Effect of recycled manure solids as bedding on bulk tank milk and implications for cheese microbiological quality

Méridie Gagnon,^{1,2} Laurie Hamelin,^{1,2} Annie Fréchette,^{2,3} Simon Dufour,^{2,3} and Denis Roy^{1,2*}

¹Département des sciences des aliments, Laboratoire de génomique microbienne, Université Laval, 2440, boulevard Hochelaga, Québec QC G1V 0A6, Canada

²Regroupement de recherche pour un lait de qualité optimale (Op+Lait), 3200, rue Sicotte, Saint-Hyacinthe, QC J2S 2M2, Canada

³Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, C. P. 5000, Saint-Hyacinthe, QC J2S 7C6, Canada

ABSTRACT

The dairy farm environment influences the raw milk microbiota and consequently affects milk processing. Therefore, it is crucial to investigate farm management practices such as the bedding materials. The aim of this study was to evaluate the effect of recycled manure solids (RMS) as bedding material on bulk tank milk and microbiological implications for cheese quality. Bulk tank samples were collected from 84 dairy farms using RMS or straw bedding. The use of RMS did not influence thermophilic and mesophilic aerobic viable counts from spores. However, straw-milk samples gave higher values for mesophilic anaerobic spore-forming bacteria (0.44 log cfu/mL) than RMS-milk samples (0.17 log cfu/mL). The presence of thermoresistant lactic acid bacteria was not increased in milk from farms using RMS. Nevertheless, taxonomic profiles of thermoresistant bacteria isolated were different between the 2 types of milk. More *Enterococcus faecalis* and *Streptococcus* spp. were identified in RMS-milk samples. Thermoresistant enterococci and streptococci could easily end up in cheese. Therefore, milk proteolytic activities of these isolates were tested. Neither *Streptococcus* spp. nor *Enterococcus faecium* isolates exhibited proteolytic activities, whereas 53% of *E. faecalis* showed some. Also, only 1 vancomycin-resistant *enterococcus* was detected. Survival of selected RMS-milk samples isolates (3 *E. faecalis* and 1 *Streptococcus thermophilus*) was evaluated during a model Cheddar cheese manufacture. Although those strains survived well, they did not modify the acidification curve of milk. However, they might cause organoleptic defects during cheese maturing.

Key words: biobedding, spore-forming bacteria, vancomycin-resistant enterococci, Cheddar

INTRODUCTION

Dairy farm management undergoes change for practical and economic reasons or for food safety. Recently, bedding from recycled manure solids (RMS) has gained popularity in Canada. This practice is widespread in United Kingdom and has been used for several years (Bradley et al., 2014). Manure solids are separated from the liquid fraction using a screw, a roller press, or high-performance slurry separation equipment (Leach et al., 2015). Manure contains a high quantity of microorganisms that would be detrimental to animal health (e.g., *Escherichia coli* vs. mastitis). Therefore, the solid fraction is often composted to modify the bacterial population from that of manure. The bacterial fermentation raises the temperature during this process. Anaerobic digestion is used instead of or as well as composting, commonly in the United States (Leach et al., 2015). A lot of potential benefits are associated with RMS, such as cost savings, availability, and cow comfort (Bradley et al., 2014; Fournel et al., 2019b). Also, this type of bedding can be considered as a strategy to mitigate pollution from livestock manure management (Petersen et al., 2007). However, using RMS includes some risks (Bradley et al., 2014). Among others, it can contain an important load of gram-negative pathogens (*Klebsiella* spp., for instance) or food spoilage microorganisms surviving pasteurization such as spore formers and thermoresistant bacteria (Sorter et al., 2014; Leach et al., 2015).

It was demonstrated that dairy farm environment influences milk microbiota (Vacheyrou et al., 2011; Skeie et al., 2019). For example, thermophilic spore-forming bacteria in milk can come from soil, silage, or bedding. A higher number of thermophilic spore-forming bacteria was observed in bulk tank milk of farms using biobedding from composted material such

Received April 16, 2019.

Accepted September 9, 2019.

*Corresponding author: denis.roy@fsaa.ulaval.ca

as municipal biowaste or wooden chips than farms using sawdust or straw (Driehuis et al., 2014). The principal spore formers in milk are *Clostridium* (*Clostridium sporogenes*, *Clostridium butyricum*, and *Clostridium tyrobutyricum*), *Bacillus* (*Bacillus cereus* and *Bacillus sporothermodurans*), and *Geobacillus* (*Geobacillus stearothermophilus*) (Quigley et al., 2013). *Clostridium tyrobutyricum* can cause significant defects in cheese such as off-flavors and late blowing (Bassi et al., 2015; Gómez-Torres et al., 2015). As for *Bacillus* spp., they can produce hydrolytic extracellular enzymes (proteases, lipases, and lecithinases) causing spoilage in pasteurized milk (De Jonghe et al., 2010). The occurrence of the genera *Enterococcus* and *Streptococcus* in milk and cheese is also important. Indeed, environmental streptococci, including *Streptococcus uberis*, *Streptococcus dysgalactiae*, and enterococci such as *Enterococcus* spp., can cause mastitis in dairy cows (Cameron et al., 2016). *Enterococcus* spp. are considered thermoresistant and are associated with the gastrointestinal tract of mammals. Enterococci are also known to be opportunistic nosocomial pathogens worldwide. Their important genome plasticity allows the dissemination of antibiotic resistance genes. There is a major concern for vancomycin-resistant enterococci (**VRE**), but more specifically for *Enterococcus faecium* and *Enterococcus faecalis* species (O'Driscoll and Crank, 2015). Moreover, enterococci are prevalent in dairy products and can have negative effects on flavor development by proteolytic and peptidolytic activities (Giraffa, 2003). *Streptococcus thermophilus* is a nonpathogenic thermophilic bacterium used in yogurt production that can have a strong acidifying activity (Shahbal et al., 1991). The rates of acidification and proteolytic activity have a critical effect on the texture of cheese (Fox et al., 2017a). In addition, some studies correlated proteolytic and acidifying activity (Giraffa, 2003). As mentioned, bacteria originating from milk can modulate cheesemaking (Fox et al., 2017b). However, to exert considerable influence, those bacteria need to survive to cheese manufacture. In pasteurized cheese production such as Cheddar, different steps can stress bacteria, for example, acidity that comes from the starter, the cooking, and the salting (Gobbetti et al., 2018). The Pearce activity test is an effective laboratory-scale Cheddar cheese simulation to assay bacteria viability (Desfossés-Foucault et al., 2014).

High concentrations of thermotolerant and thermoresistant bacteria coming from milk of farms using RMS could lead to a reduced shelf life of pasteurized milk and cheese production loss. Therefore, we first investigated, under commercial farms conditions, the effect of RMS compared with straw bedding on the occurrence of

spore-forming bacteria and thermoresistant lactic acid bacteria (**LAB**) in bulk tank milk. Second, the survival, during Cheddar cheese manufacture, of selected thermoresistant strains from RMS-milk sample strains, was evaluated using a laboratory simulation process.

