Identification and confirmation of slit defect-causing bacteria in Cheddar cheese

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Abstract

Background: Spoilage microbes remain a significant economic burden for the dairy industry. Validated approaches are needed to identify microbes present in low numbers in those foods and starting ingredients prior to spoilage. Therefore, we applied a combination of propidium monoazide treatment combined with 16S rRNA gene amplicon DNA sequencing for viable cell detection, qPCR for bacterial enumeration, and laboratory culture, isolate identification, and pilot-scale cheese production to identify the causative bacterial agents of slit defects in industrially-produced Cheddar cheese. Because spoilage cannot be predicted in advance, the bacterial composition in milk was measured immediately before and after High Temperature Short Time (HTST) pasteurization over time and on multiple days and in resulting cheese blocks.

Results: Milk was sampled over 10 h periods on ten days immediately before and after the final HTST pasteurization step prior to the initiation of cheese fermentations. HTST reduced the alpha-diversity of the viable, but not total, bacterial contents in milk and increased the proportions of thermoduric and endospore-forming bacterial taxa. There was a significant increase in viable bacterial cell numbers over the 10-h run times of the pasteurizer, including 68-fold higher numbers of Thermus. Between 0.22% to 10.9% of the bacteria in cheese were non-starter contaminants comprised mainly of Lactobacillus and Streptococcus, however, only Lactobacillus proportions increased during cheese aging. Lactobacillus, and Lactobacillus fermentum in particular, was also enriched in slit-containing cheeses and in the pre-HTST and post-HTST milk used to make them. Although some endospore-forming bacteria were associated with slits and could be isolated from milk and cheese, none were consistently associated with slit development. Pilot-scale cheeses developed slits when inoculated with L. fermentum, other heterofermentative lactic acid bacteria isolates, or with uncultured bacterial consortia collected from the pre-HTST or post-HTST milk, thus confirming that low abundance taxa in milk can negatively affect cheese quality.

Conclusions: We identified and verified that certain low-abundance, bacterial taxa in milk are responsible for causing slit defects in Cheddar cheese. The likelihood for microorganisms in milk to cause defects could be predicted based on comparisons of the bacteria present in the pre- and post-HTST milk used for cheesemaking.

Background

Unpasteurized milk contains a large diversity of bacteria originating from animal, human, and environmental sources [1–3]. The abundance and proportions of those microbes change depending on how milk is handled and from contact with storage, transport, and processing equipment [4–6]. Ultimately, the bacteria in milk, including those that survive pasteurization, impact the quality and safety of the resulting dairy products [7–9].

Cheese is a fermented dairy food that is vulnerable to variations in milk microbiota composition. Although certain bacteria are necessary for cheese manufacture, cheese is also highly susceptible to unwanted bacterial growth and enzymatic activity resulting in defects (e.g. discoloration, bitterness, open texture) even when those microbes are not highly abundant [8–10]. In Cheddar cheese, slits are one of the most common problems and are defined by open texture cracks. It is estimated that slit defects cause 180 million dollars in losses each year in the United States alone [11, 12]. Slit development is currently unpredictable and occurs even when high-quality, low-microbial count milk is used. The presence of cracks can lead to consumer rejection and additional labor, equipment maintenance, and packaging costs when the cheese is sliced or shredded.

Prior studies examining the causes of Cheddar cheese slit defects have shown that slits are caused by unwanted bacterial growth and are hypothesized to be the result of excessive gas production by several Clostridium species and heterofermentative lactic acid bacteria (LAB) [13–16]. However, those studies relied on culture-based, enrichment methods, which are now known to be insufficient for identifying the total bacterial contents of milk and other foods [17–20]. As a result, those methods also do not provide comprehensive avenues to locate the spoilage microbes.

Culture-independent approaches, such as 16S rRNA gene amplicon DNA sequencing and metagenomics methods, can enable more high-throughput and comprehensive assessments of complex microbial communities. These methods have shown that the bacterial contents of bovine milk vary significantly depending on the season and regional climate when the milk is produced as well as during pasteurization and ultrafiltration processing steps [4, 17, 21]. However, nucleic-acid based approaches tend to rely on correlative analysis to infer associations between microbes, and subsequent hypothesis testing with taxa present in complex microbial communities is frequently not possible. Therefore, combining laboratory culture isolation and DNA sequencing methods are complementary measures to study milk and cheese microbiota.

We applied culture-dependent and culture-independent methods to investigate the bacterial contents in Cheddar cheese and the raw and pasteurized milk used to make it in order to identify and verify the capacity of individual isolates and milk-associated bacterial consortia to cause slits. Because slit development is currently unpredictable, we repeatedly sampled milk and cheese at a cheese manufacturer by using extensive day-to-day sampling over a three-month period and for cheese blocks during 120 days of aging. Assessments included both the use of propidium monoazide (PMA) prior to 16S rRNA gene sequencing in order to detect viable cells [17, 22] and the isolation and identification of thermoduric bacteria and LAB from the milk and cheese. Pilot-scale Cheddar cheese fermentations were used to test the hypothesis that individual bacterial isolates and complete bacterial consortia from milk, predicted to cause slit defects based on 16S rRNA gene sequencing, were indeed necessary and sufficient to cause slits.

Methods

Milk and cheese sample collection for microbiota assessments. Milk and cheese samples were collected on ten days distributed over four months in 2015. Milk was obtained from raw milk storage silos, the inlet to the continuous-flow pasteurizer at the final High Temperature Short Time (HTST) step (pre-HTST), the outlet from that pasteurizer (post-HTST), and from cheese blocks made with the post-HTST milk (Fig. 1). Four storage silos were randomly sampled on 7/7/2015, 7/15/2015, 7/20/2015, 7/29/2015, 8/18/2015, 10/6/2015 and 10/15/2015 at 12:00 (noon), on 8/25/2015 at 14:00, and on 9/29/2015 and 10/19/2015 at 11:00. On those same dates, seven sets of pre-HTST and post-HTST milk samples were collected after cleaning-in-place (CIP) protocols were completed for the HTST pasteurizer. The sets were collected at approximately 0 h (collected at 8:00 and 20:00), 3 h (collected at 11:00 and 23:00), 6 h (collected at 14:00 and 2:00), and 9 h (17:00) after pasteurizer CIP on all dates except for 7/29/2015. On 7/29/2015, milk was collected at only 8:00, 11:00, 14:00 and 17:00. Because the length of time needed for milk to pass through the pasteurizer was known, the pre- and post-HTST samples were paired such that the milk entering the pasteurizer was collected as it exited after HTST treatment. Raw milk from the storage silos was not paired with the pre- and post-HTST samples. Cheese blocks included in the analysis were made on those same dates between 15:00 and 16:00, corresponding to pre- and post-HTST milk collected at 11:00. The cheese was sampled immediately after it was made (0 days) and after aging at 4 °C for 30, 90 and 120 days. Cheese produced on 7/15/15, 8/18/15, 9/29/15, and 10/6/15 developed slits within 90 days of aging.

All samples were shipped to the Marco lab (UC Davis) overnight on ice and were immediately processed upon arrival. We previously determined that transport in that manner did not significantly affect bacterial composition [4, 17]. Milk samples were processed as previously described [4]. For cheese samples, 30 g segments were cut randomly from 500 g blocks using a sterile knife. The segments were then placed in Whirl-Pak filter bags (Nasco, GA, US) with 270 mL 2% w/v $Na_3C_6H_5O_7$ solution and homogenized using a stomacher (Weber Scientific, NJ, US) at high speed for 10 min. Bacterial cells were separated from 25 mL milk or the cheese-$Na_3C_6H_5O_7$ homogenate by centrifuging at 13,000 g for 5 min at 4 °C. The cell pellets were washed with Phosphate Buffered Saline (PBS) pH 7.4 [4]. The cell pellets were then either directly frozen at -20 °C or were first incubated with 50 μM propidium monoazide (PMA. Biotium, CA, US) as previously described [17].

Genomic DNA extraction, PCR amplification, and DNA sequencing. Genomic DNA was extracted from the frozen cell pellets using the MagMAX Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, MA, US) according to the manufacturer's protocol with the repeat bead beating method at 6.5 m/s for 2·1 min with 1 min interval on ice. PCR was performed as previously described with ExTaq polymerase (TaKaRa, Otsu, Japan) and the F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACHVGGGTWTCTAAT) primers targeting the 16S rRNA gene V4 region with 8-bp random barcoded sequences on the 5' end of the forward primer [17]. For each DNA extraction kit used (six boxes in total), sham DNA extractions (negative controls) were performed in triplicate for the subsequent PCR amplification and DNA sequencing steps. Equal quantities of PCR products were pooled and then gel purified with the Wizard SV Gel and PCR Clean-Up System (Promega, WI, US).

