Meat Processing Plant Microbiome and Contamination Patterns of Cold-Tolerant Bacteria Causing Food Safety and Spoilage Risks in the Manufacture of Vacuum-Packaged Cooked Sausages

Jenni Hultman,[a] Riitta Rahkila,[a*] Javeria Ali,[b] Juho Rousu,[b] K. Johanna Björkroth[a]

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland[a]; Department of Computer Science, Aalto University, Espoo, Finland[b]

Refrigerated food processing facilities are specific man-made niches likely to harbor cold-tolerant bacteria. To characterize this type of microbiota and study the link between processing plant and product microbiomes, we followed and compared microbiota associated with the raw materials and processing stages of a vacuum-packaged, cooked sausage product affected by a prolonged quality fluctuation with occasional spoilage manifestations during shelf life. A total of 195 samples were subjected to culturing and amplicon sequence analyses. Abundant mesophilic psychrotrophs were detected within the microbiomes throughout the different compartments of the production plant environment. However, each of the main genera of food safety and quality interest, e.g., *Leuconostoc*, *Brochothrix*, and *Yersinia*, had their own characteristic patterns of contamination. Bacteria from the genus *Leuconostoc*, commonly causing spoilage of cold-stored, modified-atmosphere-packaged foods, were detected in high abundance (up to >98%) in the sausages studied. The same operational taxonomic units (OTUs) were, however, detected in lower abundances in raw meat and emulsion (average relative abundance of 2% ± 5%), as well as on the processing plant surfaces (<4%). A completely different abundance profile was found for OTUs phylogenetically close to the species *Yersinia pseudotuberculosis*. These OTUs were detected in high abundance (up to 28%) on the processing plant surfaces but to a lesser extent (<1%) in raw meat, sausage emulsion, and sausages. The fact that *Yersinia*-like OTUs were found on the surfaces of a high-hygiene packaging compartment raises food safety concerns related to their resilient existence on surfaces.

Refrigeration is used throughout the modern food chain to ensure the safety and quality of perishable products. Chilling has also been extended to food processing facilities to ensure that food manufacture complies with the legislative requirements governing maximum food temperature. Perishable food is usually packaged under a carbon dioxide-containing modified atmosphere to suppress bacterial growth. These modern food manufacturing practices have changed the order of prevalence of food-borne bacteria. Instead of aerobic Gram-negative bacteria, (facultatively) anaerobic Gram-positive bacteria prevail in modified-atmosphere-packaged (MAP) foods (1). In addition to refrigeration, the daily cleaning and sanitizing procedures used at the processing facilities might lead to the resilience of certain microbes and thus persistent contamination.

Hygiene in a food processing facility is monitored through an internal control procedure, including hazard analysis of critical control points. The manufacturer is responsible for guaranteeing the safety and quality of its products during the shelf life set for each respective product. In routine hygiene monitoring, the microbiological quality of the raw materials, cleanliness of the surfaces, and quality of the end products are included in these internal control protocols. Bacterial contamination within food processing facilities has been addressed in scientific studies using different molecular typing techniques enabling bacterial contamination to be tracked and traced during food manufacture (1–5). These approaches have involved massive sets of culturing, followed by the application of molecular typing methods on purified bacterial cultures.

To date, only one (6) study dealing with contamination of a meat processing environment has been conducted using 16S rRNA amplicon sequencing. Compared to studies of the microbiota in other man-made environments, such as shopping malls (7), homes (8), and hospitals (9), microbiota associated with food processing facilities have not been analyzed to the same extent using comprehensive microbial ecology approaches. When we were consulted to overcome a processing hygiene problem in a large-scale meat processing plant, we decided to approach this challenge by conducting a large-scale 16S rRNA gene amplicon sequencing study. Vacuum-packaged (VP) sausages of the manufacturer concerned had shown variable sensory spoilage changes, i.e., slime or opaque drip formation and loosening of the vacuum.

This paper presents the bacterial community composition in various processing stages of a man-made food manufacture niche. To characterize the processing plant and product microbiomes and to address the persistent hygiene problem, a total of 195 samples were taken from the processing plant environment, raw ma-

terials, and the end products. Alongside the amplicon analyses, we applied cultivation techniques and identified the main species of spoilage-causing lactic acid bacteria (LAB) using a database employing numerical analysis of 16S and 23S rRNA gene restriction patterns. We also defined the abundances and contamination patterns of some relevant cold-tolerant bacteria associated with safety and spoilage risks of vacuum-packaged, cooked meat products.

MATERIALS AND METHODS

Study design and sampling. The processing environment was sampled twice, first in October 2012 after the spoiled products had been received from customers and then a second time in early December 2012. Samples were taken both times early in the morning from cleaned surfaces before production started and during ongoing production in the afternoon (Table 1). Sampling covered both the compartments where the raw meat was handled and the packaging areas with high-hygiene practices (Table 1 and Fig. 1). Altogether, 101 samples were taken during the two sampling rounds. We also analyzed sausages, which were produced during the two visits to the processing plant, at the end of their shelf life.

In addition to the surface and sausage samples, 12 production lots from raw materials, sausage emulsion, and subsequently manufactured and cooked products were sampled during a period of 5 months after the first processing plant visit. This was done to identify the spoilage microbiota in raw materials in addition to the ones on surfaces and to understand the contamination patterns during sausage production. These production lots were sampled in a time-dependent manner according to production flow. The raw materials and sausage emulsion were analyzed within 24 h of sampling in the processing plant, and the sausages at the end of a 26-day shelf life ($\pm$1 day). All samples were subjected to cultivation, DNA extraction, and PCR. Quantitative cultivation through a dilution series of the raw meat and products was done to analyze both LAB (de Man, Rogosa, and Sharpe [MRS] agar plates; Oxoid, Hampshire, United Kingdom) and total microbial (plate count agar [PCA]) levels. Surface samples were taken with sterile cotton swabs from the processing line surfaces that the raw materials, the emulsion used in sausage production, and the sausages were in contact with (detailed information is provided in Table 1). These samples were swabbed semiquantitatively from 5- by 5-cm surface areas when possible. In some of the surfaces it was not possible to sample a 5- by 5-cm square area due to the shape of the equipment. In addition to the microbiome analyses, the sausages were evaluated for spoilage changes by a three-person expert panel judging the formation of sensory changes visually at the end of shelf life. The changes evaluated included formation of slime or drip, color of the slime or drip (clear to opaque), and potential off-odor rated on a scale of 1 to 5.