MATERIALS AND METHODS

Selection of Herds

The design was a cross-sectional observational study where milk samples from farms using RMS or straw as bedding were compared at a single point in time. To be included in the RMS group, the farms needed to have primarily used these materials as bedding for their lactating cows for at least 6 mo before the study. Almost all farms were composting the RMS before their usage (Table 1). Their herd size was between 55 and 900 cows (mean: 159; median: 111). Farms had to have their main facility within 250 km of the Faculty of Veterinary Medicine, located at the Université de Montréal (Saint-Hyacinthe, Quebec, Canada). Putative participating herds were identified by contacting (1) equipment dealers; (2) veterinary associations; and (3) via social networks. All identified farm owners using RMS were contacted and asked to participate in the study. Most of these qualified herds are also included in the regular DHI control group; however, the ones using RMS not in the latter were not excluded. Control farms (using straw as bedding) were recruited in the same area using the list provided by Valacta Inc., the Eastern Canada Dairy Herd Improvement Association. These were contacted for participation and selected based only on their exclusive use of straw bedding for the same period. Their herd size was between 43 and 229 cows (mean: 70; median: 65). A total of 84 dairy cow farms were sampled during spring 2018 (26 using RMS and 58 using straw bedding). None of these herds were provided with access to pasture during the sampling period.

Table 1. Information about recycled manure solids production

Type	No. of farms
Separator	
Roller press	8
Screw press	18
Composting	
Biodigester	1
Box	13
Tank	2
Windrow	9
None	1

Bulk Tank Milk Sampling

Before taking the milk samples from farms using RMS (RMS-milk) and farms using straw bedding (straw-milk), the bulk tank was agitated for 5 min. Then, samples were collected by the top of the tank using a straw and a syringe that were both sterilized. Subsequently, the milk was transferred in a sterile conical tube. Whenever the bulk tank milk level was too low to perform the sampling, it was obtained through the outlet valve. A little amount of milk was discarded, and then 50 mL was collected in a clean sealable bag and then transferred with a syringe into a conical tube; these last 2 items were previously sterilized. Raw milk was kept at 4°C until arrival at the laboratory. All samples were analyzed within 48 h.

Enumeration and Isolation of Heat-Resistant Bacteria

To enumerate thermophilic bacteria (spore-forming bacteria), 15 mL of raw milk was heated for 10 min in a water bath at 80°C to exclude vegetative cells, then cooled in an ice bath for 10 min. The heated milk was then plated in duplicate for aerobic spore formers on Brain Heart Infusion (BHI) Agar (BD Biosciences, San Jose, CA) supplemented with 1 mg/L of vitamin B₁₂ (Millipore Sigma, St. Louis, MO) and incubated for 24 to 48 h at 37°C (mesophilic) and 55°C (thermophilic) (Driehuis et al., 2014). For mesophilic anaerobic spore-forming bacteria enumeration, heated milk was plated on Reinforced Clostridial Medium (RCM) Agar (HiMedia Laboratories LLC, West Chester, PA) and incubated for 48 to 72 h at 37°C in an anaerobic glove box (Plas-Labs Inc., Lansing, MI) containing an atmosphere of 80% N₂, 10% H₂, and 10% CO₂ (Praxair Canada, Mississauga, ON, Canada; Gómez-Torres et al., 2015). After incubation, the colonies were counted, expressed as colony forming units per milliliter of milk. Second, heat treatment of raw milk was performed at 63°C for 10 min to enumerate thermoresistant *Enterococcus* and *Streptococcus* (Carminati et al., 2014). Heated milk and a 10-fold dilution in peptone water (1% of Bacto Peptone, BD Biosciences) were plated in duplicate on M17 Agar (Oxoid, Nepean, ON, Canada) at 37 and 42°C (for *S. thermophilus*). Colony-forming units were counted after the incubation.

Colonies selected with Harrison's disc (Ricciardi et al., 2015) for each different set of culture conditions were isolated by streaking. Then, pure isolates were grown for 24 h in the same culture conditions in liquid broth media (BHI, RCM, and M17). They were also kept at -80°C in 20% (vol/vol) glycerol (EMD Chemicals, Gibbstown, NJ).

Identification of Isolates

DNA Extraction. Genomic DNA extraction of isolates was performed with Geneaid Presto Mini gDNA Bacteria Kit (FroggaBio, Toronto, ON, Canada). One milliliter of 24-h-old culture was centrifuged at 10,000 × g, 10 min at 4°C. The cell pellet was used for the extraction. The manufacturer's protocol was modified based on Licitra et al. (2007). Briefly, to improve cell lysis, 20 mg/mL of lysozyme (Millipore Sigma) and 2.5 µL/mL of mutanolysin (20 U/µL) were added in Gram+ Buffer. Also, each incubation time in a water bath was doubled. The DNA was stored at -20°C until PCR amplification.

16S rRNA Gene Sequencing. The 16S rRNA genes of all isolates were partially sequenced (~800 bp). The amplification was performed with the primers 27F (5'-AGAGTTTGTACCTGGCTCAG-3') and 788R (5'-GGACTACCAGGGTATCTAA-3') (Rasolofa et al., 2010). The reaction for one volume of 50 µL contained 200 µM dNTP mix, 0.2 µM each primer, 2 mM MgCl₂, 1.25 U of GeneDireX Taq DNA polymerase (FroggaBio), and 25 ng of DNA. This volume was supplemented with Invitrogen nuclease-free water (Thermo Fisher Scientific, Waltham, MA). The nuclease-free water was also used as a blank. The amplification in TGradient (Biomtra, Montreal Biotech, Montreal, QC, Canada) was performed using the following program: 94°C for 5 min, 30 cycles including denaturation at 94°C for 30 s, primer annealing at 53°C for 1 min, and DNA extension at 72°C for 2 min, and a final step also at 72°C for 5 min. Next, DNA sequence analysis was carried out on both strands of the purified PCR products with the BigDye Terminator v3.1 Cycle Sequencing Kit and 3100 Genetic Analyzer System (Thermo Fisher Scientific). Sequences were aligned using Geneious Pro R6 software (Biomatters, San Francisco, CA). Identification was obtained with the Nucleotide Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/>). Further PCR amplifications were carried out for better identification of isolates belongs to *Enterococcus* species and *S. thermophilus* group.

Identification of Enterococci by Multiplex PCR. *Enterococcus* multiplex PCR was performed as described by Layton et al. (2010) to identify *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus hirae*, *Enterococcus saccharolyticus*, and *Enterococcus italicus*. Positive controls (*Enterococcus hirae* ATCC 10541, *Enterococcus faecium* 11G22K, *Enterococcus faecalis* ATCC 27275, and *Enterococcus durans* DSM-20633) and negative controls (*Lactococcus lactis* ssp. *cremoris* SK11 and *E. italicus* DSM-15952) were used. No primer was designed for *E.*

italicus. However, if isolates, identified as *E. durans*/*italicus* by 16S rRNA gene sequencing, were negative to the *E. durans* assay, they were assumed to belong to *E. italicus*. The PCR conditions and primers are shown in Supplemental Tables S1, S2, and S3 (<https://doi.org/10.3168/jds.2019-16812>). Nuclease-free water was used as blank. After the reaction, 5 µL of PCR products was analyzed with GD 100 bp DNA Ladder H3 (FroggaBio) by electrophoresis on 1.5% agarose gel (Thermo Fisher Scientific) containing Red Safe (FroggaBio). Migration was performed using a sodium boric acid buffer (Brody and Kern, 2004) 45 min at 135 mV.