Pooled and purified 16S rRNA gene V4 products were sequenced with the Ion Torrent PGM sequencer as previously described in five separate runs [23]. Milk and cheese samples from different collection locations, dates, and time points were randomly selected for each run to minimize batch effects. Additionally, in each run, a PCR amplicon mock community [23] was included in triplicate in order to examine for batch effects.

16S rRNA gene sequence analysis. Ion Torrent output BAM files were converted to FASTQ files using BEDTools [24]. Reads shorter than 200 bases were discarded. Barcode sequences were extracted by QIIME 1.9.1 [25] extract_barcodes.py script with no barcode error allowed and were subsequently analyzed in QIIME 2 version 2018.4 [26]. Specifically, sequence files were demultiplexed using the demux plugin with emp-single option. Feature table construction and chimera removal were performed for each run using the DADA2 method [27] and the denoise-single option with default settings except that the first 29 bases and low-quality bases after position 260 were truncated. Batch effects between the five sequencing runs were determined to be not significant according to the gPCA R package (data not shown) [28]. Therefore, feature tables and sequences were merged together with the feature-table merge and feature-table merge-seqs plugins, followed by de novo read alignment with MAFFT [29]. Unconserved and gapped alignments were filtered by the alignment mask plugin with default parameters. A phylogenetic tree was created with FastTree [30] using the filtered alignment method. For taxonomy assignment, a custom classifier was trained based on the truncated sequence reads (231 bases) against the Greengenes database version 13.8 [31].

The merged feature table, feature sequences, rooted phylogenetic tree, and sample information were imported to phyloseq 1.22.3 [32] in R 3.4.2 [33] and visualized with ggplot2 [34] and Superheat [35] packages. Archaea (0.04% of total sequence reads), mitochondria (0.0003% of total sequence reads), and chloroplast (0.4% of total sequence reads) ASVs as well as ASVs that were unidentified at the phylum level (0.65% of total sequence reads) were removed. ASVs comprising less than 0.005% of reads from all samples were also removed. For alpha and beta diversity analysis, DNA sequences were rarefied to a depth of 4,000 reads per sample. Significant differences in alpha diversity and sample clustering were determined as previously described [23].

Bacterial enumeration and identification with qPCR. Bacterial cell numbers in pre- and post-HTST milk were estimated by qPCR using the UniF (GTGSTGCAYGGYYGTCGTCA) and UniR (ACGTCRTCCMCNCCTTCCTC) primers and compared to a Lactobacillus casei BL23 DNA standard curve as previously described [4]. Turicibacter spp. numbers were estimated by qPCR targeting the genus-specific 16S rRNA gene region using TuriciF (CAGACGGGGACAACGATTGGA) and TuriciR (TACGCATCGTCGCCTTGGTA) primers [36] and a standard curve as previously described [17]. Lactobacillus helveticus and Lactobacillus fermentum numbers were determined by qPCR with species-specific primers (LbhelvF1: AGGTTCAAAGCATCCAATCAATATT, LbhelvR1: TCGGGACCTTGCACTACTTTATAAC, LfermF: GCACCTGATTGATTTTGGTCG, and LfermR: GTCCATTGTGGAAGATTCCC) [37, 38] and standard curves constructed using DNA isolated from known quantities of L. helveticus NRRL B-4526 and L. fermentum NRRL B-1840 cells grown in MRS (Becton,

Dickinson and Company, NJ, US). L. helveticus NRRL B-4526 and L. fermentum NRRL B-1840 genomic DNA was extracted following the same protocol used for milk and cheese DNA extractions. Reactions were performed with the SsoFast Evagreen Supermix with Low ROX (Bio-Rad Laboratories, CA, US), 400 nM primer mixtures, and 2 μL template DNA. qPCR was performed in duplicate and those with a stdev > 0.15 were repeated to confirm accuracy.

Chemical analysis of Cheddar cheese. Cheddar cheese was sampled after aging at 4 °C for 5 days and 90 days. pH and percentages (w/w) of salt, moisture, and fat were determined using previously established methods [39]. Concentrations (w/w) of galactose, lactose, L-lactate, D-lactate, and citrate were quantified using enzymatic methods with the Gallery Plus discrete photometric system following the manufacturer's protocol (Thermo Scientific, CA, USA).

Bacterial isolation and identification. Milk and cheese sampled on several dates in 2017 (4/26/2017, 7/6/2017, 10/25/2017) and 2018 (2/15/2018, 3/22/2018, and 5/23/2018) were used for bacterial isolation. Cheeses from 7/6/2017, 10/25/2017, 2/15/2018 and 5/23/2018 developed slits after aging at 4 °C for 90 days, whereas those from 4/26/2017 and 3/22/2018 did not. The cheeses were sampled after 5 and 90 days of aging. Samples were collected by randomly cutting the cheese with a sterile knife and collecting 30 g for homogenization in 270 mL 2% w/v $Na_3C_6H_5O_7$ as described above. To select for LAB, serial dilutions of the milk were plated onto MRS agar and incubated for 48 h at 30 °C under anaerobic conditions in a GasPak jar (Becton, Dickinson and Company, NJ, US). To select for endospore-forming and other thermoduric bacteria, milk and the cheese-$Na_3C_6H_5O_7$ homogenate samples were exposed to 80 °C for 20 min before plating serial dilutions on Brain Heart Infusion (BHI) agar (Thermo Scientific, MA, US) and incubating for 48 h at 30 °C. These conditions were set because they were sufficient to inactivate the high numbers of L. lactis cells (10^9 cells/mL) found in Cheddar cheese (data not shown). Bacterial colonies with distinct morphologies were streaked for isolation twice on MRS or BHI agar before preservation at -80 °C.

To identify the bacterial isolates, single colonies were transferred to sterile water and lysed in a microwave at the highest setting for 3 min. PCR amplification was performed with ExTaq DNA polymerase and primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) [40]. Amplicons were purified on a Wizard SV Gel and PCR Clean-Up System to prepare for DNA sequencing at Genewiz (https://www.genewiz.com). Species identity was determined using BLASTn [41] based on 100% nucleotide alignment to the NCBI nr/nt database and confirmed using the Ribosomal Database Project (RDP) database [42].

For testing individual isolates in cheese fermentations, rifampicin-resistant mutants of L. fermentum, Leuconostoc mesenteroides, and Leuconostoc lactis were obtained as previously described [43]. These mutants and Lactobacillus plantarum NCIMB8826-R [44] were routinely grown at 30 °C in MRS containing 50 ∝g/mL of Rifampicin (Thermo Fisher Scientific, MA, US).

CO_2 production. Gas production was detected using Durham tubes [45]. LAB were incubated in MRS broth, and thermoduric and endospore-forming bacterial isolates were incubated in both MRS and BHI broth for 48 h at 30 °C before visual inspection for gas bubbles in the Durham tube.

Heat tolerance. LAB strains were suspended in ultra high temperature (UHT) pasteurized milk (Gossner Foods, Inc., UT, CA) in levels ranging from $1.3 \cdot 10^5$ to $8.9 \cdot 10^5$ cells/mL. The cell suspensions were then exposed to 72 °C for either 15 sec, 1 min, or 5 min. Thermal tolerance was determined by plating serial dilutions of the cell suspensions onto MRS agar for incubation at 30 °C for 48 h prior to colony enumeration.

Pilot-scale Cheddar cheese. Fresh cheese curds made using Lactococcus lactis (10^9 CFU/g) and rennet were taken directly from the production line. One batch of cheese curds was used to make pilot-scale cheeses inoculated with milk consortia and the bacterial strains inoculated at a level of 10^9 CFU/g. Another batch of curds produced in the same facility was used to make pilot-scale cheeses inoculated with individual strains at a level of 10^7 CFU/g. For testing individual strains, L. plantarum NCIMB8826-R [44] and rifampicin-resistant mutants of three isolates of L. fermentum, Ln. mesenteroides, and L. lactis (Table 1) were grown overnight in MRS at 30 °C. Cells were collected by centrifugation at 13,000 g for 5 min at 4 °C, washed twice with PBS, and suspended in 5 mL sterile physiologic saline (0.85% NaCl). Bacterial numbers were quantified by microscopy and verified by colony enumeration. The suspension was mixed into 260 g cheese curds to reach a final cell number of either 10^7 or 10^9 CFU/g. Cheese curds inoculated with 5 mL saline were used as controls.

Table 1
Strains and cell consortia used for pilot-scale, Cheddar cheese production.