Microbiological analysis and identification of spoilage LAB. (i) Processing line surfaces and air. The 5- by 5-cm swabs taken from surfaces (see above) were directly streaked onto MRS plates containing amphotericin (MRS-A) as an antifungal agent (Sigma-Aldrich Co., St. Louis, MO, USA) to culture LAB. An Anderson sampler was used to sample 283 liters of air from each room into MRS-A plates both during and before production (Table 1). MRS was chosen as it is known to be selective for LAB, and the goal of the cultivation-based approach was to identify the specific spoilage organisms to the species level.

(ii) Raw materials and sausage emulsion. Bacterial levels in raw materials were analyzed quantitatively. Serial 10-fold dilution series were made from 22 g of each material with 198 ml of peptone-salt buffer (0.1% peptone, 0.9% NaCl) and homogenized by using a stomacher at medium power for 1 min (Stomacher 400; Seward, West Sussex, United Kingdom), followed by cultivation onto MRS plates (LAB). In addition, plate count agar (PCA) was used to quantify the total cultivable microbes from the raw materials and cooked sausages.

The PCA plates were incubated at 25°C for 3 to 5 days before colony enumeration. The MRS-A and MRS plates were incubated in jars made anaerobic by a commercial atmosphere generation system (AnaeroGen; Oxoid) at 25°C for 5 days, after which the CFU were calculated, and 5 to 10

colonies were randomly collected for ribotyping-based species identification (5). A total of 222 individual colonies from the MRS-A plates sampled from the processing plant surfaces were selected and purified by subculturing on MRS agar, and 175 (78%) of these were ribotyped. Similarly, 103 LAB colonies from material samples grown on MRS agar were selected for pure cultures, and 74 of these (72%) were identified using ribotyping.

LAB species identification. LAB species identification was conducted as described by Vihavainen et al. (2). In brief, selected colonies were grown in MRS broth, and DNA was extracted using the guanidium thiocyanate method complemented with mutanolysin and lysozyme (5). The DNA was sheared with HindIII, and the resulting fragments were separated by gel electrophoresis. The DNA fragments were transferred onto a nylon membrane using a vacuum blotting device. The fragments containing 16S or 23S rRNA genes were detected with a digoxigenin-labeled oligonucleotide probe mixture, Oligomix5 (10). The HindIII ribopatterns were compared to the corresponding patterns in the previously established LAB database of the Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland, with patterns from all relevant food-associated LAB in the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella* (2). For numerical analyses, the ribopatterns were normalized based on the mobility of standards, and a similarity matrix was created using the BioNumerics (version 5.10) software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation, and clustering using the unweighted-pair group method using arithmetic averages (UPGMA) was used to construct the dendrogram. Based on the use of internal controls in the database, a pattern optimization and a band position tolerance of 0.5 and 1.5, respectively, were allowed. The isolates were identified based on the locations of type and reference strains within the clusters.

DNA-based 16S rRNA gene amplicon analysis. Samples for DNA extraction were taken from the processing plant using nylon swabs (FLOQSwapos; Copan Flock Technologies, Brescia, Italy) dipped in SDS-NaCl buffer (0.15 M NaCl, 0.1% Tween 20) prior to swabbing a 5- by 5-cm area (11). The swabs were stored in 500 μl of the SDS-NaCl buffer and frozen at −20°C until DNA extraction. The raw material, emulsion, and end-of-shelf-life sausages were diluted into a peptone-salt buffer and mixed in a stomacher for 30 s at a low setting. Both a 10^{-1} and 1:1 dilution were used in DNA extraction. In order to separate the mammalian and bacterial cells, two-stage centrifugation was used. First, the supernatants from homogenization were centrifuged in 15-ml conical tubes at 200 relative centrifugal force (RCF) for 3 min. The bacterial cells from the supernatant were pelleted by centrifuging 10 ml of the supernatant from the first centrifugation at 10,000 RCF for 3 min.

DNA was extracted from the swabs and from the pelleted bacterial cells using GES (guanidium thiocyanate, 100 mM EDTA, and 0.5% [wt/vol] Sarkosyl) and phenol-chloroform extraction. First, the swabs together with the storage buffer were transferred to a FastPrep lysing matrix E tube (MP Biomedicals, Santa Ana, CA, USA), and 500 μl of denaturing buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added, followed by 500 μl of phenol-chloroform-isoamyl alcohol (pH 8.8; Sigma-Aldrich). For the bacterial cell pellets, denaturing buffer and phenol-chloroform-isoamyl alcohol were added to the tubes with the pellets, vortexed, and transferred to the lysing matrix tubes. The lysing matrix tubes were bead beaten for 40 s at 5.5 m/s in a FastPrep-24 instrument (MP Biomedicals), after which the tubes were incubated on ice for 5 min and centrifuged for 10 min at 13,000 RCF (Eppendorf, Hamburg, Germany). Chloroform (500 μl) was added to the upper layer, and the tubes were centrifuged for 10 min after vortexing. The nucleic acids in the upper layer were precipitated with a 1/10 volume of 3 M sodium acetate, 1 μl of GlycoBlue (Invitrogen, Carlsbad, CA, USA), and 3× ethanol. The pellet was washed with 70% ethanol and eluted to 50 μl of sterile nuclease-free water.

To characterize the microbiome, the V1 to V3 area of the 16S rRNA gene was amplified through PCR with primers 8f and 518r (12). The PCR

TABLE 1 Sampling sites, conducted measurements, and outcome of DNA- and cultivation-based analyses[a]