Identification of *Streptococcus thermophilus*. Isolates belonging to the taxonomic group of *S. thermophilus*/*salivarius* were distinguished by PCR amplification of the *lacZ* gene. The technique was performed as described by Lick et al. (1996). The PCR conditions and primers are shown in Supplemental Tables S4 and S5 (<https://doi.org/10.3168/jds.2019-16812>). The PCR products (968 bp) were analyzed as previously mentioned. *Streptococcus thermophilus* ATCC 19258 was used as a positive control and nuclease-free water as blank.

Proteolytic Enzyme Test

The proteolytic activity of enterococci and streptococci isolates was assayed as described by Franciosi et al. (2009). Briefly, 2 µL of 16 h culture in M17 broth, after 2 subcultures, were dropped on Skim Milk Agar [10% skim milk powder (local supplier, QC, Canada) and 2% agar (Hardy Diagnostics, Santa Maria, CA)]. The plates were incubated for 4 d at 37°C. Proteolytic activity (clear halo around the colony) were measured (Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-16812>). Halo diameter ranged from 16 to 23 mm corresponding to a strong proteolytic activity and halo diameter ranged from 9 to 16 mm corresponding to a low proteolytic activity. No halo corresponded to a negative proteolytic activity.

Detection of VRE

Multiplex PCR assay for detection of 7 types of VRE determinants (*vanA*, *vanB*, *vanC1*, *vanC2/3*, *vanD/D4*, *vanM*, and *vanN*) were performed as described by Nomura et al. (2018). The PCR conditions and primers are shown in Supplemental Tables S6, S7, and S8 (<https://doi.org/10.3168/jds.2019-16812>). The PCR products were analyzed as previously mentioned. VanB-type *E. faecalis* ATCC 51299 was used as a positive control and *E. durans* DSM-20633 as negative control. For VRE determinants detected, the MIC was determined by mi-

crodilution method in triplicate following CLSI guidelines (Clinical Laboratory Standards Institute, 2012). The MIC is the highest dilution of antibiotic showing complete inhibition of the tested strain [approximate optical density equals optical density of the blank (M17 broth) measured at 565 nm].

Pearce Activity Test

The Pearce activity test (Cheddar manufacture model) was performed as described by Desfossés-Foucault et al. (2014) to evaluate the effect of selected thermoresistant LAB associated with RMS milk. Four strains were selected [2 proteolytic *E. faecalis* (M155 and M236), 1 non-proteolytic *E. faecalis* (M390), and 1 *S. thermophilus* (M388)]. These isolates of *E. faecalis* originated from milk of different farms using RMS. Microbial population and acidification curve during the production of cheese model were monitored. From the frozen culture, the reference Cheddar starter *L. lactis* ssp. *cremoris* SK11 and the selected thermoresistant LAB were subcultured twice and were grown in microfiltered containing 3.25% fat (local supplier, ON, Canada). Briefly, during the Pearce activity test, microfiltered milk was inoculated with the starter at 10^7 cfu/mL and with the thermoresistant LAB separately at 10^6 cfu/mL. Not inoculated milk was used as the negative control. Steps and sampling times are shown in Figure 1. At each of the latter, the pH was measured, and viable counts were performed. One milliliter of milk, or 1 g of curds, was taken and mixed for 5 min at 260 rpm in Stomacher 400 Circulator (Seward Laboratory Systems Inc., West Sussex, UK) with 9 mL of sodium citrate 2% (wt/vol). Then, the appropriate dilution was plated on M17 Agar for the starter, ST

Figure 1. Temperature profile, steps, and sampling points (T0–T5) of the Pearce activity test.

Agar for *S. thermophilus* (Ashraf and Shah, 2011), and MacConkey Agar without crystal violet for *E. faecalis* (Savard et al., 2011). The experiment was performed in triplicate.

Statistical Analysis

Comparisons were made between RMS-milk and straw-milk. Analyses were conducted both including and excluding the 2 farms where RMS was not composted. This did not influence summary measures of counts or STAMP analysis or the significance of differences between RMS-milk and straw-milk. Therefore, the results including all RMS samples are presented. Differences in viable counts of thermophilic and thermoresistant bacteria between bedding types were determined with JMP 13 Software (SAS Institute Inc., Cary, NC), using nonparametric Wilcoxon test as the data did not exhibit a normal distribution. The relative abundance of taxonomic groups were compared with STAMP version 2.1.3 (Parks et al., 2014) following data transformation in the R environment (R Core Team, 2013) using Phyloseq (McMurdie and Holmes 2013) and Microbiome (Lahti et al., 2017) packages. Significance was assessed with a 2-tailed Welch's *t*-test, and the Storey's false discovery rate correction was applied on *P*-values. Chi-squared test was conducted to determine if there was difference in presence or absence of proteolytic streptococci or enterococci between RMS-milk and straw-milk. Pearce activity test viable counts and pH values were compared by an ANOVA performed with JMP 13. The means were multi-compared using the Tukey's honestly significant difference test.

RESULTS

Effect of Bedding Type on Thermophilic and Thermoresistant Bacteria Viable Counts in Milk

First, no significant difference was observed in viable counts of aerobic mesophilic spore-forming bacteria between the 2 types of milk (*Z* value = -0.88 ; *P* = 0.38; Figure 2). Similar values for aerobic thermophilic spore-forming species were determined in milk and no significant difference was observed (*Z* value = -1.12 ; *P* = 0.26). Bedding types had an effect on anaerobic mesophilic viable counts from spores (*Z* value = -2.25 ; *P* = 0.02; Figure 2). The mean values obtained for milk samples from farms using straw bedding were higher than those using RMS; they were, respectively, 0.44 and 0.17 log cfu/mL of fluid. As for thermoresistant bacteria, viable counts were not affected by the bed-

ding type during an incubation at 37°C, or at 42°C (37°C: *Z* value = 0.97; *P* = 0.33; 42°C: *Z* value = 0.34; *P* = 0.74; Figure 2).

A total of 90 mesophilic and 69 thermophilic aerobic spore-forming bacteria were isolated from milk samples. Ten different taxonomic groups of *Bacillaceae* were identified, belonging mostly to the genus *Bacillus* (Figure 3A). The other genera were *Lysinibacillus*, *Gracilibacillus*, and *Geobacillus*. *Bacillus licheniformis* was the dominant species, accounting for 68% of mesophilic and 93% of thermophilic bacteria. The other ones identified belonging to the genus *Bacillus* were *B. circulans*, *B. clausii*, *B. megaterium*, *B. pumilus/altitudinis/safensis/zhangzhouensis*, *B. subtilis/tequilensis*, and *B. thermoamylovorans*. Statistical analyses performed with STAMP indicated that *Bacillus* spp. were significantly more abundant in straw-milk samples (Figure 4A), but this difference was explained by anaerobic spore formers. As previously mentioned, milk samples from farms using RMS had a smaller number of anaerobic spore-forming bacteria than those using straw bedding. Five isolates were found in RMS-milk samples against 43 in straw-milk samples. *Bacillus licheniformis* was the most abundant taxonomic group in anaerobic spore formers. Furthermore, it was significantly more abundant in straw-milk samples (Figure 4B). On the other hand, RMS-milk samples contained more *Lysinibacillus sphaericus/halotolerans/massiliensis*.

