indicating differing community compositions. These results mirror findings from our previous study on the uncultured bacterial communities from the same wooden cheese boards [11].

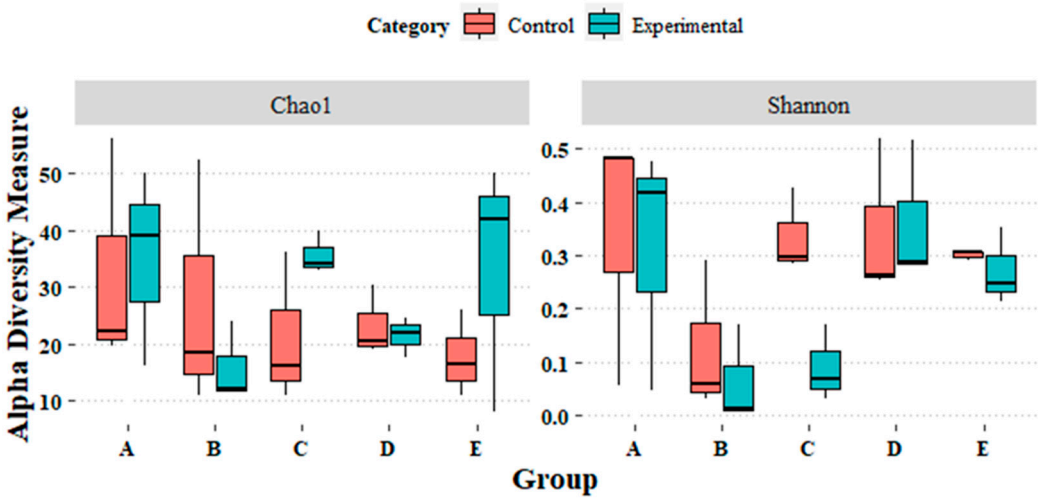

Figure 3. Box plots illustrating alpha diversity measures (Chao richness and Shannon diversity) of broth cultures of the bacterial communities from wooden cheese boards. Median values are indicated in the plots. Using Wilcoxon Rank Sum Tests (with FDR correction), it was determined that surface community alpha diversity did not significantly differ among boards (Group A–E) ($p > 0.05$), nor did they differ with exposure to *L. monocytogenes* (Category, $p > 0.05$), for either Chao's richness (Chao1) or Shannon's diversity (Shannon).

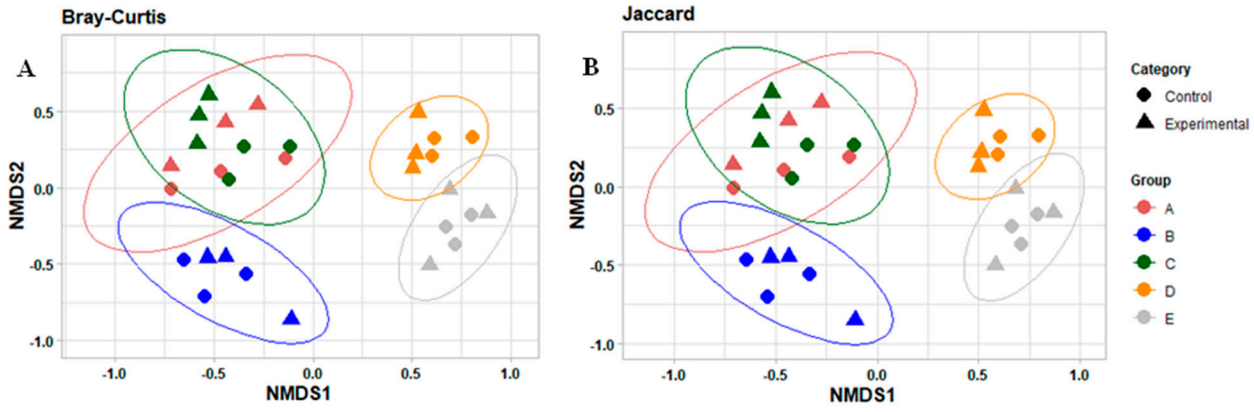

Figure 4. NMDS (nonmetric multidimensional scaling) plots depicting the β diversity of cheese-ripening boards. Beta diversity of each board's microbiota either with (experimental) or without (control) *L. monocytogenes*, assessed using (**A**) Bray–Curtis dissimilarity or (**B**) Jaccard similarity analysis. Ellipses represent standard error of all samples collected for each respective cheese-ripening board.