| | | | First sampling | | | | | | Second sampling | | | | | |
| | | | During production | | | Before production | | | During production | | | Before production | | |
Site	Sampling location	Material	Sample name	PCR result[e]	Cell abundance/plate[c]	Sample name	PCR result	Cell abundance/plate	Sample name	PCR result	Cell abundance/plate	Sample name	PCR result	Cell abundance/plate
MRU[b]	Emulsion transport belt	Plastic	Fa1	●	++				Fa69	●	+	Fa109	-	-
	Emulsion transport belt	Plastic										Fa111	-	-
	Meat grinder	Metal	Fa2	-	+++				Fa75	●	+++	Fa112	-	+
	Emulsion blender	Metal	Fa3	-	+++				Fa74	-	+	Fa113	-	-
	Emulsion storage silo door	Plastic	Fa4	●	-				Fa70	-	+	Fa110	-	-
	Emulsion storage silo door	Metal	Fa5	●	-				Fa71	-	-			
	Emulsion storage silo door	Metal	Fa6	●	-				Fa72	-	-			
	Emulsion storage silo door	Metal							Fa73	-	-			
	Air	NA[d]	Fa7	NA	+	Fa46	NA	-	Fa68	NA	++	Fa108	NA	-
	Tube on the floor	Plastic				Fa49	-	+++						
	Floor	Concrete				Fa50	-	+++						
	Cutting board	Plastic				Fa47	-	+++						
Sausage-filling machinery	Filling machine	Plastic	Fa8	●	++	Fa33	-	-	Fa77	●	+++	Fa117	●	-
	Emulsion storage silo	Metal	Fa9	●	+++				Fa76	-	++			
	Emulsion storage silo	Metal	Fa10	●	+									
	Tube to move emulsion	Metal				Fa45	●	+						
	Filling machine	Metal	Fa11	●	+++	Fa41	-	++				Fa119	-	+++
	Filling machine	Metal				Fa42	●	++				Fa118	●	+++
	Air	NA	Fa12	NA	+	–	-					Fa120	-	+
	Filling machine vacuum	Metal				Fa43	●	-				Fa121	-	-
	Sausage string hanging cart	Metal										Fa122	-	-
Packaging compartment	Link cutting drum	Plastic	Fa13	●	+++	Fa28	●	+	Fa80	-	-	Fa93	●	-
	Link cutting drum	Metal				Fa26	-	-				Fa97	-	-
	Link cutting drum	Metal				Fa27	●	-						
	Transport belt from drum	Plastic	Fa14	●	+	Fa29	●	-	Fa79	-	-	Fa95	-	-
	Transport belt from drum	Metal				Fa30	●	-	Fa81	-	-	Fa99	-	-
	Air	NA	Fa18	NA	-	Fa25	NA	-	Fa78	NA	-	Fa96	NA	-
	Air	NA	Fa19	NA	-				Fa88	NA	-	Fa101	NA	-
	Air	NA							Fa87	NA	-	Fa103	NA	-
	Air	NA							Fa92	NA	-			
	Cooling cabinet wall	Metal	Fa20	-	-				Fa89	-	-	Fa102	●	-
	Packaging tray	Metal	Fa21	●	-	Fa32	●	-	Fa85	-	-	Fa98	-	-
	Pit from transport belt to packaging tray	Metal	Fa22	●	+	Fa31	●	-	Fa84	-	++			
	Transport cart for sausage hanging sticks	Metal	Fa23	●	-	Fa40	●	-	Fa83	-	-	Fa100	-	-
	Sausage string hanging cart	Metal	Fa24	●	-									
	Transport belt from drum	Plastic							Fa90	-	-	Fa104	-	-
	Knife storage	Metal				Fa34	-	-				Fa94	-	-
	Sausage string hanging cart	Metal							Fa82	-	-	Fa105	-	++
	Sausage string hanging stick	Metal							Fa86	-	-			
	Pit from transport belt to packaging tray	Metal							Fa91	-	-			
	Wall	Metal										Fa106	-	-

[a] The numerical data for raw materials and cooked products can be found in the supplemental material.
[b] MRU, meat receiving unit.
[c] In surface microbiome samples, the numbers of colonies on MRS-A swabs are identified as follows: –, none detected; +, 1 to 10; ++, 11 to 100; +++, >100.
[d] NA, not applicable.
[e] ●, PCR successful; –, no PCR product.
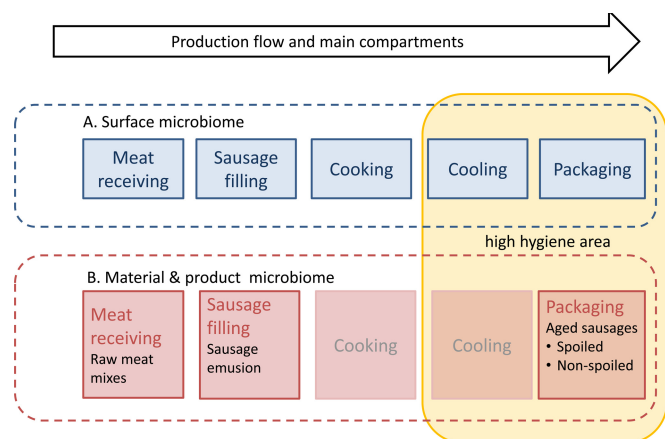
FIG 1 Schematic picture of the processing plant (blue) and the raw material flow (red) in corresponding process stages. No material samples were taken from the cooking and cooling compartments.

FIG 2 Bacterial cell numbers (log CFU/g) on PCA and MRS plates in raw materials and cooked products.

consisted of 1× Phusion GC buffer, 200 μM deoxynucleoside triphosphate (dNTP) mix, 0.2 μM each primer, 2.5% dimethyl sulfoxide (DMSO), and 50 to 250 ng of community DNA. After the PCR mix was heated to 98°C, 1 U of Phusion polymerase (Life Technologies, Carlsbad, CA, USA) was added to the reaction mixture. The PCR program was as follows: denaturation at 98°C for 30 s and 20 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 10 s, followed by 72°C for 5 min and cooling to 4°C. The PCR products were purified with 0.9× Ampure beads (Beckman Coulter, Pasadena, CA, USA) and eluted to 40 μl of 0.1× Tris-EDTA (TE) buffer. The bar codes and sequencing adapter were added to the amplified PCR fragments in a second PCR with a 1× Phusion GC buffer, 200 μM dNTP mix, 0.05 μM each primer, 2.5% DMSO, and approximately 50 ng of purified PCR product. Again, the Phusion polymerase (1 U) was added after the reaction mixture was heated to 98°C. The reverse primer had sequencing adapter A attached, and the forward primer included sequencing adapter B with a sample-specific bar code (8 bp). Three replicate PCRs were done per sample, and the products were pooled prior to purification. PCR product purification and sequencing were conducted at the Institute of Biotechnology, University of Helsinki, using the Roche 454 Titanium FLX protocol.

Sequences were analyzed with QIIME (13). First, the sequence reads were filtered for quality, and reads with a length of less than 200 bp, containing ambiguous bases, with a quality score below 30 (q30) or mismatches in the primer sequence were discarded, and the remaining reads were assigned to samples based on the sample specific bar code. OTUs were picked using the uclust (14) algorithm with 97% similarity, and the representative sequence read from each OTU was assigned to taxonomy with BLAST (15) against the Greengenes database (version 13.8.2014) (16). Chloroplast OTUs of plant origin were removed by name ("Streptophyta") based on Greengenes taxonomic classifications (average 2.3% of sequences in each sample ±7.0% standard deviation [SD]). The OTU sequences were aligned with PyNast (17), and a phylogenetic tree was built from the filtered alignments with FastTree (18). Alpha and beta diversity metrics were calculated from the rarified OTU table. Rarefaction was done to the lowest number of sequence reads used in analysis (sample Fa43 from filling machinery; 2,706 reads), with QIIME and R (19) using the Vegan package, version 1.8-2 (20). The statistical analysis was conducted with STAMP, version 2.00 (21), using two-sided Fisher's exact test with the Storey false discovery rate (FDR) correction.