In total, 432 isolates of thermoresistant bacteria were collected on M17 agar, 141 of these from RMS-milk samples (77 isolates at 37°C and 64 at 42°C) and the 291 remaining from milk samples of straw bedding (152 isolates at 37°C and 139 at 42°C). The LAB identified belonged to 4 genera [i.e., *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* (Figure 3B)]. The genus *Kocuria* is not part of LAB but of the *Micrococcaceae* family; members were also present. As expected, more isolates of *S. thermophilus* were recovered when M17 Agar preparations were incubated at 42°C. Unlike thermophilic bacteria, the profiles of thermoresistant ones from RMS- and straw-milk samples were different. According to STAMP analysis, among all the isolates recovered at both 37 and 42°C, only the relative abundance of the genus *Streptococcus* varied significantly, with nearly 7 times higher values in RMS-milk samples (20%) compared with straw-milk samples (3%; Figures 3B and 4A). At the species level, no significant difference in isolates was observed when comparing the 2 groups of farms. However, when comparing isolates from the 2 groups of farms at each incubation temperature separately, the STAMP analysis was showing that the relative abundance varied significantly only at 37°C. *Kocuria kristinae* and *Kocuria rhizophila/salsicia*

were twice as abundant as in straw-milk samples than in RMS-milk samples (Figure 4C). The abundance of *Enterococcus* spp. was comparable in both types of bedding (39%; Figure 3B). Nevertheless, in enterococci, *E. faecalis* was significantly more present in RMS-milk samples than in straw-milk samples. Unlike *E. faecalis*, *E. faecium* significantly was more abundant in the latter (16% in RMS-milk samples versus 20% in straw-milk samples; Figures 3B and 4C).

Proteolytic Activity of Streptococci and Enterococci

Out of 208 enterococci and streptococci tested, only 21 isolates identified as *E. faecalis* had positive proteolytic activities (Table 2). The prevalence of farms contained proteolytic *E. faecalis* in milk samples was

7% for farms using straw bedding (5/58) compared with 15% for farm using RMS (4/26). However, this difference was not significant ($P = 0.37$).

Vancomycin Resistance of Enterococci

Out of 171 thermoresistant enterococcal isolates, 1 strain, *E. gallinarum/casseliflavus* M530, had vancomycin-resistance associated genes. This strain was isolated from RMS-milk. The M530 strain had 2 genotypes, *vanN* (941 bp) and *vanC1* (836 bp). The *VanB* gene of *E. faecalis* ATCC 51299 was also detected (640 bp). In the present study, the MIC calculated of the vancomycin-resistant *E. faecalis* ATCC 51299 and vancomycin-sensitive *E. durans* DSM-20633 was, respectively, 62.5 and 1.95 µg/mL. Vancomycin resistance of

Figure 2. Box plot created from viable counts of thermophilic (spore of aerobic mesophilic, aerobic thermophilic, and anaerobic mesophilic bacteria) and thermoresistant bacteria in bulk tank milk from farms using recycled manure solids (RMS) or straw bedding. Thermoresistant bacteria were incubated at 2 temperatures: 37 and 42°C. The lines represent the median and the ends of the box represent the 1st and 3rd quartiles. The whiskers represent 1st quartile $- 1.5 \times$ (interquartile range) and 3rd quartile $+ 1.5 \times$ (interquartile range), and the dots represent the outliers.

E. gallinarium/casseliflavus M530 was confirmed with a MIC of 15.6 µg/mL.

Effect of Thermoresistant Bacteria from RMS in Cheddar Manufacture

Two proteolytic *E. faecalis* (M155 and M236), 1 non-proteolytic *E. faecalis* (M390), and 1 *S. thermophilus* (M388) isolates from RMS-milk samples were selected. The 4 thermoresistant strains grew well during the Pearce activity test (Figure 5A). The viable counts of *E. faecalis* and *S. thermophilus* strains were significantly different at the salting step at 285 min (sampling point T4). At this sampling point, *S. thermophilus* M388 was assessed at a higher concentration than *E. faecalis* M236 and M390 (F value = 7.2031; P = 0.0116). After the salting step, *S. thermophilus* M388 was less cultivable and *E. faecalis* M155 stayed stable. However, for the other strains M236 and M390, viable counts increased between sampling points T4 and T5. Therefore, at the 360-min time point, the 4 thermoresistant strains had the same concentration. Even if these grew during the Cheddar manufacture, they did not inhibit nor improve significantly the growth of the starter *L. lactis* ssp. *cremoris* SK11 (Figure 5B). As for the starter viable counts, the presence of the thermoresistant LAB selected did not affect the acidification curve despite their growth (P > 0.05; Figure 5C). Milk was acidified by the starter *L. lactis* ssp. *cremoris* SK11, a process

during which the pH shifted from 6.56 to 5.49 after 6 h of manufacture.

DISCUSSION

The thermoduric and thermoresistant bacteria concentrations were no higher in raw milk originating from farms using RMS compared with samples from those using straw bedding (Figure 2). This result corroborates that of Bradley et al. (2018) who showed that the bedding type (not composted RMS, sand, and sawdust) did not affect the total bacterial count in bulk tank milk, even though counts in the bedding material itself were higher when RMS was used, compared with sand or sawdust. Rowbotham and Ruegg (2015) found similar results. In their study comparing the following bedding materials: inorganic, organic nonmanure, and manure-based, no association was observed between total bacterial count in bulk tank milk and bedding type. The composting of bedding has been associated with an increase of thermophilic aerobic spore-forming bacteria in milk (Driehuis et al., 2014). However, in our study almost all farms used composting RMS but no significant differences were observed compared with straw bedding. Moreover, the inclusion or exclusion of the 2 farms that did not compost RMS did not affect the findings. This would imply that most of the bacterial population in milk originated from the mammary gland, rather than from contamination during harvest-

Figure 3. Cumulative percentage distinguishing taxonomic groups from farm using recycled manure solids (RMS) and straw as bedding of (A) spore-forming bacteria isolated from milk heated at 80°C, for 10 min, and (B) bacteria isolated on M17 agar at 37 and 42°C from milk heated at 63°C, for 10 min.

Figure 5. Pearce activity test viable counts of (A) thermoresistant *Enterococcus faecalis* M155, M236, and M390 or *Streptococcus thermophilus* M388 and (B) starter *Lactococcus lactis* subsp. *cremoris* SK11 and (C) Pearce activity test acidification curves with the starter *L. lactis* subsp. *cremoris* SK11 and inoculation of SK11 mixed cultures. The error bars represent the SE. Means with distinct capital letters were significantly different ($P < 0.05$).