	Origin	Collection date	Collection time
L. fermentum 3494-1LAB-R	Post-HTST	7/6/17	21:36
L. fermentum 3500-3LAB-R	Post-HTST	7/7/17	04:20
L. fermentum 3854-3LAB-R	Post-HTST	5/23/18	20:20
Ln.mesenteroides 3490-3LAB-R	Pre-HTST	7/6/17	12:10
Ln.mesenteroides 3502-1LAB-R	Pre-HTST	7/6/17	21:36
Ln.mesenteroides 3504-3LAB-R	Pre-HTST	7/7/17	04:20
Ln.lactis 3498-2LAB-R	Pre-HTST	7/6/17	21:36
Ln.lactis 3850-1LAB-R	Pre-HTST	5/23/18	13:00
Ln. lactis 3860-1LAB-R	Pre-HTST	5/24/18	07:00
Milk_No slits	Post-HTST	7/7/15	11:00
Milk_No slits	Post-HTST	7/20/15	11:00
Milk_No slits	Post-HTST	8/25/15	11:00
Milk_Slits	Post-HTST	8/18/15	11:00
Milk_Slits	Post-HTST	9/29/15	11:00
Milk_Slits	Post-HTST	10/6/15	11:00
Milk_Slits	Post-HTST	7/15/15	11:00

For testing cryopreserved bacterial cell pellets from post-HTST milk, four cell pellets from milk associated with cheese slits and three cells pellets associated with cheese that did not contain slits (Table 1) were suspended in 1 mL sterile physiologic saline (0.85% NaCl) and mixed with 260 g cheese curds. Cheese curds inoculated with 1 mL saline were used as controls.

After the inoculum was added, the cheese curds were mixed, transferred to cheese molds lined with two layers of cheesecloth, and pressed for 15 min using a vertical cheese press to remove extra whey and form the cheese wheel. Cheese wheels were then vacuum packed and aged at 30 °C to expedite ripening. Cheeses inoculated with bacterial cells at a level of 10^9 CFU/g were incubated for 5 d, and cheeses inoculated with 10^7 CFU/g or cryopreserved milk cell were incubated for 11 d.

Bacterial enumeration and slits quantification of pilot scale cheese. After aging, pilot cheeses were cut randomly with a sterile knife to collect 3 g of cheese for homogenization in 27 mL 2% w/v $Na_3C_6H_5O_7$ as described above. Serial dilutions of the cheese homogenates were prepared in PBS prior to plating onto

Tryptic Soy Agar (Becton, Dickinson and Company, NJ, US), MRS agar, and MRS agar containing 50 µg/mL rifampicin, for detection of the culturable bacteria, LAB, and rifampicin-resistant LAB strains, respectively. Colony enumeration was performed after incubating the plates at 30 °C for 48 h. For image quantification, three images of the randomly cut cheese cross-sections were analyzed by ImageJ [46]. Slit area was calculated as a percentage of the cheese cross-sectional slice as follows

$$: \text{slit area}\% = \frac{\text{pixels of slits}}{\text{pixels of cheese cross section}} \times 100\%.$$

Statistics For the 16S rRNA gene DNA sequencing results, differential taxa abundance between sample groups was determined between unrarefied samples using DESeq2 version 1.18.1 [47] and was considered significant when the p value after Benjamini-Hochberg correction was less than 0.1 and fold change was more than 1.5. For taxa with geometric means equal to zero, the geometric mean was replaced by pseudo counts (1) before the DESeq() function.

To determine significance differences between bacterial alpha diversity, qPCR estimated cell numbers, and slit area, Mann-Whitney test ($p < 0.05$) was used for pairwise comparison and Kruskal-Wallis with Dunn test ($p < 0.05$) was used for multiple comparisons. Odds ratio analysis was performed using the R package questionr [48].Power analysis was performed using the R package pwr [49] with 0.05 as the significance level for a single-tailed comparison (“greater”) and varying effect sizes, sample sizes, and power values.

Results

HTST pasteurization alters the proportions of viable bacteria in milk. The bacterial composition in milk contained in storage silos and transported into (pre-HTST) and out (post-HTST) of the HTST pasteurizer immediately prior to Cheddar cheese manufacture was measured on ten separate days distributed over four months (Fig. 1). Besides determining the composition of the total bacterial contents of the milk, a fraction of each milk sample was used to identify viable cells with PMA, a DNA-intercalating dye that hinders PCR amplification from bacteria with a damaged cell membrane [50]. As expected, HTST altered the milk microbiota composition (Fig. 2 and Fig. S1). Even though milk collected pre-HTST had undergone several blending and filtration steps known to cause significant changes in bacterial composition [17], the diversity of viable bacteria in milk pre-HTST was more similar to the raw, unprocessed milk in storage silos than in milk collected moments later post-HTST (Fig. 2A-C). Milk collected from storage silos and pre-HTST was enriched in Staphylococcus, Pseudomonas, Lactococcus, Enterobacteriaceae, and Bacillaceae (Fig. 3). Milk collected after pasteurization contained higher proportions of viable Clostridiales, Streptococcus, Thermus, and Turicibacter (Fig. 3). Notably, the differences in the silo, pre-HTST, and post-HTST milk microbiota were limited to the viable (PMA-treated) cell fractions. There were only slight changes in bacterial composition and no significant differences in

alpha-diversity when all bacteria (no PMA-treatment) from the three collection points were compared (Additional file 1).

Thermus cell numbers increase in milk post-HTST over pasteurizer run time. We next assessed the composition of the milk microbiota pre-HTST and post-HTST in 10-h time courses starting immediately after clean-in-place (CIP) procedures for the pasteurizer. On each of the ten production days tested, the numbers of viable bacteria in milk post-HTST increased from an average of $4.6 \cdot 10^3$ cells/mL after pasteurizer cleaning to $1.3 \cdot 10^4$ cells/mL ($p < 0.05$) 6 h later (Fig. 4A). The increase in cell numbers post-HTST was not due to higher numbers of bacteria in milk entering the pasteurizer (pre-HTST), because those quantities did not change over time (average of $3.2 \cdot 10^4$ cells/mL) (Fig. 4A). Instead, the higher cell numbers post-HTST coincided with elevated amounts of members of the Thermus genus (Fig. 4B). This taxon was present in very low quantities in milk entering the pasteurizer (average 12 cells/mL). After pasteurization, Thermus was present at a level of approximately 20 cells/mL at the start of the production period (t = 0 h). This number increased to an average of 850 and 1500 cells/mL when milk was sampled 6 and 9 h later on all production dates. Conversely, Clostridiaceae and other members of Clostridiales were present in similar amounts in milk pre-HTST and post-HTST at an average of 250 cells/mL (Clostridiaceae) and 720 cells/mL (Clostridiales) at all time points (Fig. 4C and Fig. 4D). Additionally, fewer viable Bacillus, Brevibacillus, and Enterobacteriaceae were present in milk post-HTST compared to pre-HTST and their numbers continued to decline during pasteurizer run times each of the production days (Fig. 4E-H).

Non-starter Lactobacillus cell numbers increase over time during Cheddar cheese aging. The microbial contents of Cheddar cheese made from milk that was pasteurized 3 h after HTST pasteurizer cleaning was monitored for 120 days (Fig. 5A). As expected, L. lactis, the organism used as the cheese starter culture, was very abundant and comprised, on average, 96% of the viable bacteria present in the aged cheese according to DNA sequence analysis. The non-starter, viable bacterial contaminants in the cheese constituted the remaining 0.22–10.09% of all DNA sequence reads. The non-starter bacteria were predominantly Lactobacillus (44.3% ± 31.1%), Streptococcus (29.2% ± 15.8%) and Staphylococcus (10.4% ± 9.8%) (Fig. 5B). The levels of Streptococcus declined overtime, whereas Staphylococcus populations did not change. Only the proportions of Lactobacillus increased over the 120 days of aging (Fig. 5B).

Lactobacillus and Turicibacter are associated with Cheddar cheese slit defects. Within 90 days of aging, four out of the ten cheese blocks developed slits. The other six blocks did not develop slits. The cheeses were similar according to the general quality characteristics of pH (average pH of 5.12), fat content (33.2%), moisture (38.1%), lactose (0.19%), and L-lactate (1.01%) (Additional file 2). An exception to this finding was that cheeses which developed slits contained modest but significant reductions in salt content after 5 days (1.81% ± 0.09% in normal cheese vs. 1.74% ± 0.09% in cheese with slits, p value = 0.015) and 90 days of aging (1.79% ± 0.07% in normal cheese vs. 1.70% ± 0.09% in cheese with slits, p value = 0.017) (Additional file 2).

Cheeses that developed slits contained an altered non-starter microbiota. Comparisons of the non-starter bacteria in the cheese blocks during aging showed that blocks with slits contained higher proportions of viable Lactobacillus and Turicibacter after 30 days of aging (Fig. 5C). Lactobacillus and Turicibacter were also enriched at either 90 or 120 days of aging, respectively (Fig. 5C). The trend towards higher numbers of Turicibacter spp. in cheese with slits was confirmed by qPCR (Additional file 3A). The same was found for L. fermentum, the species identified as the dominant Lactobacillus ASV in the cheese (Additional file 3B).