Microarray data accession number. The 16S rRNA gene sequences were deposited in the Sequence Read Archive (SRA) of the NCBI under BioProject number PRJNA293141. In order to characterize the clustering of sequences assigned as *Yersinia*, these sequence reads were aligned with PyNast (17) with selected reference sequences. The subsequent phylogenetic analysis was conducted with FastTree (18).
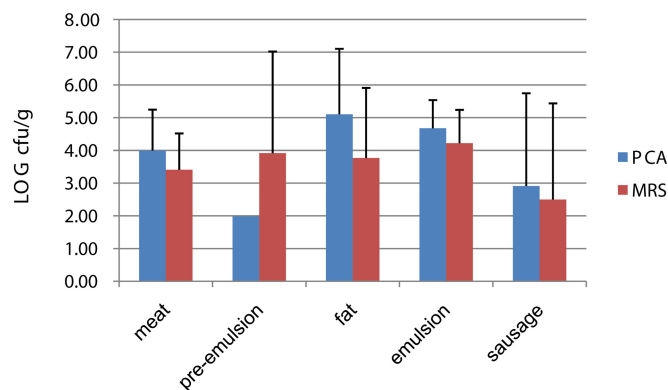
RESULTS

Results from cultivation and the outcome of DNA extraction of the surface samples from the processing plant are shown in Table 1. Figure 2 and Table S1 in the supplemental material present the results of similar analyses from 94 samples of raw materials and cooked sausages manufactured from them. Thirty, 63, and 9 samples yielded PCR products from the processing plant, raw material, and products, respectively, and these were subsequently sequenced. 16S rRNA analysis produced a total of 766,686 sequence reads. Since sequencing produced a varying number of good-quality reads from each sample (average, 4,819 ± 1,742 reads; in total, 443,335 reads), the data were rarified to 2,707, the lowest number of reads in samples, before diversity analysis.

16S rRNA gene sequencing showed the bacterial community in the sausages to be comprised mainly of *Firmicutes* (Fig. 3) and, in detail, of the genus *Leuconostoc* (Fig. 4A). The OTUs classified as leuconostocs were detected in low relative abundance (2% ± 5%) in raw meat and emulsion, as well as on the processing plant surfaces (<4% relative abundance) (Fig. 5). In the processing plant, surface samples with *Leuconostoc* levels of >2% of the sequence reads were obtained from the high-hygiene compartment where the sausages were packaged. Despite the low abundance on the processing plant surfaces and in raw materials, in the cooked sausages the *Leuconostoc* sequence abundance increased up to >98% (Fig. 5), with an average of 40% relative abundance (Fig. 4A).

Bacterial communities in the compartments dealing with raw products were mainly composed of bacteria from the orders *Pseudomonadales*, *Actinomycetales*, *Bacillales*, and *Lactobacillales* (Fig. 3). In the high-hygiene packaging area, the main bacterial orders were *Actinomycetales*, *Pseudomonadales*, *Lactobacillales*, *Clostridiales*, and *Enterobacteriales*. Detected microbial diversity was between 53 and 393 OTUs (average, 216 ± 78 OTUs) throughout the processing plant, and there was no substantial difference in diversities between the main compartments (see Table S1 in the supplemental material). Although phylogenetic assignment of OTUs showed that the microbiomes of different processing compartments differed, the processing plant samples did not show site-specific clustering in the UniFrac analysis (Fig. 6). Similar OTUs were found throughout the facility from both standard and high-hygiene compartments although differences in the relative abundances were detected. Based on the UniFrac results, the processing plant samples clustered together with the raw meat sam-
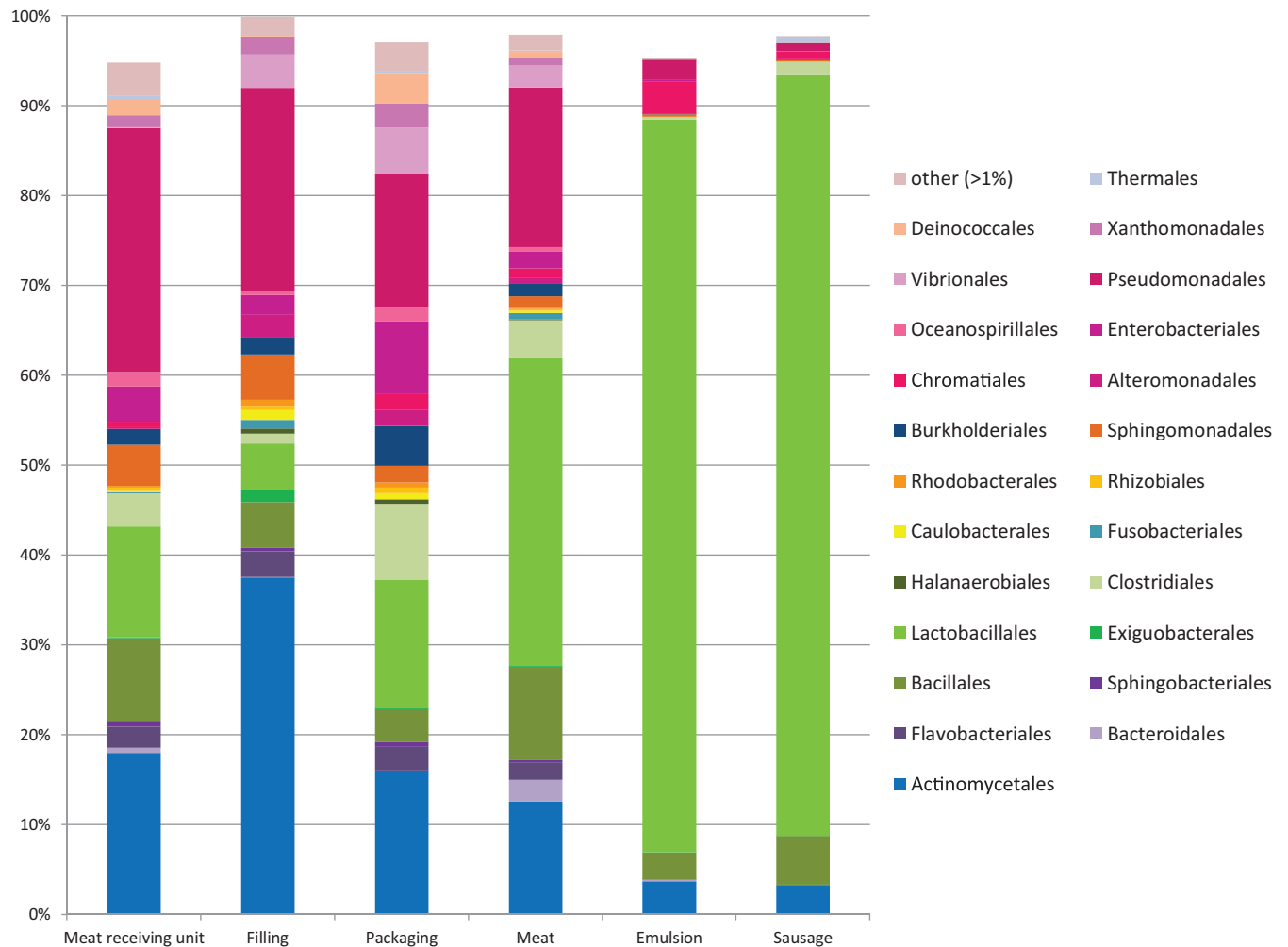
FIG 3 Order-level classification of the sequence reads by main compartment or material. Other, minor orders with abundance of <1%.

ples, whereas the sausage emulsion samples were more similar to the samples from cooked sausages (Fig. 6).