Table 2. Thermoresistant *Enterococcus faecalis* with positive proteolytic activity isolated from bulk tank milk collected from farms using straw or recycled manure solids (RMS) as bedding

Isolate code	Farm code	Proteolytic activity ¹
Straw		
M097	1243	++
M285	1261	+
M284		++
M282		++
M283		++
M287		++
M436	1272	+
M437		+
M450		++
M447		++
M441	1273	++
M680	1288	++
RMS		
M155	1104	++
M242	1109	+
M239		+
M240		+
M237		+
M234		++
M236		++
M500	1123	+
M689	1127	++

¹++ = halo diameter ranged from 16 to 23 mm; + = halo diameter ranged from 9 to 16 mm.

ing. Bradley et al. (2018) demonstrated a difference in thermophilic spore counts between 3 bedding materials (RMS, sand, and sawdust) but no influence of bedding type on the thermophilic spore counts in the milk produced. The thermophilic viable count from spores was higher (~2 log cfu/mL) than in the present study. This could be explained by the difference in climate between the United Kingdom and Canada. Also, in the present study, samples were collected only in spring in comparison to December to March. Our results are more similar to those of Buehner et al. (2014). The spores count in raw milk collected in winter in the Midwest of the United States were, respectively, 0.69 and 0.39 log cfu/mL for mesophilic spores and thermophilic spores. The authors showed an increase of spores count during summer. Other factors, for example use of silage could influence the concentration of spore-forming bacteria in milk (Driehuis, 2013; Buehner et al., 2014), but no information about feeding type was collected in the present study.

The identification of isolates in the current study has shown that aerobic spore-forming bacteria are similar in milk samples regardless of the bedding type (Figure 3A). *Bacillus licheniformis* appeared to be adapted to different culture conditions, as it was the most abundant species among the aerobic (thermophilic and mesophilic) and anaerobic spore formers. Our results are in agreement with the study of Coorevits et al. (2008)

who demonstrated the dominance of *B. licheniformis* in milk from organic and conventional dairy farms. In the present study, anaerobic *B. licheniformis* was more abundant in milk from farm using straw bedding. This species has already been reported to grow in anaerobic conditions (Clements et al., 2002). *Bacillus licheniformis* anaerobic respiration using nitric oxide reductase is possible (Rey et al., 2004). This type of enzyme is associated with denitrification (Hendriks et al., 2000) and could explain the presence of anaerobic *B. licheniformis* in soil, and transfer to straw bedding. Another difference in anaerobic spore formers was the presence of *Lysinibacillus sphaericus/halotolerans/massiliensis* only in RMS-milk samples. Members of the genus *Lysinibacillus* have previously been isolated in bulk tank milk but was not linked to particular management practices (Miller et al., 2015). Moreover, *Lysinibacillus* spp. are common in dairy cow fecal microbiota (Mao et al., 2012) and could be associated with RMS. However, this is to be taken lightly considering the limited number of isolates, especially since they did not show strong proteolytic activities in milk, neither cytotoxicity nor toxigenic activities (De Jonghe et al., 2010). Anaerobic spore-forming *C. butyricum* was a major concern regarding the use of RMS for cheese defects (Driehuis et al., 2013). Fortunately, we did not detect cultivable *C. tyrobutyricum* in RMS- or straw-milk samples. *Bacillus cereus*, an important food pathogen, was not found in bulk tank milk. Based on our results, using RMS does not seem to affect the occurrence of bacterial spores in milk.

The identification of bacteria isolated on M17 Agar demonstrated that this medium was not specific to LAB because numerous strains of *Kocuria* spp. were collected (Figure 3B). Some studies indicated that their growth can be supported on M17 medium (Renyé et al., 2011; Yunita and Dodd, 2018). Therefore, we obtained thermoresistant bacteria profiles rather than thermoresistant LAB profiles. These were affected by milk origin. *Kocuria* spp. were more abundant in straw-milk samples. This genus is common in the dairy environment (Quigley et al., 2013) and isolates have already been reported in pasteurized milk samples (Coorevits et al., 2008). *Kocuria* spp. were found in farm air and hay (Lis et al., 2008; Vacheyrou et al., 2011). Furthermore, *K. kristinae* has been isolated from biofilms covering wooden vats collected in a farm (Licitra et al., 2007). However, it is not clear if the higher abundance of this genus, more specifically *K. rhizophila/salsicia* and *K. kristinae*, is related directly to straw bedding.

The use of RMS was associated with a higher abundance of thermoresistant *Streptococcus* spp. and *E. faecalis* in milk (Figures 3B and 4). Zdanowicz et al. (2004) revealed a positive correlation between bacterial

populations, including *Streptococcus* spp., in the sand bedding and on the teat skin. Indeed, some studies indicated a high quantity of *Streptococcus* spp. in RMS [e.g., 9 (Sorter et al., 2014) and 6 log cfu/g of DM (Fournel et al., 2019b)]. Fournel et al. (2019b) demonstrated that solid-liquid separation methods and composting time did not affect their number in RMS. Also, *Streptococcus* spp. can grow in the RMS obtained by composting, from 4.8 to 8.7 log cfu/g of DM (Cole and Hogan, 2016). Therefore, higher levels in RMS-milk samples could be linked to their presence in the bedding. This could be an issue since environmental streptococci other than *S. uberis* and *S. dysgalactiae*, such as *S. gallolyticus* and *S. lutetiensis*, can cause mastitis in dairy cows (Cameron et al., 2016). This infection increases the number of somatic cells in raw milk. A high count of those reduces the product yield of Cheddar cheese (Auldist et al., 1996). However, it is not clear in the literature that RMS increase the prevalence of clinical mastitis (Leach et al., 2015). Future investigations will be needed on this point. Despite the fact that in our study *S. thermophilus* M388 did not affect the Cheddar manufacture (Figure 5), the presence of this species in RMS-milk samples is also worrying for cheesemakers. Indeed, some strains can form biofilms on stainless steel. These can cause many problems in cheese manufacturing plants such as undesirable contamination of dairy products and uncontrolled milk acidification during food production (Bassi et al., 2017).

The equivalent abundance of enterococci in the 2 types of milk is not surprising as they are present not only in cow feces, but also in soil and plants (Gelsomino et al., 2002). In comparison to environmental streptococci having a continued susceptibility to β -lactam antimicrobials (Cameron et al., 2016), the higher abundance of *E. faecalis* in RMS-milk sample could be of concern, given that this species has been identified as resistant to the glycopeptide antimicrobial vancomycin (O'Driscoll and Crank, 2015). Moreover, this species has a high potential for pathogenicity. However, we have not detected any vancomycin-resistant determinants belonging to this species. The low VRE strain, *E. gallinarum/casseliflavus* M530, collected in a milk sample from a farm using RMS, had the *vanC* and *vanN* genotype. The chromosomal *vanC* gene is intrinsic to *E. gallinarum* and *E. casseliflavus* species and corresponds to a low resistance to vancomycin (MIC between 2 and 32 μ g/mL). Like *vanC*, the presence of *vanN* gene produces a low vancomycin-resistant phenotype (MIC of 15 μ g/mL). The presence of this gene can be problematic as it is in plasmid (O'Driscoll and Crank, 2015). VanN-type *E. faecium* strains were reported in clinical settings in Canada and in chicken meat in Japan (Nomura et al., 2018). However, we can-

not link the *E. gallinarium/casseliflavus* M530 strain directly to the use of RMS. The multiplex PCR test used in this study could be used to detect VRE in RMS directly in future studies.