Lactobacillus in milk pre-HTST and post-HTST correspond with Cheddar cheese slit defects. The pre-HTST and post-HTST milk used to make the cheese blocks that developed slits contained a different microbial composition compared to milk that resulted in good quality cheese (Fig. 6A and B). These differences accounted for most of the variation observed between milk samples (pre-HTST milk, Adonis R^2 = 0.29, p-value = 0.018; post-HTST milk, Adonis R^2 = 0.21, p-value = 0.046). The Shannon index of the viable bacteria was significantly higher in post-HTST milk associated with slits in cheese (Fig. 6C), and those milk samples tended to have lower cell numbers (p = 0.087) (Fig. 6D). Lactobacillus, Brevibacillus, and Bacillus were significantly enriched in pre- and post-HTST milk associated with cheese slits (Fig. 6E). Clostridium was also similarly enriched but only in milk samples collected post-HTST (Fig. 6E).

Lactobacillus was the only member of the milk microbiota that was consistently enriched in both slit-containing cheese (Fig. 5) and the pre- and post-HTST milk used to make it (Fig. 6E). Odds ratio analysis also supported this result (Additional file 4). At the species level, L. fermentum was the most abundant Lactobacillus ASV in milk both pre- and post-HTST, and this species was enriched in milk resulting in slit-containing cheese blocks (Additional file 3C). Power analysis showed that based on an 0.92 effect size of L. fermentum populations (estimated by qPCR) in slit-associated, post-HTST milk, 100 milk samples would be needed to use L. fermentum numbers to predict slit formation in cheese at an accuracy of 95% (Additional file 5).

Diverse LAB and thermoduric bacteria are present in pasteurized milk and cheese. Milk collected pre-HTST and post-HTST on several dates in 2017 and 2018 and the resulting corresponding Cheddar cheese blocks were used to isolate culturable bacteria. As found in 2015, several of the cheese blocks tested developed slits (Additional file 6). Because of the association of LAB and endospore-forming and other thermoduric bacteria with slits, those groups of bacteria were the focus of culture-based enrichments. A total of 69 LAB were isolated including L. plantarum (16 isolates), L. fermentum (11 isolates). Leuconostoc lactis (6 isolates), and Ln. mesenteroides (6 isolates) (Additional file 6). Bacteria able to survive at 80 °C for 20 min encompassed 207 isolates belonging to 43 different bacterial species (Additional file 6). Most of the isolates were identified as members of the Bacillus genus and included high numbers of representatives from the Bacillus licheniformis (41 isolates) and Bacillus paralicheniformis (30 isolates) species. Besides Bacillus, other endospore-forming bacteria were isolated including Paenibacillus sp. and Brevibacillus brevis. Several species from the Proteobacteria phylum including Serratia liquefaciens, Pseudomonas fragi, and Pseudomonas psychrophila were also isolated from milk pre-HTST but not from milk post-HTST or from cheese.

Because slit formation is caused by the accumulation of gas (CO_2) in cheese, the capacity of the bacterial isolates to produce gas during growth was measured. The heterofermentative LAB, L. fermentum, Ln. mesenteroides, and Ln. lactis, produced gas during growth in MRS broth containing Durham tubes. Gas production was not visible for homofermentative Lactobacillus species L. delbrueckii subsp. lactis, L. paracasei, L. paraplantarum, L. plantarum, L. rhamnosus as well as thermoduric bacteria (data not shown). Additionally, L. fermentum survived HTST simulated conditions for 15 sec at 72 °C (Additional file 7, three isolates tested). By comparison, none of the Ln. mesenteroides and only one of the three Ln. lactis isolates survived for that length of time (Additional file 7).

Heterofermentative LAB and milk consortia cause slit formation in Cheddar cheese. L. fermentum, Ln. mesenteroides, and Ln. lactis isolates (n = 3 isolates of each species) were tested for their role in the development of Cheddar cheese slit defects. Rifampicin-resistant variants of each of the isolates along with L. plantarum NCIMB8826-R were separately inoculated at either moderate (10^7 CFU/g) or high (10^9 CFU/g) levels into freshly made cheese curds prior to pressing and aging. The lower cell quantities are in the range of Lactobacillus cell numbers found in Cheddar cheese (2.5% ± 1.2% out of the 10^9 CFU/g viable bacteria in the 2015 samples).

After aging, culturable bacterial cell numbers decreased from 10^9 CFU/g to 10^8 CFU/g (Additional file 8–10). The quantities of the rifampicin-resistant LAB inoculants remained unchanged in cheese inoculated with 10^7 CFU/g) and decreased by 10-fold in cheese into which 10^9 CFU/g were added (Additional file 8–10). Slits were found in all cheese blocks inoculated with either L. fermentum, Ln. mesenteroides, or Ln. lactis (Fig. 7A-B, 8A-B). Cheese containing the L. fermentum isolates exhibited the highest levels of slit damage. The surface area of those cheeses contained 4- to 5-fold higher levels of slits compared to the controls (Fig. 7A-B, 8A-B). A dose-dependent effect was also observed such that cheese inoculated with L. fermentum at a level of 10^9 CFU/g contained more slits (2.5% ± 0.6% cheese area) than cheese inoculated with 10^7 CFU/g (1.1% ± 0.4% cheese area) (Fig. 7A and 7B). Culture-independent assessments of the cheese microbiota by DNA sequencing showed that L. fermentum was the dominant ASV (Additional file 11 A and B). Notably, cheese blocks inoculated with the Ln. mesenteroides and Ln. lactis isolates also contained elevated levels of in non-starter L. fermentum (Additional file 11 A and B), potentially indicating that those bacteria altered the cheese in a manner that promoted L. fermentum growth.

We also tested whether the inoculation of cryopreserved bacterial consortia collected from milk post-HTST and not cultured in laboratory medium could cause slit defects. Consortia were selected because they were either associated with good quality cheese (no slits) or cheese with slit defects. Adding those bacteria to cheese curds and then pressing and aging confirmed that slit-associated milk consortia are sufficient to cause slits (Fig. 7C and Fig. 8C). Importantly, because the inocula consisted of only approximately $1.8 \cdot 10^4$ cells, this finding showed that slit-production was reproducible and only low numbers of contaminating bacteria are needed to cause this defect. DNA sequencing confirmed that the proportions of L. fermentum ASVs were higher in cheese made using slit-associated milk consortia,

compared to cheese inoculated with bacteria from milk that yielded good quality cheese (Additional file 11 C).

Discussion

Food quality defects are significant contributors to the global problem of food waste. This issue is particularly problematic for fermented foods. Detecting the cause of a microbial-induced, fermented food defect is particularly challenging because of the expected large number and frequently diverse microorganisms present. Herein, we showed that culture-independent microbiota assessments with 16S rRNA gene sequencing are useful for the identification of low-abundance taxa responsible for slit-defects in Cheddar cheese and that thermotolerant bacterial contaminants in milk used to make that cheese are responsible for those flaws. These findings demonstrate that although there are significant challenges to the consistent manufacture of high-quality cheeses, microbiome-based assessments of fermented food products have both predictive and diagnostic value.

HTST pasteurization results in reductions in viable cell numbers and bacterial diversity as well as changes to the proportions of individual bacterial taxa. Notably, these changes were limited to the PMA-treated milk, a result that was consistent with our prior observation that pasteurization does not change the total bacterial DNA contents of milk but rather alters the proportions of the remaining surviving cells [17]. Also as found previously, pasteurization resulted in reduced proportions of Staphylococcus and Pseudomonas and higher levels of endospore-forming bacteria including Turicibacter and Clostridiales [17].

Bacterial populations change rapidly and repeatedly in milk upon transfer from tanker trucks to storage silos and in milk undergoing initial pasteurization and blending steps for cheese production, even in individual pieces of equipment [4, 17]. Focusing on the final HTST pasteurizer step prior to cheese manufacture here, we showed that the viable cell numbers and individual taxa cell quantities changed in milk post-HTST, but not pre-HTST, over pasteurizer run times. These changes were consistently detected even when the milk was sampled on different days spanning a time-period of four months. This result might have been due to either the retention or growth of certain taxa in the pasteurizer or clearance due to changing conditions in that piece of equipment. The most notable change was the increase in members of the Thermus genus during the production periods. Thermus, a member of the Deinococcus-Thermus phylum, was previously found in milk [51, 52], and at least one species, Thermus thermophilus, is a thermophile that can grow between 47 °C to 85 °C with an optimum growth range between 65 °C and 72 °C [53]. Because the HTST temperature was at 71.7 °C (161 °F) (personal communication), it is possible that Thermus grow inside the pasteurizer. However, this should be confirmed by testing the surfaces in that piece of equipment. Previous studies confirmed that the microbial populations on processing equipment surfaces are highly correlated with the microorganisms present in the final food contents [5, 6, 54]. Because T. thermophilus can cause a pink discoloring defect [14], such testing could be useful for preventing other types of cheese spoilage.

