Microbial background flora in small-scale cheese production facilities does not inhibit growth and surface attachment of *Listeria monocytogenes*

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ABSTRACT

The background microbiota of 5 Norwegian small-scale cheese production sites was examined and the effect of the isolated strains on the growth and survival of *Listeria monocytogenes* was investigated. Samples were taken from the air, food contact surfaces (storage surfaces, cheese molds, and brine) and noncontact surfaces (floor, drains, and doors) and all isolates were identified by sequencing and morphology (mold). A total of 1,314 isolates were identified and found to belong to 55 bacterial genera, 1 species of yeast, and 6 species of mold. *Lactococcus* spp. (all of which were *Lactococcus lactis*), *Staphylococcus* spp., *Microbacterium* spp., and *Psychrobacter* sp. were isolated from all 5 sites and *Rhodococcus* spp. and *Chryseobacterium* spp. from 4 sites. Thirty-two genera were only found in 1 out of 5 facilities each. Great variations were observed in the microbial background flora both between the 5 producers, and also within the various production sites. The greatest diversity of bacteria was found in drains and on rubber seals of doors. The flora on cheese storage shelves and in salt brines was less varied. A total of 62 bacterial isolates and 1 yeast isolate were tested for antilisterial activity in an overlay assay and a spot-on-lawn assay, but none showed significant inhibitory effects. *Listeria monocytogenes* was also co-cultured on ceramic tiles with bacteria dominating in the cheese production plants: *Lactococcus lactis*, *Pseudomonas putida*, *Staphylococcus equorum*, *Rhodococcus* spp., or *Psychrobacter* spp. None of the tested isolates altered the survival of *L. monocytogenes* on ceramic tiles. The conclusion of the study was that no common background flora exists in cheese production environments. None of the tested isolates inhibited the growth of *L. monocytogenes*. Hence, this study does not support the hypothesis that the natural background flora in cheese production environments inhibits the growth or survival of *L. monocytogenes*.

Key words: flora, cheese, small scale, *Listeria monocytogenes*

INTRODUCTION

To produce safe food for consumers, food producers have a strong focus on hygiene during production. Cleaning and disinfection are the most common means of obtaining safe production environments, but even though daily cleaning and disinfection will significantly reduce the bacterial load in the production environment, surfaces are rarely sterile (Langsrud et al., 2012). At the same time, although most food products (including meat, fish, fruits, and vegetables) are produced or processed in clean environments, food types exist that are produced in the presence of a rich microbial background flora. In the production of cheese, disinfection is often avoided or performed rarely in the storage facilities to preserve a bacterial background flora, as it is considered to be important for the quality of the product (Retureau et al., 2010).

In recent years, cheese and other milk products have been the source for several outbreaks of listeriosis (Carrique-Mas et al., 2003; Gaulin et al., 2003; Makino et al., 2005; Bille et al., 2006; Swaminathan and Gerner-Smidt, 2007; Vít et al., 2007; Fretz et al., 2010; Johnsen et al., 2010; Gaulin et al., 2012). *Listeria monocytogenes*, the causative agent of listeriosis, is known to survive and grow well under conditions that inhibit a range of other bacteria, including low temperatures and high salt concentrations (Larson et al., 1999; Lado and Yousef, 2007). It does, however, not grow well at low pH (Lado and Yousef, 2007) and the presence of lactic acid bacteria, both naturally occurring and added during production, would hence inhibit further growth of *Listeria* in the cheese during the ripening period. However, as surface cultures (molds or bacteria) are commonly added during ripening of the cheese, a rise in pH may occur and conditions may allow for growth of *Listeria* to high numbers, especially close to the cheese surface (Bell and Kyriakides, 2005).

As *Listeria* is killed by pasteurization (Lado and Yousef, 2007), the main cause for the presence of vi-

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able *Listeria* in pasteurized milk and cheese from pasteurized milk is recontamination from the processing environment. It has been shown that *L. monocytogenes* is able to survive and persist in food production environments (Romanova et al., 2002; Di Bonaventura et al., 2008) and due to this, may pose a threat to food safety. Unpasteurized milk may contain *Listeria* even if no source for recontamination from the cheese production environment exists (Bemrah et al., 1998) but it has been shown that the bacterial background flora in raw milk may inhibit the growth of *L. monocytogenes* (Schvartzman et al., 2011). It is hence of interest to investigate whether the naturally occurring background flora in the cheese production environment may also have a protective effect against *Listeria*.