Next, cultured board bacterial community compositions were compared at the genus level. Relative abundances of the 10 most abundant genera were found to be similar for

boards A, B and C, which were distinct from boards D and E, which exhibited similar microbiota to each other (Figure 5). The microbiota of boards D and E were dominated by *Leuconostoc* (>85–95% relative sequence abundance in each) with lesser contributions from *Carnobacterium*, *Staphylococcus* and *Enterococcus*. The microbiota of boards A, B and C were dominated by *Carnobacterium* (>87–99% relative sequence abundance) with lesser contributions from *Brevibacterium*, *Enterococcus*, *Nocardiopsis*, *Psychrobacter*, *Prevotella* and *Staphylococcus*.

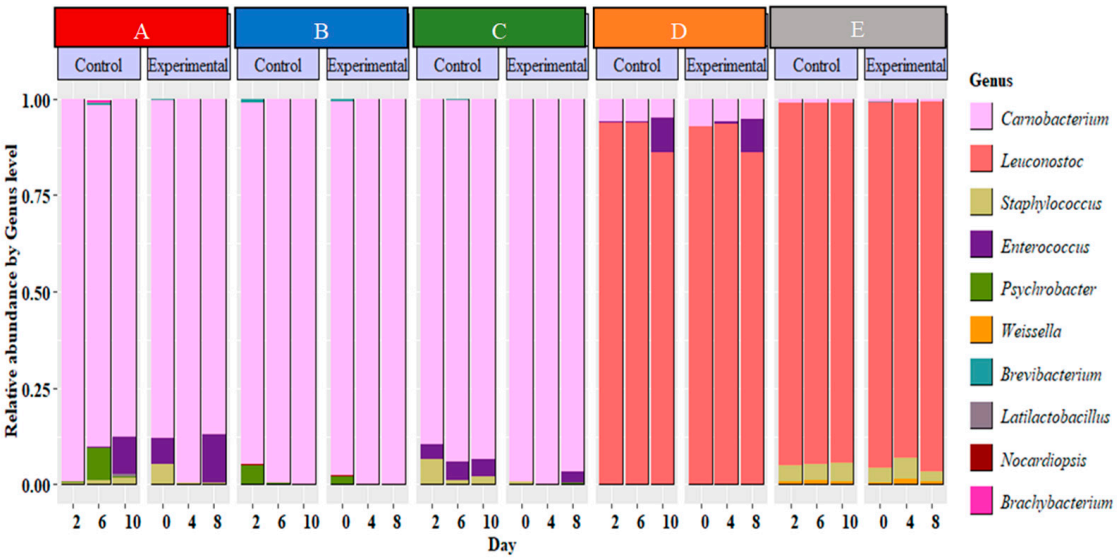

Figure 5. Relative abundance of the 10 most abundant genera in broth cultures from the wooden boards. Samples were assessed using 16S rRNA sequencing. The x-axis labels indicate number of days of sample collection for each board community (**A**–**E**) incubated with (experimental) or without (control) *L. monocytogenes*.

3.5. Impact of L. monocytogenes 2203 on Cheese Board Bacterial Communities

To evaluate the response of the cultured board communities to *L. monocytogenes*, bacterial community alpha and beta diversity was compared between experimental (with *L. monocytogenes*) and control (without *L. monocytogenes*) samples from the five board communities (A–E). The alpha diversity of the board communities was not found to significantly differ ($p > 0.05$) between the experimental and control samples for either Shannon's diversity or Chao's richness (Figure 3, Table A1). For beta diversity, the Bray–Curtis dissimilarity and Jaccard index metrics were also found to not differ ($p > 0.05$) between the experimental and control groups (Figure 4, Table A1).

When considering bacterial community composition, we found no substantial change in the abundances of the 10 most abundant OTUs in the experimental samples, compared to the controls using SIMPER (similarity percentages) (Figure 5) (asteinberger9, 2020). We note that OTUs classified in the genus *Listeria* were not recovered from any sample that was not inoculated with *L. monocytogenes* 2203, and plating of these samples on MOX agar plates yielded no colonies, further verifying the absence of *L. monocytogenes* in the uninoculated samples.