However, Thermus was not associated Cheddar cheese slit-defects. Cheese blocks with those defects were enriched in Lactobacillus and Turicibacter within 90 days. Importantly, the milk used to make that cheese already showed differences in bacterial contents prior to cheese production. These differences were even found for milk pre-HTST, suggesting that the spoilage bacteria were not effectively inactivated by HTST nor were they introduced after the pasteurization step. In particular, Lactobacillus, Brevibacillus, Bacillus, and Clostridium were detected in higher numbers in pre- and/or post-HTST milk associated with slits. Although clostridia like Clostridium tyrobutyricum are known to cause slits [55, 56], only Lactobacillus was enriched in both slit containing cheese and the milk used to make it. The lack of an undesired odor or flavor from butyric acid, an end-product of C. tyrobutyricum growth [16, 55, 57], is further support that C. tyrobutyricum was not the spoilage agent of the cheese studied here.

We also isolated LAB, endospore forming bacteria and other thermoduric bacteria from milk and cheese. These bacteria were collected during sampling performed in 2017 and 2018, as opposed to the prior culture-independent, collection points in 2015. Importantly, several of the cheese blocks collected during those later time points also developed slits, a result that is indicative of how slits are a very common problem in Cheddar cheese production. The isolates included a variety Bacillales including members of the Bacillus, Brevibacillus, and Paenibacillus genera which have been frequently detected in milk, dairy processing plants, and cheese [58–60]. Included were B. licheniformis, Bacillus subtilis, and Bacillus amyloliquefaciens, among which are strains known to produce biogenic amines [61, 62] and cause off-flavors due to lipolysis or proteolysis [59, 63, 64]. Other recovered Bacillales were Ornithinibacillus scapharcae, Paucisalibacillus globulus and Sporosarcina soli species,previously only isolated from only soil and marine environments [65–67]. Although Turicibacter was enriched in slit containing cheese produced in 2015, members of this genus were not isolated under the growth conditions used here, potentially because strict anaerobic conditions were not applied [68]. Although members of this genus have yet to be isolated from milk and only three isolates from human and mice intestinal contents are known [68], Turicibacter appear to be pervasive in milk and have been detected in proportions over 1% in numerous studies investigating the microbiota of bovine milk and dairy products [4, 69–72].

Lactobacillus, primarily represented by heterofermentative LAB and specifically L. fermentum, were the only members of the milk and cheese microbiota that were consistently associated with slit defects. Therefore, pilot cheeses were inoculated with isolates of heterofermentative LAB isolated from pre-HTST and post-HTST milk. Between the three species tested (L. fermentum, Ln. mesenteroides, and Ln. lactis), L. fermentum resulted in cheese with the most slit damage. The likelihood that L. fermentum was the primary spoilage agent is supported by the high thermal tolerance levels observed for the strains tested here, thereby indicating their potential to survive HTST pasteurization. L. fermentum was also present at higher proportions in slit-containing, pilot cheese inoculated with the cryopreserved bacterial consortia. Those consortia were collected from milk used to make Cheddar that showed slit defects during aging. Remarkably, those pilot-scale cheeses developed slits even though the consortia contained low numbers of bacterial cells (10^4 cells per milk consortia).

Lastly, although most of the general quality characteristics of the commercial cheeses were indistinguishable, there was a modest but significant reduction in the salt content of cheeses that developed slits. This result indicates that L. fermentum might be controlled by maintaining an adequate salt level above 1.8% at the beginning of cheese fermentations. This possibility is consistent with prior reports that L. fermentum is sensitive to NaCl concentrations above 2% [73, 74]. Hence, targeted detection of heterofermentative LAB combined with heightened scrutiny over salt content could be useful to reduce the incidence of the pervasive slit defect in Cheddar cheese.

Conclusions

Food production involves numerous control points for microorganisms to ensure both food quality and safety. These control points (e.g. HTST pasteurization) are especially challenging for fermented foods wherein the microbial contents are also expected to provide positive contributions to the final food product. By examining the non-starter bacteria in Cheddar cheese, we showed that bacterial identification by marker gene sequencing to identify spoilage microorganisms was successful even when those spoilage contaminants constituted less than 0.8% of the total bacteria present. The inoculation of heterofermentative LAB and total consortia from milk post-HTST were sufficient to cause slit formation in cheese, validating that DNA sequencing of viable cell fractions is a useful approach for determining whether there are certain microorganisms present in the food ingredients (e.g. milk) that may increase the risk for quality defects. In order to prevent slit-defects, screening methods such as qPCR or targeted DNA sequencing methods for *L. fermentum* and other heterofermentative LAB should be incorporated in quality control protocols for milk to exclude or modify that milk during cheese-making. Evaluating this approach for other cheese types and spoilage defects should result in robust methods for the identification of microbial-associated, spoilage risk factors and new opportunities for controlling those microorganisms in food.

Declarations

Availability of data and materials. DNA sequences were deposited in the Qiita database [75] under study ID 12366 (https://qiita.ucsd.edu/study/description/12366#) and in the European Nucleotide Archive (ENA) under accession number ERP114733 (https://www.ebi.ac.uk/ena/data/view/PRJEB32097).

Competing interests. JH and JM are employed by Hilmar cheese company.

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Authors' contributions. ZX, MEK, JH, JM, and MLM designed the study and sampling plans. ZX, JTB, ZQ, ETS, and JH collected and processed samples. ZX and ZQ performed DNA extraction, PCR, and sequencing of all samples. JTB and ETS cultured and isolated bacteria from milk and cheese. ZX, JTB, and JH made pilot-scale Cheddar cheese. ZX performed bioinformatics and statistical analyses. ZX and MLM interpreted the data and wrote the manuscript. All authors edited and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

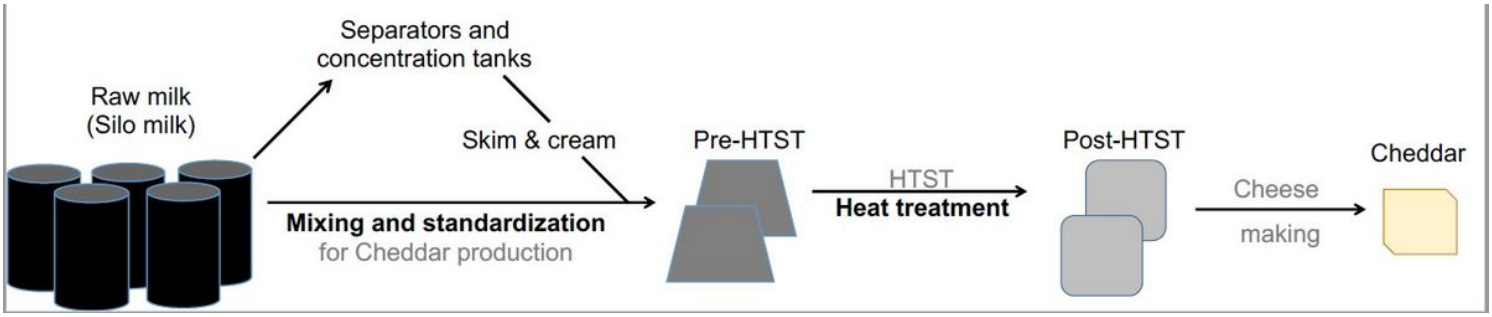

Figure 1

Diagram of the sampling plan. Milk was sampled from storage silos and at the terminal HTST pasteurization step. The microbial shifts that occur during milk mixing and standardization in separators and concentration tanks were documented in a previous study [17]. Milk streams entering (pre-HTST) and leaving (post-HTST) the pasteurizer were sampled multiple times throughout 10-h Cheddar production periods and on multiple dates. Cheddar cheese blocks were made using the post-HTST milk and sampled at 0, 30, 90 and 120 days of aging. Slits in cheese were visually examined after aging at 4 □C.