As the UniFrac analysis indicated, the microbial community profile of the raw materials (meat cut selections as well as rind and fat) used for the sausage manufacture was similar to that in the meat receiving unit (Fig. 3). The raw materials contained a significantly ($P \leq 0.001$) higher proportion than the meat receiving unit of bacterial sequences from the genera *Pseudomonas*, *Brochothrix*, *Carnobacterium*, *Arthrobacter*, and *Lactobacillus*. In the meat receiving unit, *Psychrobacter* and *Rhodococcus* were found to be more abundant ($P \leq 0.001$). The bacterial communities in the sausage emulsion and sausages at the end of the shelf life were composed mainly of *Lactobacillales* with lower relative abundance of gammaproteobacterial orders and *Actinomycetales* (Fig. 3; see also Fig. S1 in the supplemental material). However, at the genus level, the abundance of leuconostocs was found to be significantly ($P \leq 0.001$) higher in the sausages than in the sausage emulsion. In sausage emulsion, bacteria from the genera of *Streptococcus*, *Lactococcus*, and *Lactobacillus* were more abundant (Fig. 4A).

OTUs classified as *Yersinia* were observed in rather high abundance (up to 28%) on the processing plant surfaces but to a lesser extent in the raw meat, sausage emulsion, and cooked sausages

(relative abundance of <1%) (Fig. 4B and 5). The largest phylogenetic cluster was similar to that of the pathogenic *Yersinia pseudotuberculosis* (see Fig. S2 in the supplemental material), with one OTU comprising a total of 3,438 sequences (OTU denovo2230). When the abundance of this OTU on the processing line was studied, the highest relative abundance was found from the packaging area, with relative abundance of over 27% in two samples. The relative abundance in raw meat was low, below 1% (see Table S2 in the supplemental material). The other *Enterobacteriaceae* found from the sequenced OTUs included representatives from the genera of *Klebsiella* (0.02% to 1.2%), *Enterobacter* (0.1% to 0.3%), and *Morganella* (0.001% to 0.3%), but representatives from the genus *Yersinia* were by the far most abundant (Fig. 4B).

More detailed analysis of sequences assigned as *Bacillales* (Fig. 4C) revealed the *Listeriaceae* to be the main bacterial family. However, the genus *Brochothrix* was found to be the most abundant genus in the raw meat (Fig. 4C and 5), whereas bacteria from the genus *Listeria* were rare and detected only in low abundance (<0.01%) in all samples. Thus, the risk of *Listeria monocytogenes* contamination appeared low. Sequences assigned as *Clostridiales* were found throughout the processing plant and from the raw material and sausages. However, there were differences among the
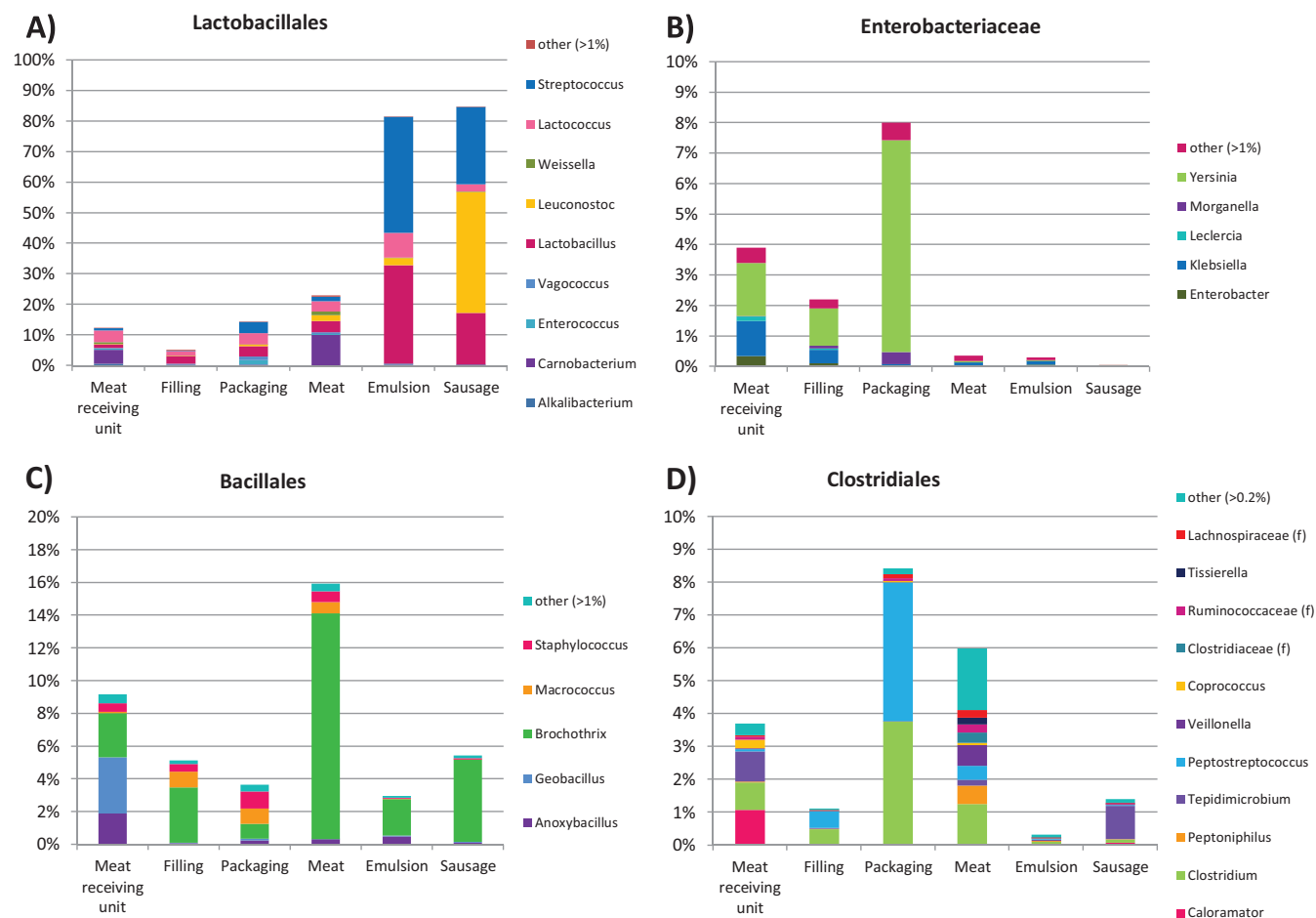
FIG 4 Relative abundances of sequence reads assigned as indicated. f, family-level classification.

abundant genera in this order: surface samples harbored *Peptostreptococcus*, *Clostridium*, and *Tepidimicrobium*. The meat receiving unit and raw meat had similar profiles with large diversity (Fig. 4D), and the clostridia found in the cooked sausages consisted mainly of *Tepidimicrobium*.