3.6. Inhibition of L. monocytogenes 2203 by Specific Cheese Board Microbes

To determine which individual members of these cheese board microbial communities might be inhibitory to *L. monocytogenes* 2203, we chose to focus on the community from board E (dominated by *Leuconostoc* and *Staphylococcus*), which was found to be the most inhibitory of the five board communities (Figures 2 and 5). One isolate from community E was identified by MALDI-TOF MS (School of Veterinary Medicine diagnostic microbiology service) as a probable *Leuconostoc mesenteroides* (score of 1.860) and a second as *Staphylococcus equorum* (score of 1.840). Broth cultures of these two bacterial species, along

with the total microbial community of board E as a positive control, were assessed for inhibition of *L. monocytogenes* 2203 growth. As shown in Figure 6, the isolate identified as a probable *Leuconostoc mesenteroides* completely inhibited the growth of *L. monocytogenes* 2203, similar to the total microbial community from board E. In contrast, the isolate identified as *S. equorum* was less inhibitory for the growth of *L. monocytogenes* 2203 (approximately 1 $\log_{10}$ fewer CFUs than control cultures). We acknowledge that there might be other inhibitory bacterial species present in the community from board E that we did not isolate.

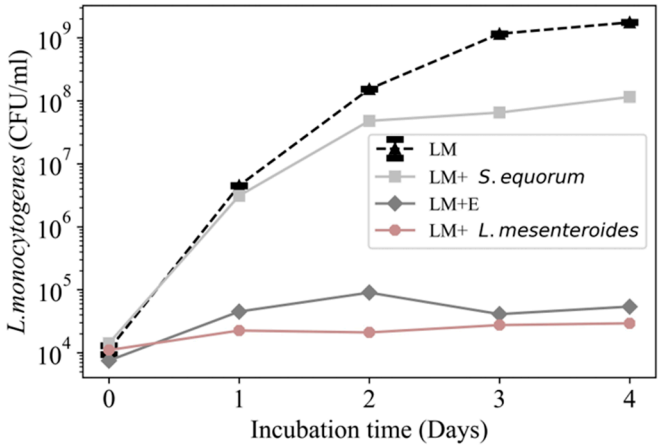

Figure 6. Inhibition of *L. monocytogenes* 2203 (LM) by *L. mesenteroides* and *S. equorum* isolated from an inhibitory microbial community (board E). LM corresponds to LM 2203 alone (negative control); LM+E corresponds to growth of LM 2203 in presence of microbial community of board E (positive control). LM+*S. equorum* and LM+*L. mesenteroides* illustrate growth of LM 2203 in the presence of these species that were isolated from the microbiota of board E. Symbols represent the mean ± SEM CFU/mL from a single experiment.

4. Discussion

In this study, we demonstrated that broth cultures of the surface microbiota of wooden boards obtained from three cheese-ripening processes are inhibitory towards *L. monocytogenes* 2203. In addition, we characterized the resident inhibitory microbiota communities and showed that specific members of these communities may participate in the observed inhibitory effects. These results confirm the "Jameson effect" proposed by Mellefont et al. [27], which posits that a well-established indigenous microbial community can inhibit the growth of *L. monocytogenes*, and further verify other findings [28], which reported the inhibitory effect of the surface microbiota of smear cheese wooden shelves on *L. monocytogenes*. We attempted to recreate a condition similar to that used in cheese ripening by performing our experiments in a low-temperature environment. This likely compounds the challenges that *L. monocytogenes* must overcome when colonizing and establishing on wooden cheese boards. As such, we expected that any persistence of *L. monocytogenes* in the presence of these inhibitory microbiota would likely be at low numbers, relative to other members of the microbial community. This is supported by our 16S rRNA analysis of the cheese board communities, where samples that were not inoculated with *L. monocytogenes* 2203 did not contain any OTUs classified as *Listeria*.