To establish whether the microbial background flora in the cheese production environment may have any protective effect against *L. monocytogenes*, it is necessary to know the composition of this flora. While many studies have focused on the bacterial flora in raw milk or cheese (Retureau et al., 2010; Fricker et al., 2011), few have studied the background flora in the whole cheese production environment, despite the fact that the flora on materials in direct contact with cheese has been shown to vary at different locations (Licitra et al., 2007; Mariani et al., 2007). Several studies have shown that various bacterial species can influence survival, growth, and adherence of *L. monocytogenes* to surfaces (Leriche et al., 1999; Leriche and Carpentier, 2000; Carpentier and Chassaing, 2004; Guðbjörnsdóttir et al., 2005), but no direct connection has been made between the common background flora in cheese-producing environments and the growth and survival of *L. monocytogenes* in production environments. This study shows in detail the composition of the microbial background flora in 5 Norwegian small-scale cheese production sites and how the isolated strains influence the growth of *L. monocytogenes* in a model system and in co-culture biofilms.

MATERIALS AND METHODS

Sampling and Microbial Culture Conditions

Five small-scale cheese-producing farms (ranging from 1 to 7 employees and production volumes from 2 to 10 t per year) in Norway were visited in the period between February and May 2010. All farms had their own milk production and used one set of production equipment to produce several types of cheese, including at least 1 type of soft cheese (Camembert or Brie type). Ripening rooms contained more than 1 type of cheese at all producers, but sampling was conducted in areas adjacent to soft cheeses. Four farms used pasteurized

milk in their production, whereas 1 farm (producer 4) used unpasteurized milk. All farms produced cheeses that were salted in brines and inoculated with surface molds. At each farm, 8 sites were sampled (Supplemental Table S1, available online at <http://dx.doi.org/10.3168/jds.2012-6395>) using sampling swabs in Lethen broth (3M Norge AS, Skjetten, Norway) as well as at least 1 out of 2 *Listeria*-selective all-in-one swabs (InSite *Listeria* Test; Labolytic, Trondheim, or the Path-Chek Hygiene *Listeria*; Interfarm AS, Asker, Norway). In addition, plate count agar (PCA) plates (Oxoid Ltd., Basingstoke, UK) were placed without lids for 10 min in the production rooms and the ripening rooms for air samples. Brines (1 mL) were sampled directly by pipetting. For each selected location, 2 areas of 5 × 5 cm within 10 cm distance were swabbed thoroughly and the swabs were kept in a cooling bag until they reached the laboratory within 24 h after sampling. Appropriate dilutions of the Lethen broth and the brine were plated on PCA and the plates were incubated aerobically at 20°C for 5 d. The *Listeria*-selective swabs were kept following the manufacturer's instructions and examined for color changes after 1 and 2 d as indicated in the manuals.

Determination of the Microbial Flora: Bacteria

From each sample, 24 colonies were picked randomly from PCA plates and subjected to partial sequencing of the 16S ribosomal DNA (rDNA) gene to identify the isolated strains. All colonies were recultured on PCA for 3 d at 20°C to rule out contaminations. From these plates, 1 to 15 colonies (15 for small colonies to ensure enough cell material) were picked and mixed with Tryptone soy broth (TSB; 45 µL; Oxoid Ltd.), guanidine thiocyanate (GTC; 4 mol/L, 135 µL; Merck KGaA, Darmstadt, Germany) and Mag Prep Silica Particles (10 µL; Merck KGaA) in wells of a 96-well Greiner U plate (Greiner Bio-One GmbH, Frickenhausen, Germany). The DNA was isolated on a Biomek 2000 Workstation (Beckman Coulter Inc., Fullerton, CA) using magnetic silica particles and sarkosyl (Skånseng et al., 2006). Polymerase chain reaction of the 16S rDNA was conducted using DyNAzyme II Hot Start Polymerase (Finnzymes, Espoo, Finland) with primers MangalaF and MangalaR (Nadkarni et al., 2002) according to the producer's instructions. The PCR products were cleaned using ExoSAP-IT (USB Europe GmbH, Stauf, Germany). Sequencing PCR was carried out using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions, with primer MangalaF. Samples were prepared for sequencing by using the Applied Biosystems BigDye XTerminator

Purification Kit (Applied Biosystems Inc.) according to the producer's instructions and sequenced by a 3130xl Genetic Analyzer (Applied Biosystems Inc.). Acquired sequences (350–450 readable bp) were compared with known sequences using the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp, 06/2010) and bacteria were identified to the genus level and the species level where possible.