Differences were observed in the surface sample microbiotas before and after daily cleaning procedures. The highest *Leuconostoc* levels detected (up to 3.3%) were in samples taken after daily cleaning before the production started from the high-hygiene packaging compartment. In the samples taken during production, the relative abundance did not reach 1%. Similarly, the highest *Yersinia* and *Clostridia* abundances were found in the high-hygiene compartment before production. In the surface samples taken during production, the *Proteobacteria* levels were the highest (27% ± 26.7%).

On the processing plant surfaces, the highest LAB levels based on the MRS-A swabs (>100 colonies) (Table 1) were in the samples from the meat receiving unit of the plant during and before production at both of the sampling times. The number of colonies in the sausage-filling machinery ranged from 0 to >100 both before and during production. Intriguingly, the high-hygiene packaging compartment had LAB levels ranging from low (>10) to high (>100) both during production and before production was started. The high LAB levels were found during production from

the device separating the sausages from a string (sample Fa13), and lower levels were found from the transport belt and from the pit from the transport belt to the packaging tray (Table 1). Before production, the detectable bacterial growth was found in sample Fa28 from the same cutting drum blade.

In the raw materials and cooked sausages, both LAB (MRS) and total microbial (PCA) levels varied among different lots (Fig. 2). In raw meat and in the rind and associated fat, the total microbial levels (PCA) were 4.0 ± 1.3 and 5.1 ± 2 log CFU/g, respectively. In emulsion, the log CFU/g for total microbes was 4.7 ± 0.9, decreasing to 2.9 ± 2.8 in cooked sausages. The LAB log CFU/g levels on MRS plates varied from 3.4 to 3.8 (±1.1 to 3.1) in raw meat, increasing to 4.2 (±1.0) in emulsion. Based on cultivation, the LAB levels in the sausages at the end of shelf life varied from 0.0 to 9.7 log CFU/g (see Table S3 in the supplemental material). In 8 of the 14 batches, the sausages were found to have clear drip around them at the end of shelf life, and in these eight production lots the LAB levels were <10,000 CFU/g, except in two lots, where the levels had reached log 5.4 CFU/g (see Table S4 in the supplemental material). In two of the lots, the drip around the sausages was mildly turbid and contained 0.0 to 8.0 log CFU/g LAB cells. Four of the lots had high (7.4 to 9.7 log CFU/g) LAB levels, and the packages contained gray and turbid drip on the last day of shelf life. Additionally, in one of these the vacuum was loosened.
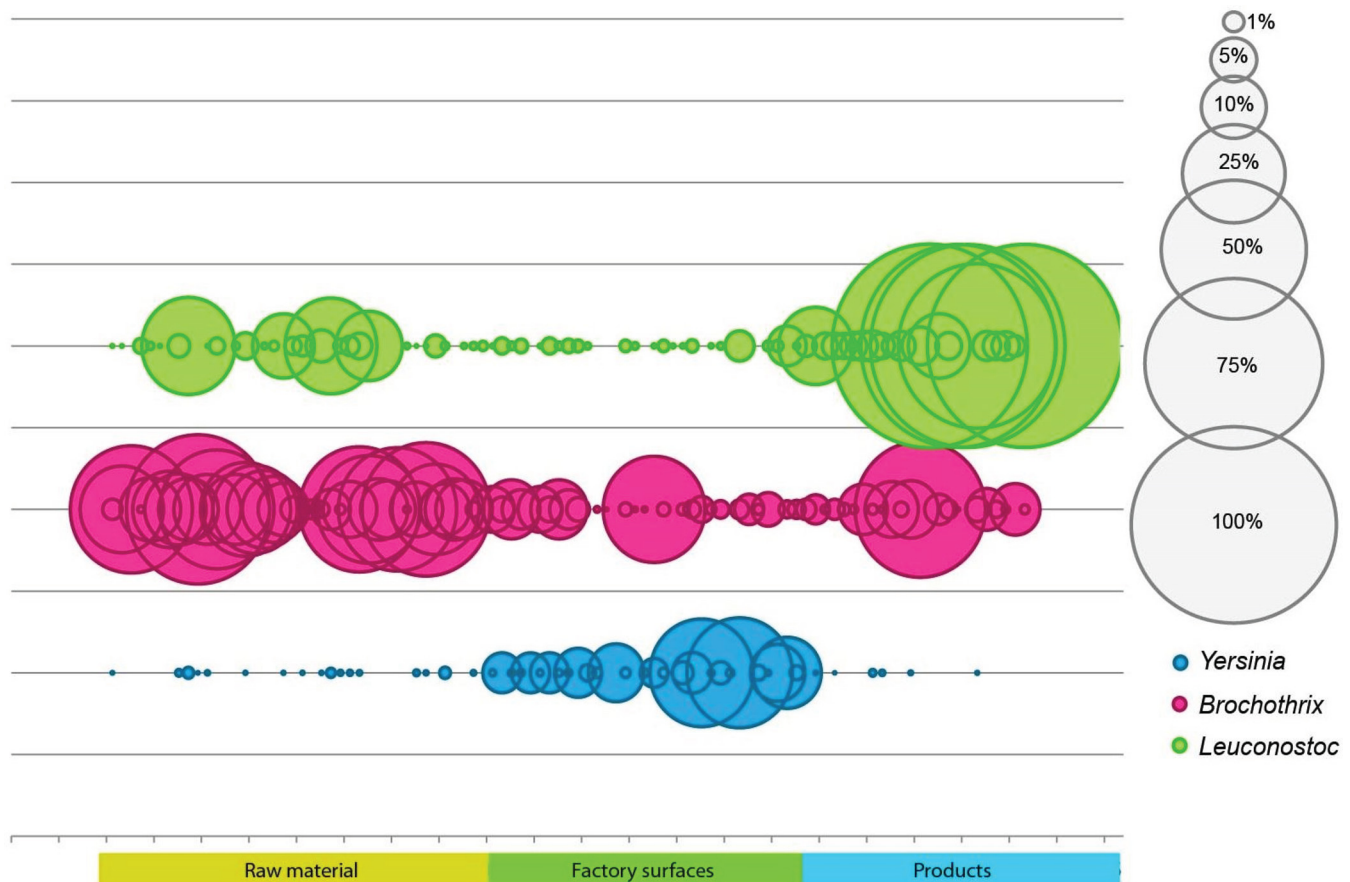
FIG 5 Relative abundance of *Leuconostoc*, *Yersinia*, and *Brochothrix* in raw materials, on processing plant surfaces, and in cooked sausages. Sphere size corresponds to relative abundance.