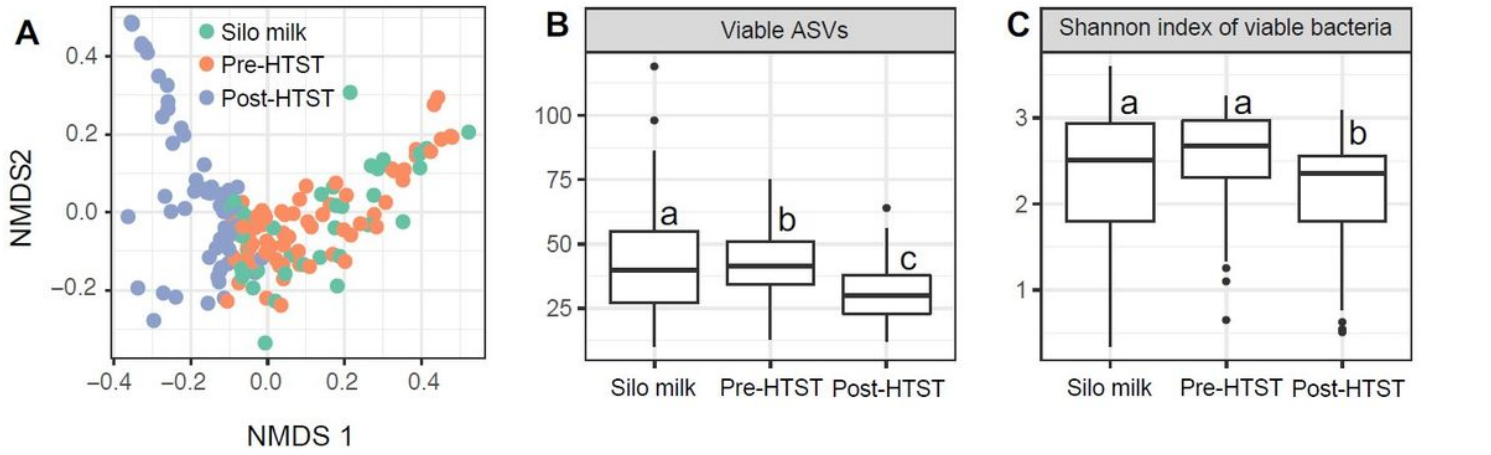

Figure 2

Pasteurization alters the diversity of viable bacteria in milk. (A) Non-metric multidimensional scaling analysis of individual milk samples. (B) Number of total observed ASVs and (C) Shannon diversity of each milk type after PMA treatment. Significant differences (Kruskal-Wallis with Dunn test, $p < 0.05$) are indicated by the presence of different lower-case letters above the boxplots.

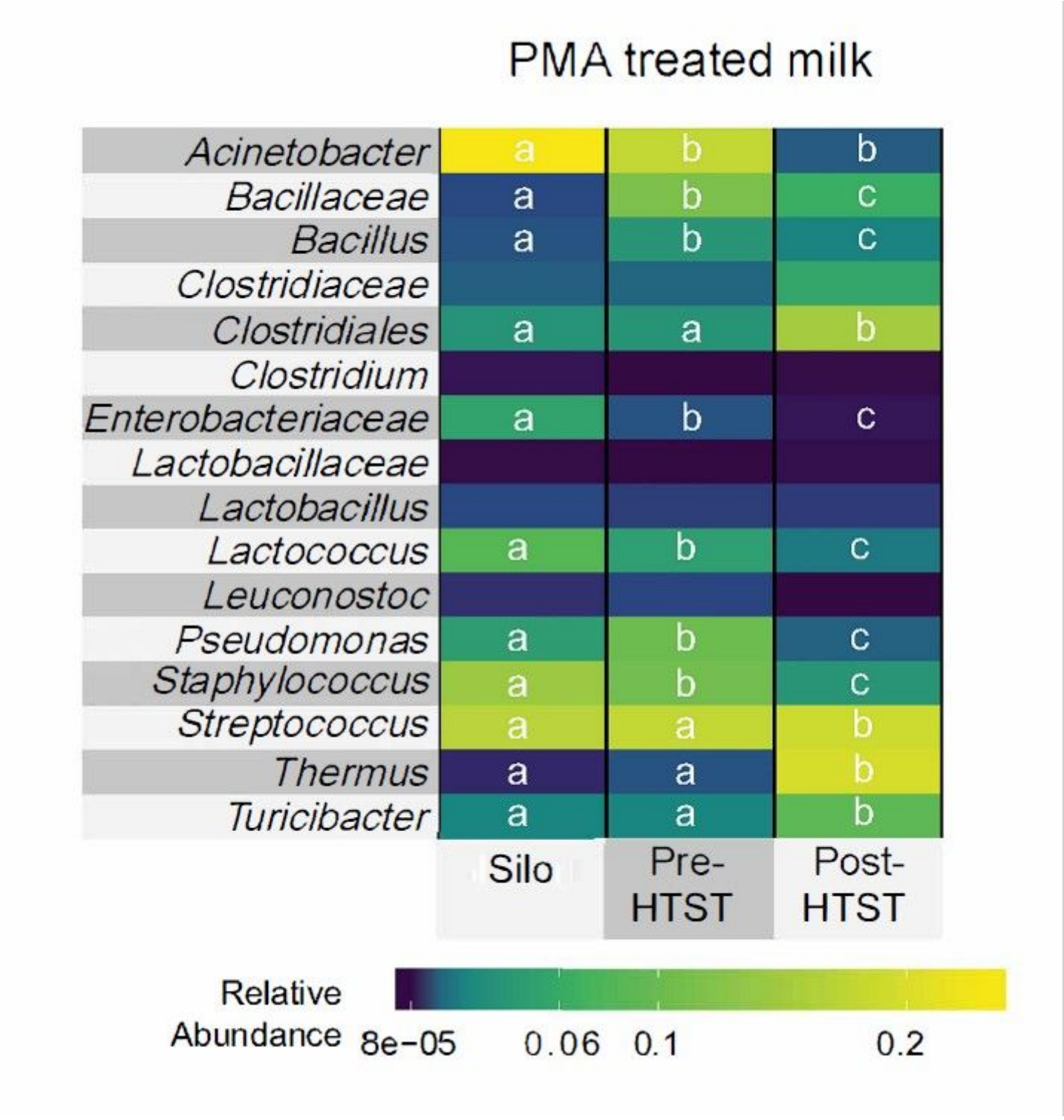

Figure 3

Pasteurized milk is enriched in thermoduric and endospore-forming bacteria. The 16 most abundant bacterial taxa identified from PMA treated samples are shown. The heat map projects the average proportions for 40 samples from silo, 67 samples from pre-HTST, and 67 samples from post-HTST. Significant differences in the proportions of taxa between collection points (silo, pre-HTST, and post-HTST) are indicated by lower-case letters (DESeq2 adjusted $p < 0.1$ and log2 fold change > 1.5).