Determination of the Microbial Flora: Yeast

Samples that did not yield PCR products after 16S rDNA PCR were by microscopy confirmed to be yeasts. Fresh yeast colonies were picked from PCA plates and subjected to a thermal treatment of 30 min at -80°C and 60 min at 65°C . The 26S rDNA PCR was carried out using AmpliTaq Gold Hot Start Polymerase (Roche Diagnostics GmbH, Penzberg, Germany) and primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') at the following cycling conditions: 10 min at 95°C , 38 cycles of 30 s at 95°C , 30 s at 56°C , and 1 min at 72°C , followed by a final extension period of 7 min at 72°C . The PCR products were treated as described for bacteria, and sequencing PCR was carried out using primer NL1. Sequencing and database searches were carried out as described for bacteria.

Determination of the Microbial Flora: Mold

Mold colonies were transferred to malt extract agar (MEA) and potato dextrose agar (PDA; Samson et al., 2010). The inoculated agar media were incubated for 7 d in the dark at $25 \pm 1^{\circ}\text{C}$ and inspected for genus identification using macro- and microscopic morphological characters. The identified genera were then subcultured on suitable agar plates for species identification. Isolates of *Penicillium* were plated on MEA, Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES), creatine sucrose agar (CREA), and nitrite sucrose agar (NO_2), as described in Samson et al. (2010). Other isolates were plated on MEA and PDA. The MEA, CYA, YES, and PDA were incubated in the dark at $25 \pm 1^{\circ}\text{C}$, whereas CREA and NO_2 at were incubated at $20 \pm 1^{\circ}\text{C}$ for 7 d.

The mold isolates were identified at species level using a polyphasic approach (Samson and Frisvad, 2004). To differentiate certain species of *Penicillium*, the Ehrlich test was performed (Samson et al., 2010). All the isolates were identified according to Pitt (1979), Pitt (1999), Samson and Frisvad (2004), and Samson et al. (2010).

Molecular identification by sequencing the internal transcribed spacer (ITS) regions of fungal DNA was

performed for some molds when the traditional method of identification was judged to be inefficient. Sequencing was also used for verifying the identification of the dominant mold isolates. The DNA was extracted by the cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Murray and Thompson, 1980). Briefly, one 4-mm loopful of mold cells from a pure culture was transferred to a 1.5-mL Eppendorf tube containing 600 μL of CTAB buffer. The cell mixture was frozen at -80°C for about 20 min, homogenized, and resuspended in 400 μL of phenol-chloroform. After centrifuging the mixture at $17,530 \times g$ for 10 min, the upper aqueous phase was transferred to a new Eppendorf-tube. The DNA was then precipitated by adding 300 μL of isopropanol by centrifuging at $17,530 \times g$ for another 10 min. The DNA pellet was washed once in 70% ethanol, resuspended in 100 μL of Milli-Q water (EMD Millipore Corp., Billerica, MA) before being used as a template.

The fungus-specific universal primers ITS1 and ITS4 were used to amplify genes encoding the ITS region (White et al., 1990). Primers encoding the β -tubulin gene, Bt2a and Bt2b (Glass and Donaldson, 1995), were used in addition. Ready-to-go-PCR beads (GE Healthcare, Buckinghamshire, UK) containing PuRe-Taq polymerase were used and PCR was carried out at the following conditions: 10 min at 95°C , 38 cycles of 30 s at 95°C , 30 s at 56°C , and 1 min at 72°C , and a final extension at 72°C for 7 min. The PCR products were purified by ExoSAP-IT PCR Clean-Up Kit (GE Healthcare). Sequencing was performed using an ABI Prism automated DNA sequencer (Applied Biosystems Inc.). A BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems Inc.) was used following the manufacturer's manual on both strands by the same primers. Sequence comparisons were performed using the basic local alignment search tool (BLAST) in GenBank (<http://www.ncbi.nlm.nih.gov/blast>) after editing and trimming the sequences by BioEdit sequence alignment editor (version 7.0.0; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Overlay Agar Assay