Enterococcus faecalis isolated from RMS-milk did not affect the Cheddar manufacture as the strains tested did not modify the acidification curve. Nevertheless, the number of those bacteria increased during the 6 h of cheese production. This suggests that their presence would be elevated at the beginning of the ripening, and as such, given the proteolytic activity of species like *E. faecalis*, noxious effects could occur during this step. The addition of salt in the curd helps to control microbial metabolism, such as proteolysis (Fox et al., 2017b). However, 2 *E. faecalis* strains (M236 and M390) and *E. faecium* M361 isolated from RMS-milk samples have shown a NaCl tolerance. Uncontrolled proteolysis of casein can have a major effect on the Cheddar texture (Lawrence et al., 2004). Also, the breakdown of proteins followed by the catabolism of AA can produce off-flavor volatile compounds (Singh et al., 2003; Lawrence et al., 2004). As bulk tank samples from farms using RMS contained more thermoresistant *Streptococcus* spp. and *E. faecalis*, the milk could be prioritized for fresh cheese (no ripening step). Otherwise, it could be microfiltered before cheesemaking. Moreover, the RMS production is not standardized in Canada. Further studies on RMS production could help to limit spoilage and pathogen bacteria such as enterococci and streptococci (Fournel et al., 2019a,b).

CONCLUSIONS

Our study investigated under farm conditions the effect of RMS bedding used in dairy farms on thermotolerant and thermoresistant milk microbiota in comparison to conventional straw bedding. Aerobic spore formers and thermoresistant bacteria concentrations in milk were not higher in milk samples from farms using RMS. However, RMS bedding introduces an increased risk of having thermoresistant streptococci and enterococci in milk. Potentially, *E. faecalis* strains with proteolytic activity and NaCl tolerance could be noxious for Cheddar cheese ripening. Therefore, it remains important to assess the effect of new farm management practices on milk quality. This knowledge could help cheesemakers to select milk (e.g., use of milk from RMS farms for fresh cheese with no ripening step). To assure the milk producers and the cheesemakers that the RMS bedding is safe, future work using metagenomics to also target viable but nonculturable bacteria will have to be done. Moreover, because the use of RMS is relatively new, the different production methods will have to be compared in combination with milk quality.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada (NSERC), Novalait, Agriculture and Agri-Food Canada, and the Fonds de recherche du Québec–Nature et technologies (FRQNT) for their financial contribution. They also give special thanks to the Op⁺Lait research group which allowed the creation and realization of this project. The authors also thank all members of S. Dufour's and D. Roy's teams for their technical assistance and scientific advice, particularly Alexandre Jules Kennang Ouamba (Faculté des sciences de l'agriculture et alimentation, Université Laval, Québec, Canada) in regard to the statistical analysis and Caroline Forest (Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Canada) for technical assistance during the collection of samples. The authors sincerely thank Dominique Fournier (www.serviceslinguistiquesdf.com) for editing and improving the text of the manuscript.

REFERENCES

- Ashraf, R., and N. P. Shah. 2011. Selective and differential enumerations of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt — A review. *Int. J. Food Microbiol.* 149:194–208. <https://doi.org/10.1016/j.ijfoodmicro.2011.07.008>.
- Auld, M. J., S. Coats, B. J. Sutherland, J. J. Mayes, G. H. McDowell, and G. L. Rogers. 1996. Effects of somatic cell count and stage of lactation on raw milk composition and the yield and quality of Cheddar cheese. *J. Dairy Res.* 63:269–280. <https://doi.org/10.1017/S0022029900031769>.
- Bassi, D., F. Cappa, S. Gazzola, L. Orrù, and P. S. Cocconcelli. 2017. Biofilm formation on stainless steel by *Streptococcus thermophilus* UC8547 in milk environments is mediated by the proteinase PrtS. *Appl. Environ. Microbiol.* 83:e02840–16. <https://doi.org/10.1128/AEM.02840-16>.
- Bassi, D., E. Puglisi, and P. S. Cocconcelli. 2015. Understanding the bacterial communities of hard cheese with blowing defect. *Food Microbiol.* 52:106–118. <https://doi.org/10.1016/j.fm.2015.07.004>.
- Bradley, A. J., K. A. Leach, S. C. Archer, J. E. Breen, M. J. Green, I. C. Ohnstad, and S. Tuer. 2014. Scoping study on the potential risks (and benefits) of using recycled manure solids as bedding for dairy cattle. Report prepared for DairyCo. Accessed Apr. 15, 2019. <https://dairy.ahdb.org.uk/resources-library/technical-information/buildings/rms-bedding>.
- Bradley, A. J., K. A. Leach, M. J. Green, J. Gibbons, I. C. Ohnstad, D. H. Black, B. Payne, V. E. Prout, and J. E. Breen. 2018. The impact of dairy cows' bedding material and its microbial content on the quality and safety of milk—A cross sectional study of UK farms. *Int. J. Food Microbiol.* 269:36–45. <https://doi.org/10.1016/j.ijfoodmicro.2017.12.022>.
- Brody, J. R., and S. E. Kern. 2004. Sodium boric acid: A Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36:214–216. <https://doi.org/10.2144/04362BM02>.
- Buehner, K. P., S. Anand, G. D. Diira, and A. Garcia. 2014. Prevalence of thermotolerant bacteria and spores on 10 Midwest dairy farms. *J. Dairy Sci.* 97:6777–6784. <https://doi.org/10.3168/jds.2014-8342>.
- Cameron, M., M. Saab, L. Heider, J. T. McClure, J. C. Rodriguez-Lecompte, and J. Sanchez. 2016. Antimicrobial susceptibility patterns of environmental streptococci recovered from bovine milk