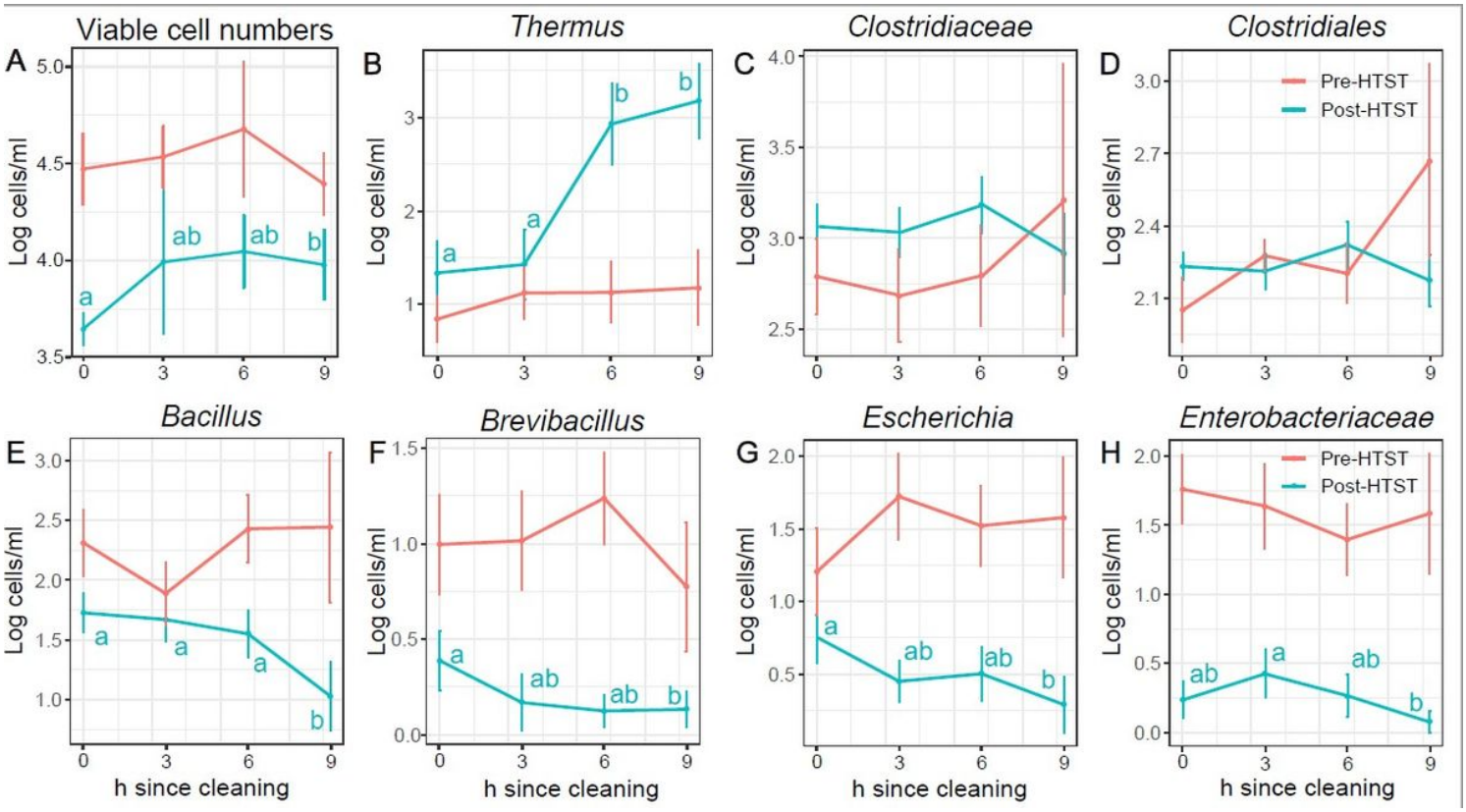

Figure 4

Thermus populations increase over time after pasteurizer cleaning. (A) Total bacterial cell numbers (log10 transformed) were determined by qPCR on DNA isolated from milk following PMA treatment. (B to H). Cell quantities were estimated by relating the total viable bacterial cell numbers in a milk sample (A) to the proportions of individual bacterial taxa determined by 16S rRNA gene sequencing. The taxa shown were selected based on their total abundance in milk and variation in cell numbers over time. Lower-case letters indicate significant differences between pre-HTST or post-HTST milk samples according to Kruskal-Wallis with Dunn test ($p < 0.05$).

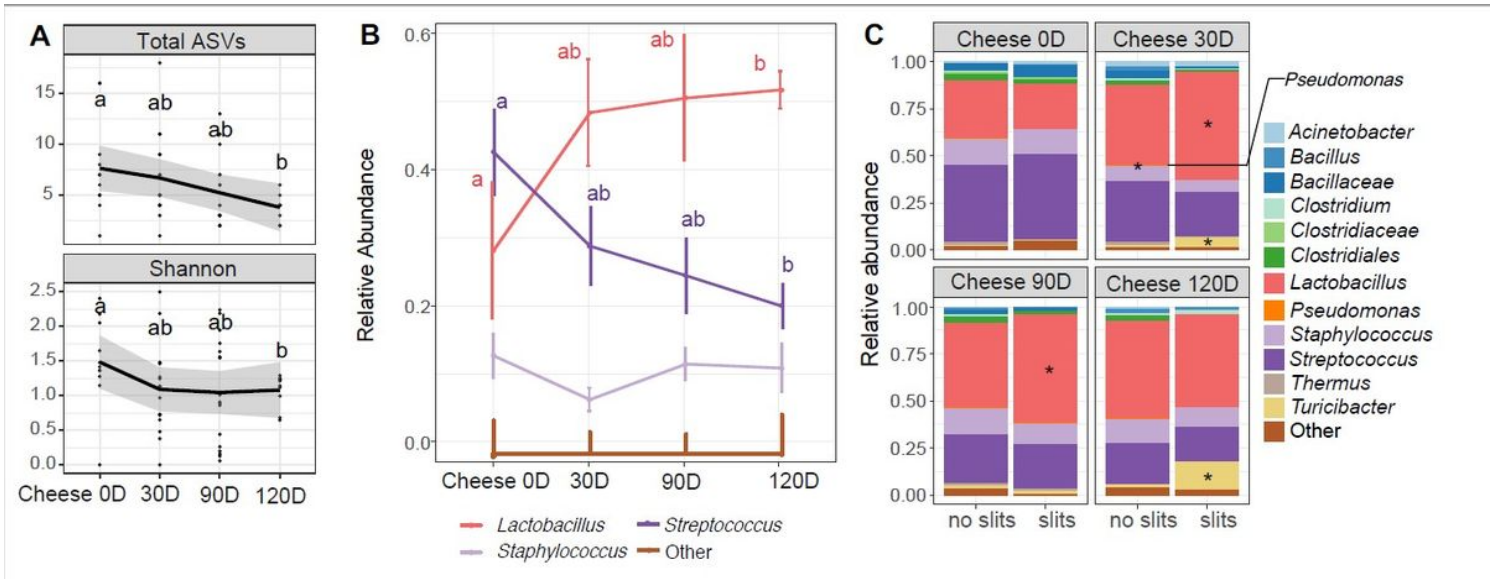

Figure 5

Lactobacillus cell numbers increase with cheese aging are associated with cheese slits. (A) Alpha diversity, plotted as observed total ASVs and Shannon index, and (B and C) proportions of the viable bacteria in Cheddar cheese during aging. Starter-culture Lactococcus DNA sequences were removed prior to analysis. Bacteria in PMA treated cheese samples are shown. In B and C, the most abundant bacterial taxa are named and all the other bacteria are grouped in the “other” category. Significant differences are indicated by the different lower-case letters or asterisks and determined by the (A) the Kruskal-Wallis with Dunn test ($p < 0.05$) and (B and C) DESeq2 adjusted $p < 0.1$ and log2 fold change > 1.5.

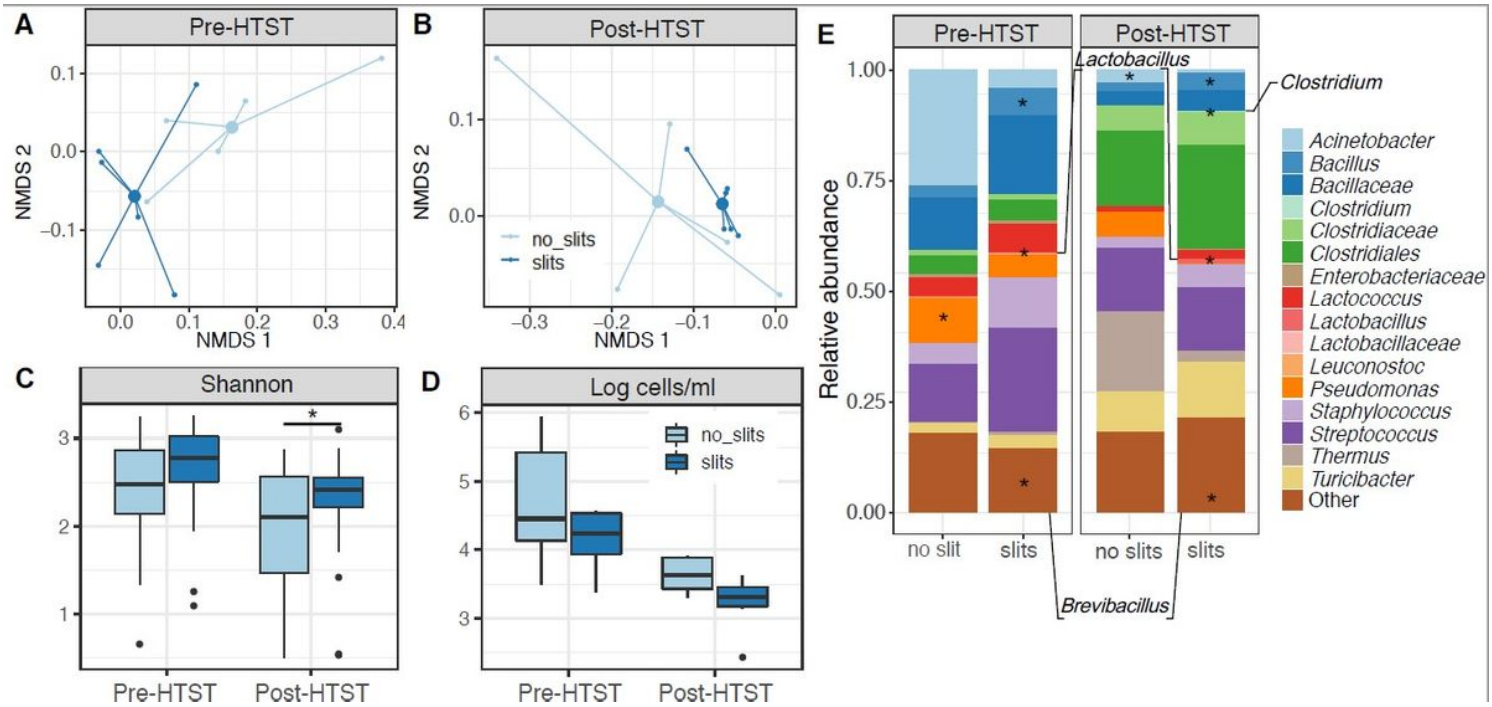

Figure 6

Pre-HTST and post-HTST milk microbiota are associated with Cheddar cheese slit defects. (A and B) Non-metric multidimensional scaling analysis, (C) Shannon diversity, (D) log10 transformed total bacterial numbers, and (E) log10 transformed bacterial abundances of each taxon. PMA treated milk samples were used for bacterial identification. In (E), the 16 most abundant bacterial taxa are named and all the other taxa are grouped under the “other” category. Asterisks (*) indicate significant differences between milk samples according to the (C and D) Mann-Whitney test ($p < 0.05$) and (E) DESeq2 adjusted $p < 0.1$ and log2 fold change > 1.5 values. In pre-HTST and post-HTST milk not associated with cheese slits, Pseudomonas (average of 10.15%) and Acinetobacter (average of 2.59%) were enriched, respectively. In cheese slit-associated, pre-HTST milk, Bacillus (average of 6.22%), Lactobacillus (average of 0.26%), and Brevibacillus (average of 0.69%) were enriched. In cheese slit-associated, post-HTST milk, Bacillus (average of 3.92%), Clostridium (average of 0.56%), Lactobacillus (average of 0.87%), and Brevibacillus (average of 0.32%) were enriched.