A total of 63 microbial isolates (at least 1 of each identified genus; Table 1) were tested individually for inhibitory effects on the growth of *L. monocytogenes* MF3638 [serotype 1/2a; Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima; Ås, Norway) strain collection], the strain isolated from the Norwegian outbreak in 2007 (Johnsen et al., 2010). Each strain to be tested was cultured in plate count broth (prepared manually as PCA but without the agar) at 25°C for 2 d. The PCA plates were inoculated

Table 1. Overview of strains that were tested for antilisterial activity in an overlay assay¹

Species	Producer	Sampling point ²	MF no. ³
<i>Achromobacter</i> spp. ⁴	3	RF	3808
<i>Acidovorax</i> spp.	4	PF	3771
<i>Acinetobacter</i> spp.	2	PD	3737
<i>Aerococcus</i> spp.	2	I	3739
<i>Arthrobacter</i> spp.	1	I	3738
<i>Brachybacterium</i> spp.	2	PD	3766
<i>Brevibacterium</i> spp.	4	I	3753
<i>Brevundimonas</i> spp.	4	PD	3743
<i>Carnobacterium maltaromaticum</i>	1	RD	3744
<i>C. maltaromaticum</i> ⁴	1	RD	3801
<i>Carnobacterium</i> spp.	2	I	3745
<i>Cellulosimicrobium</i> spp.	3	I	3765
<i>Chryseobacterium</i> spp.	3	RD	3740
<i>Curvibacter</i> spp.	4	PF	3768
<i>Debaryomyces hansenii</i>	5	RS	3774
<i>Dermacoccus</i> spp.	4	I	3752
<i>Devosia</i> spp.	3	RF	3764
<i>Dyadobacter</i> spp.	5	RF	3786
<i>Escherichia coli</i>	2	PD	3733
<i>Enterobacter</i> spp.	1	PF	3761
<i>Enterococcus casseliflavus</i>	2	PD	3749
<i>Enterococcus faecalis</i> ⁴	4	RS	3800
<i>Enterococcus</i> spp.	2	PF	3750
<i>Enterococcus</i> spp. ⁴	2	PF	3798
<i>Ewingella americana</i> ⁴	3	RF	3804
<i>Flavobacterium</i> spp.	1	RD	3751
<i>Geobacillus stearothermophilus</i>	2	PF	3732
<i>Haloanella</i> spp.	5	RF	3782
<i>Janibacter</i> spp.	2	I	3763
<i>Janthinobacterium</i> spp.	3	RD	3734
<i>Kocuria</i> spp.	4	RF	3731
<i>Lactococcus lactis</i>	3	S	3718
<i>Lactococcus lactis</i> ⁴	5	S	3792
<i>Leifsonia</i> spp.	3	PF	3756
<i>Leuconostoc mesenteroides</i>	5	RF	3783
<i>Leuconostoc pseudomesenteroides</i> ⁴	5	S	3793
<i>Listeria</i> spp.	5	RS	3773
<i>Methylobacterium</i> spp.	4	PF	3769
<i>Microbacterium gubbeenense</i> ⁴	3	RS	3807
<i>Microbacterium</i> spp.	3	RD	3724
<i>Microbacterium</i> spp.	5	RF	3781
<i>Nesterenkonia</i> spp.	4	RF	3736
<i>Ochrobactrum</i> spp.	3	PD	3757
<i>Paenibacillus</i> spp.	5	RS	3772
<i>Pedobacter</i> spp.	3	PD	3758
<i>Pelomonas</i> spp.	1	PD	3762
<i>Plantibacter</i> spp.	3	RF	3755
<i>Propionibacterium</i> spp.	4	PF	3770
<i>Pseudomonas putida</i>	3	RF	3720
<i>Pseudomonas</i> spp. ⁴	4	RS	3796
<i>Psychrobacter</i> spp.	4	RD	3729
<i>Psychrobacter</i> spp.	5	RF	3778
<i>Rhodococcus erythropolis</i>	3	RD	3727
<i>Rothia</i> spp.	5	RF	3785
<i>Sanguibacter</i> spp. ⁴	3	RF	3809
<i>Shewanella putrefaciens</i>	3	I	3742
<i>Sphingobacterium</i> spp.	1	RF	3754
<i>Sphingomonas</i> spp.	2	I	3759
<i>Staphylococcus equorum</i>	2	I	3723