A total of 175 colonies from processing plant surfaces and 74 from material samples were ribotyped. The majority (41% from processing microbiome and 72% from materials) of these colonies represented species of the genus *Leuconostoc* (see Table S5 and Fig. S3 in the supplemental material). Additionally, bacterial cells were found in 3 of the 15 air samples (Table 1). The LAB species identified were *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc gelidum* subsp. *gasicomitatum*, *Lactobacillus curvatus*, *Lactococcus lactis*, and *Brochothrix* sp. (see Table S5 in the supplemental material).

DISCUSSION

The finding that bacteria from the phylum *Firmicutes* dominated the cooked sausage microbiome was expected since LAB are common spoilage organisms in MAP meat products (1). However, we did not anticipate that they would dominate the sausage emulsion microbiome as well (Fig. 3) since *Firmicutes* were not abundant in the raw materials. It is thus likely that the emulsification step with harsh mechanical mixing and the addition of salt and nitrite led to the selection of the Gram-positive bacteria. Exploration of the main genera (Fig. 3 and 4) within the processing plant 16S rRNA gene amplicons showed that all of the abundantly found genera contained mesophilic psychrotrophs. This is understandable as approximately 80% of the earth is cold, and most of the psychrotolerant microbes (optimal temperatures between 20°C and 25°C) on earth live in fluctuating temperatures (22). The surface samples

had high levels of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Bacteria from these phyla have been detected also in previous indoor microbiome studies (23–25). Intriguingly, of these phyla, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* comprise known cold-tolerant food spoilage bacteria (Fig. 3). Furthermore, the orders *Pseudomonadales* and *Actinomycetales* have been found in a butchery environment, beef carcass, and aerobically stored beef products (6). These microbes are thus likely to tolerate conditions such as the recurrent cleaning and sanitizing procedures prevailing in the processing plants.

There were dramatic differences between the 16S rRNA microbiomes of raw materials, emulsion, surfaces, and sausages (Fig. 3). *Firmicutes* were found in high abundance in all raw materials and sausage lots studied (Fig. 3; see also Fig. S1 in the supplemental material). The similarity of the emulsion and cooked sausage microbiomes was visualized by the UniFrac distances (Fig. 6), where, apart from the processing plant samples and most of the raw meat samples, the emulsion and sausage samples clustered together. The few raw meat samples clustering with the emulsion and sausages were from assorted meat chunks and chopped meat. The processing plant surface samples did not cluster based on the sampling location in the present study. Similar results were seen in a cooking center microbiome study (24), where the sampling location did not affect the clustering, and consequently samples from the same location had different microbiomes.
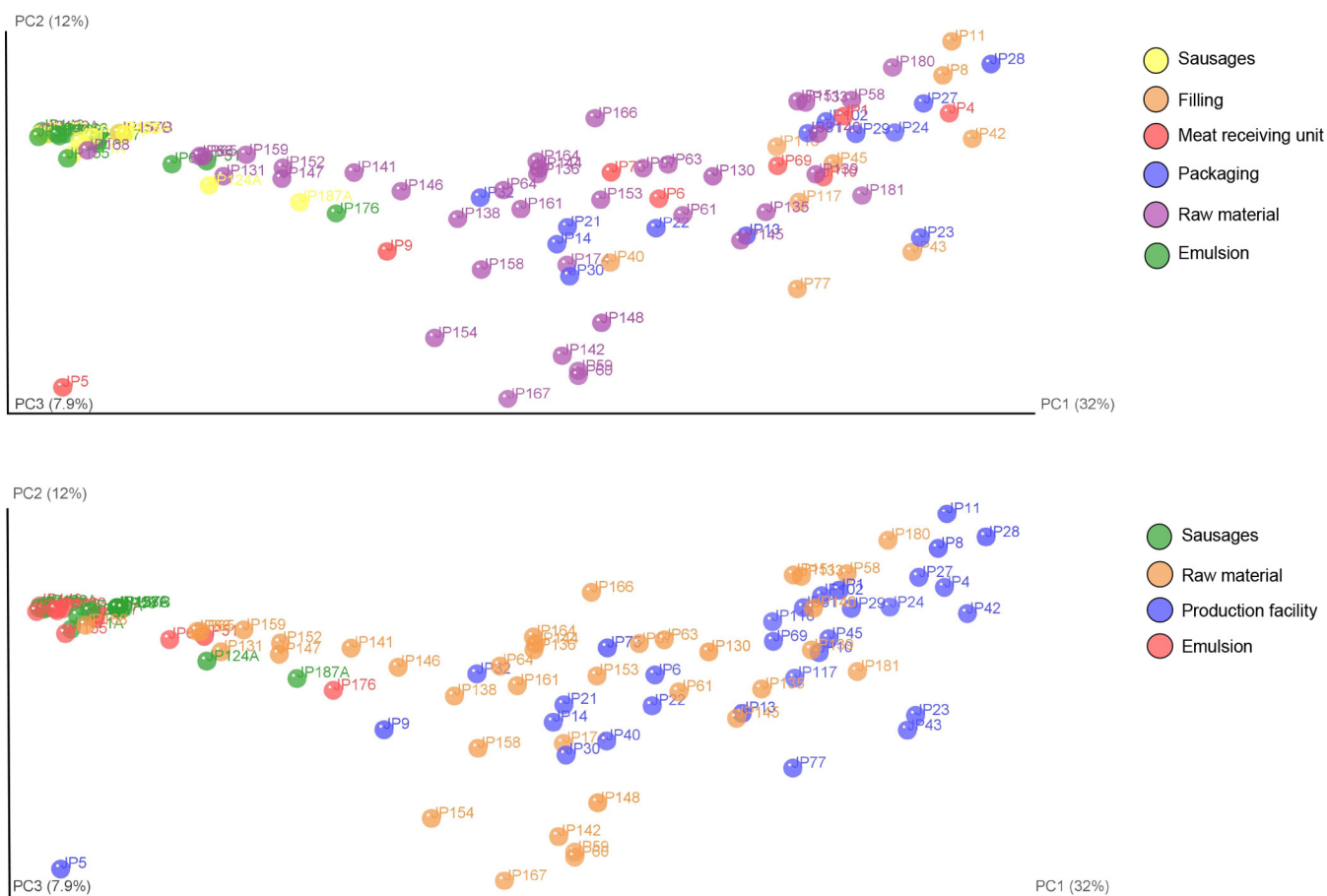
FIG 6 UniFrac analyses of the microbiomes of raw materials, sausage emulsion, and cooked sausages for facility compartments and material source (top panel) and source alone (bottom panel).