Our analyses of five cheese board microbiotas inoculated with *L. monocytogenes* 2203 revealed communities dominated by a handful of genera. Two distinct microbiotas were observed, which were dominated by either *Carnobacterium* or *Leuconostoc*. Additionally, our diversity analyses indicated that control and experimental sets of all cultured communities were not significantly different from one another (Figures 3–5). Several studies have reported that bacteriocins produced by various species of *Carnobacterium* and *Leuconostoc* are capable of inhibiting *L. monocytogenes*. A previous report [29] demonstrated that *Carnobacterium* can persist at refrigeration temperatures and produce Carnobacterial class IIa bacteriocins that inhibit the growth of various *Listeria* spp. These authors suggested that

Carnobacterium could be used as a protective culture against *L. monocytogenes*. Surprisingly, we did not isolate colonies of *Carnobacterium* spp. from our TSB cultures, despite our 16S rRNA analysis indicating that it was a dominant member of the community. This suggests that either it is not a dominant member of our cultures (16S rRNA analyses cannot differentiate live from dead bacterial cells) or our culture conditions were incapable of isolating *Carnobacterium*.

Leuconostoc is also known to produce bacteriocins with inhibitory effects on *L. monocytogenes*. For example, ref. [30] showed the antimicrobial effects of a *Leuconostoc mesenteroides* isolate from goat's milk and demonstrated that bacteriocins produced by this isolate had a narrow inhibitory spectrum limited to *Listeria* spp. Our finding that a *Leuconostoc mesenteroides* isolate from board E was capable of strongly inhibiting *L. monocytogenes* 2203 (Figure 6) may explain, in part, the inhibitory activity of the microbiota from board E on *L. monocytogenes* 2203.

Overall, our findings are consistent with studies by other investigators on the use of wood in the production of artisan cheese. An analysis of wooden boards used to produce traditional Sicilian cheeses showed they contained a complex microbiota on their surfaces [31,32], and pathogens such as *L. monocytogenes* were not detected. Although the use of wood has been called into question for putative hygienic reasons, there have not been foodborne outbreaks associated with its use [33]. Nor does wood appear to be effective at transferring pathogens to cheese. Fresh blocks of wood inoculated with *L. monocytogenes* poorly transferred the organism to cheese [34], and *L. monocytogenes* can be eliminated from wood when subjected to heat and chemical sanitization [8,35].

We acknowledge that our study did not directly examine the inhibitory activity of the microbiota on the intact boards. Nor does it provide insights into the inhibitory mechanisms that these communities and isolates employ to limit the growth of *L. monocytogenes* 2203. Future studies should investigate these mechanisms. We also chose to use a single cheese-associated outbreak isolate of *L. monocytogenes* for this initial study. Subsequent studies should examine the generalizability of our findings to other relevant isolates of *L. monocytogenes*.

Despite the limitations of our study, these data provide evidence that the complex microbial communities present on the surface of wooden boards used for cheese ripening might be inhibitory for *L. monocytogenes*, while also contributing to the desired sensory character of aged cheese. Although these results are intriguing, it is important to note they do not negate the potential risk from undesirable microbes that might be present on a poorly maintained wooden surface.

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Appendix A

Table A1. Statistical analysis of alpha (Shannon and Chao) and beta (Bray–Curtis and Jaccard) diversity. Kruskal–Wallis and permutational analysis of variance (PERMANOVA) tests of different sample sets were used. * < 0.05.

Diversity	Analysis	Sample Sets	*p*-Value
Shannon	Kruskal	Group	0.06
		Category	0.13
		Days	0.5
Chao	Kruskal	Group	0.6
		Category	0.4
		Days	0.2
Bray-Curtis	Betadisper	Group, days, category	>0.05
	PERMANOVA	Group	<0.05 *
		Category	0.32
		Days	<0.05 *
Jaccard	Betadisper	Group, days, category	>0.05
	PERMANOVA	Group	<0.05 *
		Category	0.29
		Days	<0.05 *

Table A2. Pairwise comparison of groups (A-E; control and experimental). FDR correction method for both metrics (Bray–Curtis and Jaccard) was used. * $p < 0.05$.

Group	Analysis: Corrected Method	Adjusted *p*-Value (Bray-Curtis)	Adjusted *p*-Value (Jaccard)
A vs. B	Pairwise Adonis: fdr	0.023	0.023
A vs. C		0.239	0.238
A vs. D		0.003 *	0.003 *
A vs. E		0.003 *	0.003 *
B vs. C		0.022	0.021
B vs. D		0.003 *	0.003 *
B vs. E		0.003 *	0.003 *
C vs. D		0.003 *	0.003 *
C vs. E		0.003 *	0.003 *
D vs. E		0.003 *	0.003 *

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