Continued

Table 1 (Continued). Overview of strains that were tested for antilisterial activity in an overlay assay¹

Species	Producer	Sampling point ²	MF no. ³
<i>Staphylococcus</i> spp.	4	RS	3722
<i>Stenotrophomonas maltophilia</i> ⁴	4	PD	3805
<i>Streptococcus</i> spp.	2	PD	3735
<i>Tsukamurella</i> spp.	4	I	3746

¹Strains in bold characters showed slight inhibition (zones of 2 to 3 mm) of *Listeria monocytogenes* MF3638.

²RF = floor in the ripening room; PF = floor in the production room; PD = drain in the production room; I = rubber insulation around doors; RD = drain in the ripening room; RS = ripening room shelves; S = salt brines.

³MF no. refers to the Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima; Ås, Norway) strain collection.

⁴Strains isolated from *Listeria*-selective enrichment media, not directly from swab platings.

with spots of 1 μ L each of the strains to be tested and incubated for 48 h at 25°C. An overnight culture of *L. monocytogenes* in plate count broth (300 μ L) was mixed with 8 mL of PCA soft agar. The mixture was deposited on the spotted plates and the plates were incubated at 37°C for 24 h before measuring the inhibition zone.

Spot-on-Lawn Assay

The method was adapted from Larsen and Knøchel (1997). The PCA plates were inoculated with a diluted overnight culture of *L. monocytogenes* (500 μ L; approximately 10^7 cfu/plate) and allowed to dry on a sterile workbench for 15 min. Overnight cultures (5 μ L) of the strains to be tested, as described for the overlay assay, were spotted onto this layer of *L. monocytogenes* and the plates were incubated at 20°C for 3 d before measuring the inhibition zone.

Co-Culture Biofilm Assay

The method was adapted from Carpentier and Chassaing (2004). Pressed ceramic stoneware tiles (catalog no. 1050/12; Höganäs Céramique France SA, Thouaré-sur-Loire, France) were cut to pieces (2.5 $\times$ 4 cm) and a wall of transparent bathroom silicone (silicone bâtiment translucide; Fischer SAS., Strasbourg, France) was applied, resulting in a working surface of approximately 7.5 cm². Five bacterial isolates from the production sites were selected for the experiments: *Lactococcus lactis* (MF3719), *Pseudomonas putida* (MF3721), *Staphylococcus equorum* (MF3723), *Rhodococcus* spp. (MF3727), and *Psychrobacter* spp. (MF3729). With the exception of the *Pseudomonas* strain, the isolates belonged to genera or species found in at least 4 of the 5 visited sites and were part of the dominating flora in the storage environments.

The strains were cultured in brain heart infusion (BHI; Oxoid Ltd.) for 3 d at 20°C, with the exception of *L. monocytogenes*, which was cultured for 24 h at 37°C. All bacterial suspensions used in the biofilm assay were made by diluting the respective cultures in 1% skim milk (Merck) to a bacterial concentration of approximately 10^6 cfu/mL. One milliliter of bacterial test suspension of each of the isolates from cheese environments was added to 6 to 8 coupons and left at 12°C for 3 h for cell attachment. The bacterial suspension was removed carefully by pipetting and the coupons were stored at 12°C and 98% relative humidity (RH) for 3 d and rinsed carefully by pipetting sterile water (3 $\times$ 25 mL) on the coupons to remove unattached cells. After rinsing, 1 mL of freshly prepared *L. monocytogenes* suspension (also 10^6 cfu/mL in 1% skim milk) was added to the coupon and left at 12°C for 3 h. The remaining suspension was removed carefully by pipetting to remove unattached cells and the coupons were stored at 12°C and 98% RH for 9 d. On the last day, coupons were again rinsed with 3 $\times$ 25 mL of sterile water to remove unattached cells and swabbed with a cotton swab. The swab was stored in 1 mL of peptone water and vortexed vigorously to remove cells from the swab. Suitable dilutions of the peptone water were plated on Brilliance *Listeria* Agar (Oxoid Ltd.) and BHI to obtain *L. monocytogenes* numbers and total bacterial numbers, respectively. The experiment was carried out 3 times with freshly prepared cultures each time.