Leuconostoc spoilage is common in cold-stored, modified-at-mosphere-packaged (MAP) foods (1, 2, 26), and representatives from this genus have been causing severe food spoilage cases in Finland since 1996 (4, 27, 28). Recently, the same phenomenon has been detected in refrigerated ready-to-eat (RTE) foods, as well as in meat and vegetable products in Central Europe (29), although *L. gelidum* has not been detected abundantly in raw materials and production facility surfaces (30). Some *L. gelidum* strains have also been found to adhere well to food contact surfaces (31). In the present study, OTUs from the genus *Leuconostoc* prevailed in the products at the end of shelf life, as determined by both cultivation-dependent and -independent approaches. Nevertheless, the abundance on the surfaces based on the sequence data was low (<4% relative abundance), and thus the high abundance of leuconostocs in the product at the end of shelf life can be considered to be due to the efficient growth ability this species has on vacuum-packaged sausages (Fig. 2; see also Table S3 in the supplemental material).

These leuconostocs can evidently outcompete the other bacteria present in packaged foods. This is supported by growth ability in cold temperatures and low-oxygen MAP (28) and the ability to adhere to surfaces (31), which may help the bacteria tolerate the washes and disinfectant treatments. Similarly, several contaminating *Leuconostoc carnosum* strains were recovered from a meat processing environment, but only a single strain was found to

prevail in the spoiled ham produced at that plant (28). The predominance of this spoilage strain was concluded to be due to its physiological characteristics improving adaptation and competence in cooked, VP ham. The LAB identified by culturing from the production plant surfaces were considered to be *Leuconostoc gelidum* subsp. *gasicomitatum* and subsp. *gelidum*, *Lactobacillus sakei*, and *Carnobacterium maltaromaticum* (see Table S5 and Fig. S3 in the supplemental material), all known as food spoilage bacteria (1). The same species were retrieved using culturing also in both the raw materials and in the cooked sausages, supporting the view that they originated in the raw material (see Table S5).

The bacterial richness in the processing plant varied from 53 to 393 OTUs (see Table S1 in the supplemental material), showing much lower diversity in this controlled environment than in nature, as in temperate and cold soils microbial richness has been found to range from 338 to 725 OTUs (32). Some of the processing plant samples were interesting when the focus was on the estimated microbial diversity. The sample from a cart used for transporting sausages from the filling stage through cooking to the packaging had the highest Chao1 diversity values. This is the only device shared between the regular and high-hygiene compartments of the processing plant and could have acted as a vehicle for contamination. The microbial growth on the MRS-A swabs from this device was not detectable (Table 1), indicating the viable microbiota to be other than LAB, which is supported by the amplicon

sequencing (Fig. 3 and 4). Other interesting sites in the packaging area included the conveyor belt and the packaging tray, where the number of observed OTUs was higher, 313 and 301, respectively, than in the packaging area as a whole. The first sample had 1 to 10 colonies in the MRS-A plate, showing viable LAB in the site.

The high abundance of *Yersinia* bacteria in the processing plant surfaces was unexpected. *Yersinia* is usually associated with raw pork meat (33), and especially *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* have been detected in both pigs and surface samples from pig farms (34). As the OTUs from this genus were detected in only a few of the meat assortments, it can be considered that they had adhered well to the processing plant surfaces and might have formed biofilms. This is supported by the finding of these OTUs both before and after daily cleaning procedures. The fact that *Yersinia*-like OTUs were found from the packaging compartment raises concerns regarding food safety, but the sausage cooking and storage conditions seemingly prevented the growth of these species since they were not found in the cooked sausages. *Yersinia* bacteria are known to have from 5 to 6 copies of rRNA operons, and this could bias the results. However, the other main groups have several copies of the ribosomal operons (*Brochothrix* with 9 and *Leuconostoc* with 4). Thus, this bias can be considered not to have an impact on the results.

Like the *Enterobacteriales* order, to which *Yersinia* belongs, the other main bacterial orders dominating the processing plant surfaces and raw meat were not observed in the sausage emulsion or cooked sausages, based on the 16S amplicons. Also *L. monocytogenes*, a typical biofilm-forming bacterium in food processing plants with hygiene problems (35), was not among the sequenced OTUs. However, *Brochothrix thermosphacta*, a bacterium from the same family as *Listeria monocytogenes*, was detected in the raw meat (Fig. 5) but not in the cooked products on the last day of shelf life. This meat spoilage bacterium is commonly associated with the spoilage of fresh meats (1) and is capable of growing under both aerobic and anaerobic conditions (36). However, under anaerobic conditions, *B. thermosphacta* has not been observed at the end of the shelf life (37) since during microbial succession the abundance of LAB increases.

To conclude, the microbiota in this food processing plant had a different characteristic microbiome from the one grown in the cooked sausages during product shelf life. The main bacterial phyla colonizing this processing plant, the raw materials, and cooked sausages comprised known food-spoilage-causing psychrotrophs. Despite the different hygiene practices, microbiota in the standard and high-hygiene compartments did not differ drastically (Fig. 3 and 4). Only the genera of *Yersinia* and *Peptostreptococcus* were significantly more abundant in the high-hygiene compartments. The microbes on the processing plant surfaces can be occasional contaminants, or, as in the case of *Yersinia*, they may have become resilient. Nevertheless, the main genera observed with food safety and quality interest had their own characteristic patterns of contamination, suggesting different food safety risk profiles. *Yersinia* bacteria were detected on the surfaces but not in the cooked sausages, yet in the raw materials, *Brochothrix* was one of the most abundant genera, whereas *Leuconostoc* was scarcely present in the surfaces and raw materials. Based on the observed abundance patterns, the leuconostocs do not cause spoilage of the products due to high initial contamination levels since they were not abundant in the raw materials or on surfaces but are evidently able to grow during microbial succession under the packaging and storage con-

ditions used. This shows that there is a need to develop total enumeration-based hygiene monitoring tools toward more specific ones. The abundance patterns revealed here increased our understanding of different contamination patterns of psychrotrophic processing plant microbiota and their implications for food spoilage and safety.

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