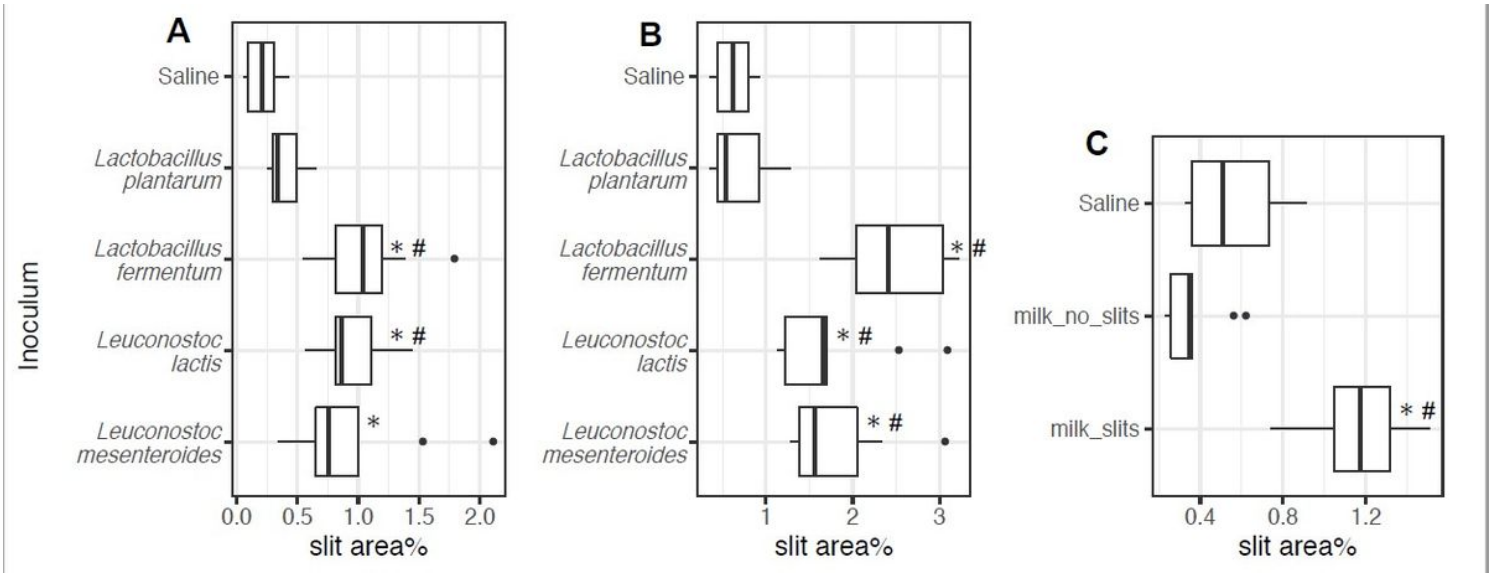

Figure 7

Inoculation of heterofermentative LAB or slit-associated milk consortia results in Cheddar cheese with slits. The percentage (%) of surface area containing slits is shown for cheese inoculated with either (A) 107 CFU/g or (B) 109 CFU/g of the indicated LAB species or with (C) cryopreserved milk bacterial consortia. For each cheese sample, three cross-section surfaces were randomly selected for slit analysis. The Kruskal-Wallis with Dunn test ($p < 0.05$) was used to assess for significant differences in slit area compared to controls with added saline (*) (A, B, and C), homofermentative LAB L. plantarum (#) (A and B), or bacterial consortia from milk not associated with cheese slit formation (*) (C).

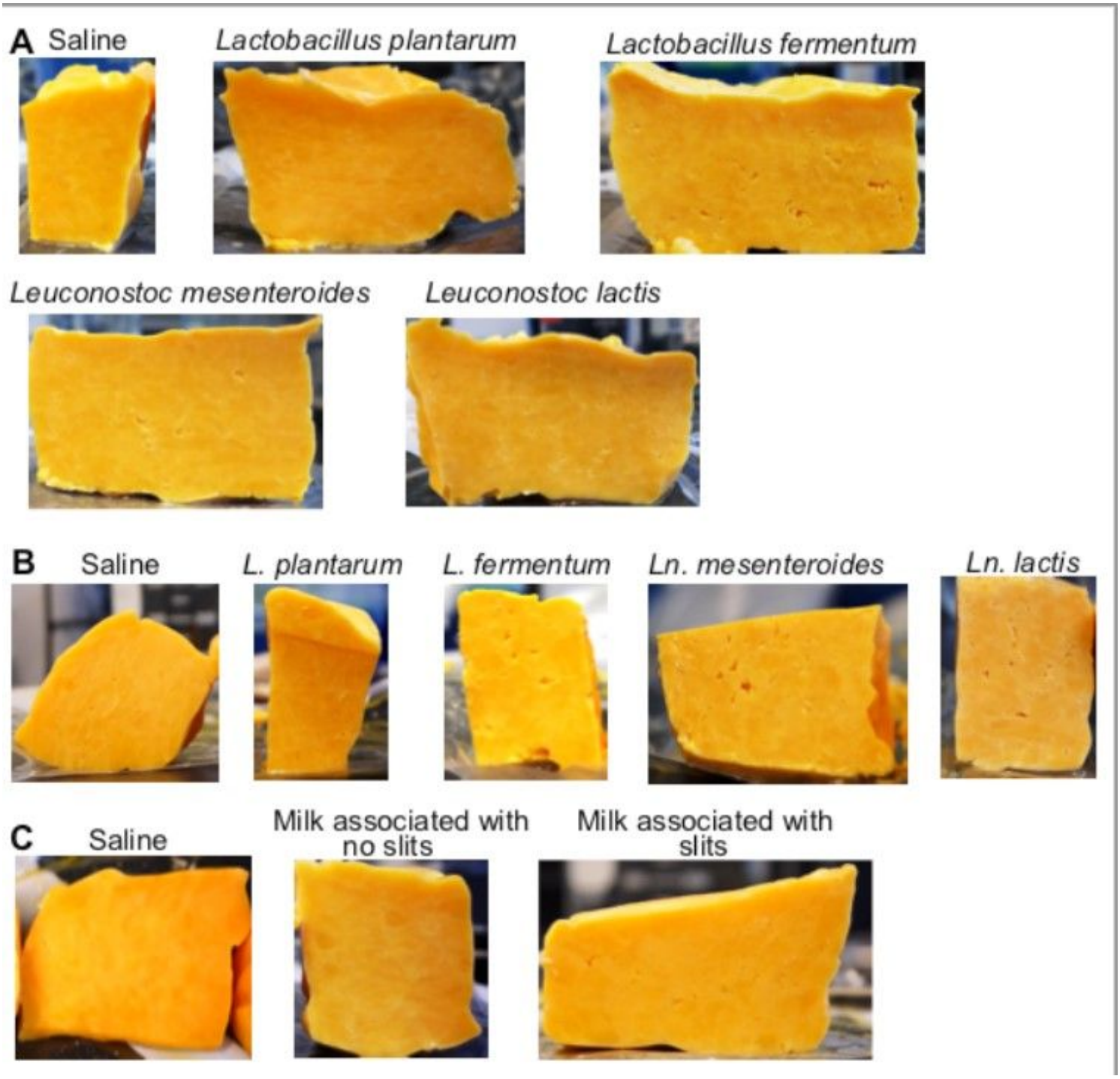

Figure 8

Representative photos of pilot cheese cross-sections. Cheese was inoculated with (A) 107 CFU/g or (B) 109 CFU/g of the selected bacterial strains or (C) with cryopreserved milk consortia. Saline was added to serve as an inoculum control.

Supplementary Files

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