RESULTS

Characterization of the Microbial Background Flora

Listeria monocytogenes was not found in any of the production sites. Total bacterial counts generally appeared to be higher in the ripening room than in the production room (1–4 log₁₀ cfu/cm²) and higher in drains than on floors (1–2 log₁₀). Direct quantita-

tive comparisons were, however, difficult due to uneven surfaces, especially in drains, that made it hard to standardize the sampling areas. No bacteria were detected on the ready-to-use plastic cheese molds. A total of 1,314 isolates were identified. The complete results of the microbial survey are presented in Supplemental Table S1 (available online at <http://dx.doi.org/10.3168/jds.2012-6395>). Fifty-five bacterial genera, 1 species of yeast (*Debaryomyces hansenii*), and 6 species of mold (*Penicillium camemberti*, *Penicillium roqueforti*, *Penicillium commune*, *Penicillium echinulatum*, *Cladosporium cladosporioides*, and *Lecanicillium lecanii*) were isolated. Only 4 of the bacterial genera (*Lactococcus* spp., *Staphylococcus* spp., *Microbacterium* spp., and *Psychrobacter* spp.) were found in all 5 sites, but more than 80% of the total bacteria found comprised 11 genera (*Pseudomonas* spp., *Rhodococcus* spp., *Kocuria* spp., *Geobacillus* spp., *Escherichia coli*, *Brevibacterium* spp., and *Janthinobacterium* spp. in addition to the aforementioned 4). Thirty-two genera were found in single facilities only. Results showed great variations in the microbial composition both between and within all sampled production sites. Figures 1 and 2 illustrate the differences in microbial background flora at corresponding sampling points at various producers. Within most production sites, the dominating bacterial genera were similar in drains and on corresponding floors, as illustrated in Figure 3. In general, the background flora

was less diverse in the salt brine and on cheese storage surfaces than in other sampling sites. The material of the cheese storage surfaces did not appear to have any influence on the bacterial composition, as no significant differences were found between wooden and synthetic shelves. Also, no significant differences were observed between metal and stoneware drains.

Effect of Single Strains on the Growth of *Listeria monocytogenes*

A total of 63 strains, at least 1 of each bacterial and yeast species or genus isolated from the production sites, were tested for antilisterial activity in an overlay assay and in a spot-on-lawn assay. Twenty-four out of the 63 tested strains showed slight inhibition of the growth of *L. monocytogenes* (Table 1). However, although a known bacteriocin producer (*Lactobacillus plantarum* Alc01; Danisco, Copenhagen, Denmark) yielded inhibition zones with a radius of 1.2 to 1.5 cm, none of the tested mold, yeast, or bacteria strains yielded inhibition zones with a radius larger than 2 to 3 mm, indicating that the inhibitory effect was comparably small.

Co-Culture Biofilms

Listeria monocytogenes was cultured on ceramic tiles that had previously been inoculated with a strain of *Lc.*

Figure 1. Microbial background flora profile on the storage surfaces in contact with cheese in the storage rooms of 5 different producers [n = 14 (producer 1), n = 48 (producer 2), n = 48 (producer 3), n = 34 (producer 4), n = 46 (producer 5)]. *E. coli* = *Escherichia coli*.

Figure 2. Microbial background flora profile on the floor of the storage rooms of 5 different producers [n = 46 (producer 1), n = 24 (producer 2), n = 48 (producer 3), n = 46 (producer 4), n = 48 (producer 5)]. *E. coli* = *Escherichia coli*.

Figure 3. Microbial background flora profile at various sampling points at producer 1. Sampling points were as follows: RS = ripening room shelves, RF = floor in the ripening room, RD = drain in the ripening room, PF = floor in the production room, PD = drain in the production room, I = rubber insulation around doors, S = salt brines [n = 14 (RS), n = 46 (RF), n = 42 (RD), n = 43 (PF), n = 48 (PD), n = 48 (I), n = 24 (S)].

lactis, *Pseudomonas putida*, *Staphylococcus equorum*, *Rhodococcus* spp., or *Psychrobacter* spp. Test strains reached concentrations between 3.7×10^4 and 1.1×10^5 cfu/cm² before *L. monocytogenes* was added to the coupons. The initial *L. monocytogenes* concentration after the remaining suspension had been removed was 2 to 3×10^3 cfu/cm². Results showed that *L. monocytogenes* grew to concentrations of between 3.5×10^3 and 6.7×10^4 cfu/cm² within 9 d of storage, regardless whether it was cultured alone or together with one of the tested single strains.