- samples in the maritime provinces of Canada. *Front. Vet. Sci.* 3:79. <https://doi.org/10.3389/fvets.2016.00079>.
- Carminati, D., F. Tidona, M. E. Fornasari, L. Rossetti, A. Meucci, and G. Giraffa. 2014. Biotyping of cultivable lactic acid bacteria isolated from donkey milk. *Lett. Appl. Microbiol.* 59:299–305. <https://doi.org/10.1111/lam.12275>.
- Clements, L. D., B. S. Miller, and U. N. Streips. 2002. Comparative growth analysis of the facultative anaerobes *Bacillus subtilis*, *Bacillus licheniformis*, and *Escherichia coli*. *Syst. Appl. Microbiol.* 25:284–286. <https://doi.org/10.1078/0723-2020-00108>.
- Clinical Laboratory Standards Institute. 2012. Performance standards for antimicrobial susceptibility testing; twenty-second international supplement. Volume 32, Number 3. CLSI document M100–S22. Wayne, PA.
- Cole, K., and J. Hogan. 2016. Short communication: Environmental mastitis pathogen counts in freestalls bedded with composted and fresh recycled manure solids. *J. Dairy Sci.* 99:1501–1505. <https://doi.org/10.3168/jds.2015-10238>.
- Coorevits, A., V. De Jonghe, J. Vandroemme, R. Reekmans, J. Heyrman, W. Messens, P. De Vos, and M. Heyndrickx. 2008. Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst. Appl. Microbiol.* 31:126–140. <https://doi.org/10.1016/j.syapm.2008.03.002>.
- De Jonghe, V., A. Coorevits, J. De Block, E. Van Coillie, K. Grijspeerdt, L. Herman, P. De Vos, and M. Heyndrickx. 2010. Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *Int. J. Food Microbiol.* 136:318–325. <https://doi.org/10.1016/j.ijfoodmicro.2009.11.007>.
- Desfossés-Foucault, É., G. LaPointe, and D. Roy. 2014. Transcription profiling of interactions between *Lactococcus lactis* ssp. *cremoris* SK11 and *Lactobacillus paracasei* ATCC 334 during Cheddar cheese simulation. *Int. J. Food Microbiol.* 178:76–86. <https://doi.org/10.1016/j.ijfoodmicro.2014.03.004>.
- Driehuis, F. 2013. Silage and the safety and quality of dairy foods: A review. *Agric. Food Sci.* 22:16–34. <https://doi.org/10.23986/afsci.6699>.
- Driehuis, F., E. Lucas-van den Bos, and M. H. J. Wells-Bennik. 2013. Risico's van het gebruik van gescheiden mest as beddinmateriaal voor de melkwaliteit: sporen van *Bacillus cereus* en boterzuurbacteriën. (Risks of the use of cattle manure solids as bedding material for milk quality: *Bacillus cereus* and butyric acid bacteria). NIZO-Rapport E 2013/180 pp. NIZO Food Research BV, Ede, the Netherlands.
- Driehuis, F., E. Lucas-van den Bos, and M. H. J. Wells-Bennik. 2014. Sporen van thermofiele aërobe sporenvormers in compost en andere beddingmaterialen bij melkveebedrijven met een vrijloop- of ligenboxenstal. (Spores of thermophilic aerobic sporeformers in compost and other bedding materials used by dairy farmers). NIZO-Rapport E 2014/045 pp. NIZO Food Research BV, Ede, the Netherlands.
- Fournel, S., S. Godbout, P. Ruel, A. Fortin, K. Duquette-Lozeau, V. Létourneau, M. Généreux, J. Lemieux, D. Potvin, C. Côté, C. Duchaine, and D. Pellerin. 2019a. Production of recycled manure solids for use as bedding in Canadian dairy farms: II. Composting methods. *J. Dairy Sci.* 102:1847–1865. <https://doi.org/10.3168/jds.2018-14967>.
- Fournel, S., S. Godbout, P. Ruel, A. Fortin, M. Généreux, C. Côté, C. Landry, and D. Pellerin. 2019b. Production of recycled manure solids for bedding in Canadian dairy farms: I. Solid-liquid separation. *J. Dairy Sci.* 102:1832–1846. <https://doi.org/10.3168/jds.2018-14966>.
- Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2017a. Overview of cheese manufacture. Pages 11–25 in *Fundamentals of Cheese Science*. Springer US, Boston, MA.
- Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2017b. Microbiology of cheese ripening. Pages 333–390 in *Fundamentals of Cheese Science*. Springer US, Boston, MA.
- Franciosi, E., L. Settanni, A. Cavazza, and E. Poznanski. 2009. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *Int. Dairy J.* 19:3–11. <https://doi.org/10.1016/j.idairyj.2008.07.008>.
- Gelsomino, R., M. Vancanneyt, T. M. Cogan, S. Condon, and J. Swings. 2002. Source of enterococci in a farmhouse raw-milk cheese. *Appl. Environ. Microbiol.* 68:3560–3565. <https://doi.org/10.1128/aem.68.7.3560-3565.2002>.
- Giraffa, G. 2003. Functionality of enterococci in dairy products. *Int. J. Food Microbiol.* 88:215–222. [https://doi.org/10.1016/s0168-1605\(03\)00183-1](https://doi.org/10.1016/s0168-1605(03)00183-1).
- Gobbetti, M., R. Di Cagno, M. Calasso, E. Neviani, P. F. Fox, and M. De Angelis. 2018. Drivers that establish and assembly the lactic acid bacteria biota in cheeses. *Trends Food Sci. Technol.* 78:244–254. <https://doi.org/10.1016/j.tifs.2018.06.010>.
- Gómez-Torres, N., S. Garde, Á. Peirotén, and M. Ávila. 2015. Impact of *Clostridium* spp. on cheese characteristics: Microbiology, color, formation of volatile compounds and off-flavors. *Food Control* 56:186–194. <https://doi.org/10.1016/j.foodcont.2015.03.025>.
- Hendriks, J., A. Oubrie, J. Castresana, A. Urbani, S. Gemeinhardt, and M. Saraste. 2000. Nitric oxide reductases in bacteria. *Biochim. Biophys. Acta Bioenerg.* 1459:266–273. [https://doi.org/10.1016/S0005-2728\(00\)00161-4](https://doi.org/10.1016/S0005-2728(00)00161-4).
- Lahti, L., S. Shetty, et al. 2017. Tools for microbiome analysis in R. Version 1.5.23. <http://microbiome.github.com/microbiome>.
- Lawrence, R., J. Gilles, L. Creamer, V. L. Crow, H. A. Heap, C. G. Honoré, K. A. Johnston, and P. K. Samal. 2004. Cheddar cheese and related dry-salted cheese varieties. Pages 71–102 in *Cheese: Chemistry, Physics and Microbiology*. P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, ed. Elsevier.
- Layton, B. A., S. P. Walters, L. H. Lam, and A. B. Boehm. 2010. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *J. Appl. Microbiol.* 109:539–547. <https://doi.org/10.1111/j.1365-2672.2010.04675.x>.
- Leach, K. A., S. C. Archer, J. E. Breen, M. J. Green, I. C. Ohnstad, S. Tuer, and A. J. Bradley. 2015. Recycling manure as cow bedding: Potential benefits and risks for UK dairy farms. *Vet. J.* 206:123–130. <https://doi.org/10.1016/j.tvjl.2015.08.013>.
- Licitra, G., J. C. Ogier, S. Parayre, C. Pediliggieri, T. M. Carnemolla, H. Falentin, M. N. Madec, S. Carpino, and S. Lortal. 2007. Variability of bacterial biofilms of the “tina” wood vats used in the ragusano cheese-making process. *Appl. Environ. Microbiol.* 73:6980–6987. <https://doi.org/10.1128/AEM.00835-07>.
- Lick, S., M. Keller, W. Bockelmann, and J. Heller. 1996. Rapid identification of *Streptococcus thermophilus* by primer-specific PCR amplification based on its lacZ gene. *Syst. Appl. Microbiol.* 19:74–77. [https://doi.org/10.1016/S0723-2020\(96\)80012-9](https://doi.org/10.1016/S0723-2020(96)80012-9).
- Lis, D. O., J. Z. Pacha, and R. L. Górný. 2008. Bacterial contamination of the air in farmhouses. *Indoor Air* 8:17–22.
- Mao, S., R. Zhang, D. Wang, and W. Zhu. 2012. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC Vet. Res.* 8:237. <https://doi.org/10.1186/1746-6148-8-237>.
- McMurdie, P. J., and S. Holmes. 2013. phyloseq: An R Package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. <https://doi.org/10.1371/journal.pone.0061217>.
- Miller, R. A., D. J. Kent, M. J. Watterson, K. J. Boor, N. H. Martin, and M. Wiedmann. 2015. Spore populations among bulk tank raw milk and dairy powders are significantly different. *J. Dairy Sci.* 98:8492–8504. <https://doi.org/10.3168/jds.2015-9943>.
- Nomura, T., Y. Hashimoto, J. Kurushima, H. Hirakawa, K. Tanimoto, B. Zheng, G. Ruan, F. Xue, J. Liu, J. Hisatsune, M. Sugai, and H. Tomita. 2018. New colony multiplex PCR assays for the detection and discrimination of vancomycin-resistant enterococcal species. *J. Microbiol. Methods* 145:69–72. <https://doi.org/10.1016/j.mimet.2017.12.013>.
- O'Driscoll, T., and C. W. Crank. 2015. Vancomycin-resistant enterococcal infections: Epidemiology, clinical manifestations, and optimal management. *Infect. Drug Resist.* 8:217–230. <https://doi.org/10.2147/IDR.S54125>.