DISCUSSION

The microbial background flora was investigated at 5 small-scale cheese production sites that were spread across Norway. The aim of the study was to determine if cheese production environments have a common background flora and whether this flora can potentially affect survival, growth, or surface colonization of *L. monocytogenes*. Of the 55 different bacterial genera and genera that were isolated from the production sites, only 4 were present in all 5 sites, whereas 32 were only found in 1 of the sites. It is hence not likely that the latter occur widely as a regular part of the background flora in cheese-producing environments. Because *Lc. lactis* is commonly used as a starter culture, its presence was expected.

Plate count agar was used as the growth medium, as it contains casein and has been shown to support the growth of most dairy-related bacteria (Bore and Langsrud, 2005). Still, this study was limited to culturable cells. Unculturable cells may have effects on the growth and survival of *L. monocytogenes* that cannot be accounted for by this study (Quigley et al., 2011). Among the 11 bacterial genera that comprised more than 80% of all bacteria isolated, 7 were isolated from at least 3 farms. Notably, more than 90% of all isolated *E. coli* were found at the one farm that used unpasteurized milk for cheese production. Also, 44% of all *Staphylococcus* isolates and 71% of all *Kocuria* isolates were found at this farm. As only 1 of the included farms used unpasteurized milk, it was not possible to conclude that the presence of these bacteria is common at this kind of producer. It is, however, possible that the source of these bacteria was the raw milk, as these types of bacteria have been reported to be present in raw milk (Fricker et al., 2011; Van Kessel et al., 2011). *Lactococcus lactis*, which was frequently found at most sampling points at all sites using pasteurized milk, was found only in the brine at the site using unpasteurized milk, but not in any other environmental sites.

As expected, the greatest diversity in the flora was found in places where cleaning was less thorough. This included drains and rubber insulations of doors where 35 and 26 different bacterial genera were found, respectively. The smallest diversity in the bacterial flora was found in the brines, where only 6 different bacterial species or genera (*Lc. lactis*, *Staphylococcus* spp., *Psychrobacter* spp., *Microbacterium* spp., *Geobacillus stearothermophilus*, and *Leuconostoc pseudomesenteroides*) were present and on the storage shelves, where 9 bacterial species or genera could be found in addition to 1 species of yeast and 6 species of mold.

The small variation of the bacterial composition in the brines was most likely caused by the harsh conditions (pH ranging from 4.5 to 6.0 and salt concentrations between 15 and 27%) that did not allow for growth of many of microorganisms. *Staphylococci* are known to tolerate high salt concentrations (Bergdoll, 1989), as well as strains of *Microbacterium gubbeenense* (Bockelmann et al., 2003) and various *Leuconostoc* strains (Nieto-Arribas et al., 2010). Some *Psychrobacter* species have been related to survival at low temperatures and low water activity, corresponding to high salt concentrations (Ponder et al., 2008). However, none of these species are expected to grow at salt concentrations above 15%. With the exception of *L. pseudomesenteroides*, all strains that were found in the brines were also found in the surrounding environment. It is hence likely that these strains were also present on the product, and the brines are replenished with these bacteria from the cheese over time. *Lactococcus lactis* dominated the flora in 4 of the 5 brines. In the fifth brine, *Staphylococcus* spp. dominated. Although the salt concentration in this brine was within the range of the other 4 brines, measurements showed that the brine dominated by *Staphylococcus* spp. had the highest pH value (6.01, whereas the others ranged from 4.54 to 5.52). This may be the reason for why *Staphylococcus* spp. were able to survive and compete with the lactic acid bacteria.