- Parks, D. H., G. W. Tyson, P. Hugenholtz, and R. G. Beiko. 2014. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30:3123–3124. <https://doi.org/10.1093/bioinformatics/btu494>.
- Petersen, S. O., S. G. Sommer, F. Béline, C. Burton, J. Dach, J. Y. Dourmad, A. Leip, T. Misselbrook, F. Nicholson, H. D. Poulsen, G. Provolo, P. Sørensen, B. Vinnerås, A. Weiske, M.-P. Bernal, R. Böhm, C. Juhász, and R. Mihelic. 2007. Recycling of livestock manure in a whole-farm perspective. *Livest. Sci.* 112:180–191. <https://doi.org/10.1016/j.livsci.2007.09.001>.
- Quigley, L., O. O'Sullivan, C. Stanton, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, and P. D. Cotter. 2013. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 37:664–698. <https://doi.org/10.1111/1574-6976.12030>.
- R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
- Rasolofo, E. A., D. St-Gelais, G. LaPointe, and D. Roy. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int. J. Food Microbiol.* 138:108–118. <https://doi.org/10.1016/j.ijfoodmicro.2010.01.008>.
- Renye, J. A., G. A. Somkuti, D. L. Van Hekken, and V. M. Guerrero Prieto. 2011. Short communication: Characterization of microflora in Mexican Chihuahua cheese. *J. Dairy Sci.* 94:3311–3315. <https://doi.org/10.3168/jds.2011-4177>.
- Rey, M. W., P. Ramaiya, B. A. Nelson, S. D. Brody-Karpin, E. J. Zaretsky, M. Tang, A. de Leon, H. Xiang, V. Gusti, I. G. Clausen, P. B. Olsen, M. D. Rasmussen, J. T. Andersen, P. L. Jørgensen, T. S. Larsen, A. Sorokin, A. Bolotin, A. Lapidus, N. Galleron, S. D. Ehrlich, and R. M. Berka. 2004. Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. *Genome Biol.* 5:r77. <https://doi.org/10.1186/gb-2004-5-10-r77>.
- Ricciardi, A., A. Guidone, R. G. Ianniello, S. Cioffi, M. Aponte, D. Pavlidis, E. Tsakalidou, T. Zotta, and E. Parente. 2015. A survey of non-starter lactic acid bacteria in traditional cheeses: Culture dependent identification and survival to simulated gastrointestinal transit. *Int. Dairy J.* 43:42–50. <https://doi.org/10.1016/j.idairyj.2014.11.006>.
- Rowbotham, R. F., and P. L. Ruegg. 2015. Association of bedding types with management practices and indicators of milk quality on larger Wisconsin dairy farms. *J. Dairy Sci.* 98:7865–7885. <https://doi.org/10.3168/jds.2015-9866>.
- Savard, P., B. Lamarche, M.-E. Paradis, H. Thiboutot, É. Laurin, and D. Roy. 2011. Impact of *Bifidobacterium animalis* ssp. *lactis* BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. *Int. J. Food Microbiol.* 149:50–57. <https://doi.org/10.1016/j.ijfoodmicro.2010.12.026>.
- Shahbal, S., D. Hemme, and M. Desmazeaud. 1991. High cell wall-associated proteinase activity of some *Streptococcus thermophilus* strains (H-strains) correlated with a high acidification rate in milk. *Lait* 71:351–357. <https://doi.org/10.1051/lait:1991327>.
- Singh, T. K., M. A. Drake, and K. R. Cadwallader. 2003. Flavor of Cheddar cheese: A chemical and sensory perspective. *Compr. Rev. Food Saf. Food Saf.* 2:166–189. <https://doi.org/10.1111/j.1541-4337.2003.tb00021.x>.
- Skeie, S. B., M. Håland, I. M. Thorsen, J. Narvhus, and D. Porcellato. 2019. Bulk tank raw milk microbiota differs within and between farms: A moving goalpost challenging quality control. *J. Dairy Sci.* 102:1959–1971. <https://doi.org/10.3168/jds.2017-14083>.
- Sorter, D. E., H. J. Kester, and J. S. Hogan. 2014. Short communication: Bacterial counts in recycled manure solids bedding replaced daily or deep packed in freestalls. *J. Dairy Sci.* 97:2965–2968. <https://doi.org/10.3168/jds.2013-7814>.
- Vacheyrou, M., A.-C. Normand, P. Guyot, C. Cassagne, R. Piarroux, and Y. Bouton. 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. *Int. J. Food Microbiol.* 146:253–262. <https://doi.org/10.1016/j.ijfoodmicro.2011.02.033>.
- Yunita, D., and C. E. R. Dodd. 2018. Microbial community dynamics of a blue-veined raw milk cheese from the United Kingdom. *J. Dairy Sci.* 101:4923–4935. <https://doi.org/10.3168/jds.2017-14104>.
- Zdanowicz, M., J. A. Shelford, C. B. Tucker, D. M. Weary, and M. A. G. von Keyserlingk. 2004. Bacterial populations on teat ends of dairy cows housed in free stalls and bedded with either sand or sawdust. *J. Dairy Sci.* 87:1694–1701. [https://doi.org/10.3168/jds.S0022-0302\(04\)73322-6](https://doi.org/10.3168/jds.S0022-0302(04)73322-6).

ORCIDS

Mérlie Gagnon <https://orcid.org/0000-0002-0631-4946>
 Simon Dufour <https://orcid.org/0000-0001-6418-0424>