Mariani et al. (2007) isolated bacteria from wooden shelves and found leuconostocs, enterococci, pseudomonads, and lactobacilli, in addition to staphylococci, at 8 out of 8 tested farms in France. In an Italian study, streptococci, lactobacilli, enterococci, and lactococci were found in 4 out of 4 tested wooden Tina vats (Lortal et al., 2009). In our study, lactococci, streptococci, staphylococci, and pseudomonads were also found on cheese contact surfaces, whereas the other mentioned species were not detected. In addition, other genera, including *Geobacillus* spp., *Brevibacterium* spp., and *E. coli* were found in high proportions at single farms. This shows that the flora on contact surfaces varies between

sampling locations. This may be due to environmental factors and may also reflect variations in the flora on the surface of the product in contact with the storage surface.

Penicillium camemberti and *P. roqueforti* are commonly used as starter cultures, and are hence expected to be found in cheese production environments. *Penicillium commune* and *C. cladosporioides* are common indoor fungi in temperate regions (Samson et al., 2010). *Penicillium commune*, the wild form of *P. camemberti*, and *P. echinulatum* both belong to the associated flora of cheeses (Samson and Frisvad, 2004). They are hence not surprising to find in such environments. *Penicillium echinulatum* is also associated with wood and wood products (Samson and Frisvad, 2004), and wooden storage shelves for cheeses are consequently perfect environment for this species. *Cladosporium cladosporioides* grows very well in environments with fluctuating temperatures and is associated with water-damaged wood (Samson et al., 2010) and is commonly found in storage rooms for food and feed. *Lecanicillium lecanii* is considered to be pathogenic to insects and may be a contamination from insects in the production facility.

It is believed that the presence of an active microflora in the food-processing environment may inhibit the growth or reduce the total number of pathogenic bacteria, including *L. monocytogenes*, both in complex biofilms (Guillier et al., 2008; Mariani et al., 2011) and as single strains (Zhao et al., 2004). However, other studies have shown that the presence of other bacteria may increase the ability of *Listeria* to establish itself on surfaces (Leriche and Carpentier, 2000; Bremer et al., 2001; Carpentier and Chassaing, 2004; Rieu et al., 2008). The current study investigated the effects of microbial strains that were most commonly isolated from the production environment of small cheese farms on the growth of *L. monocytogenes*. Only slight inhibition compared with a known inhibiting strain was observed in an overlay assay testing 63 bacterial and yeast isolates. None of the 5 tested bacterial strains showed conclusive inhibition of *L. monocytogenes* when cultivated together on a ceramic surface at conditions relevant for cheese ripening (milk-based soil, 12°C, and 98% RH). Previous studies have indicated that biofilm formation on surfaces may be reduced in the presence of nisin-producing *Lc. lactis* (Leriche et al., 1999; Zhao et al., 2004) or *P. putida* (Carpentier and Chassaing, 2004; Saá Ibusquiza et al., 2012) and by some, but not all *Staphylococcus* spp. (Leriche and Carpentier, 2000; Carpentier and Chassaing, 2004; Rieu et al., 2008; Zameer et al., 2010). The effect is, however, highly dependent on the experimental conditions such as strains, temperature, and inoculum concentration (Leriche et al. 1999; Zhao et al., 2004; Saá Ibusquiza et al., 2012)

and using relevant strains and conditions mimicking the production environment in question is, therefore, crucial. It must also be taken into account that the current study was limited to the effect of single strains from the background flora. It does not consider the possibility of several bacterial strains acting together to inhibit the growth of *Listeria*. Studies exist that have shown that complex bacterial mixtures may increase the attachment of *L. monocytogenes*, whereas single strains do not (Guðbjörnsdóttir et al., 2005). This may, however, be due to nutrient consumption by competing microflora and not the production of inhibitory substances (Guillier et al., 2008).

CONCLUSIONS

This study shows that no common background flora exists in small-scale cheese production environments. Only 4 of the isolated species could be found at all 5 production sites and none of these could be found at all sampling points within the farms. Isolates from these species did not show significant antilisterial activity in biofilm and growth-inhibition assays. Additional selected isolates comprising all bacterial species identified among the 5 producers showed only slight growth inhibitory activity to *Listeria*. This study also shows that species and strains with significant activity against *L. monocytogenes* growth and survival are not widespread in small-scale cheese production facilities. It was hence not possible to demonstrate that the environmental background flora of small-scale cheese production sites provides a significant contribution for controlling growth and survival of *L. monocytogenes* in the plants.

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