Coliform detection in cheese is associated with specific cheese characteristics, but no association was found with pathogen detection

A. Trmčić, K. Chauhan, D. J. Kent, R. D. Ralyea, N. H. Martin, K. J. Boor, and M. Wiedmann¹

Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, NY 14850

ABSTRACT

Coliform detection in finished products, including cheese, has traditionally been used to indicate whether a given product has been manufactured under unsanitary conditions. As our understanding of the diversity of coliforms has improved, it is necessary to assess whether coliforms are a good indicator organism and whether coliform detection in cheese is associated with the presence of pathogens. The objective of this study was (1) to evaluate cheese available on the market for presence of coliforms and key pathogens, and (2) to characterize the coliforms present to assess their likely sources and public health relevance. A total of 273 cheese samples were tested for presence of coliforms and for *Salmonella*, *Staphylococcus aureus*, Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and other *Listeria* species. Among all tested cheese samples, 27% (75/273) tested positive for coliforms in concentrations >10 cfu/g. Pasteurization, pH, water activity, milk type, and rind type were factors significantly associated with detection of coliforms in cheese; for example, a higher coliform prevalence was detected in raw milk cheeses (42% with >10 cfu/g) compared with pasteurized milk cheese (21%). For cheese samples contaminated with coliforms, only water activity was significantly associated with coliform concentration. Coliforms isolated from cheese samples were classified into 13 different genera, including the environmental coliform genera *Hafnia*, *Raoultella*, and *Serratia*, which represent the 3 genera most frequently isolated across all cheeses. *Escherichia*, *Hafnia*, and *Enterobacter* were significantly more common among raw milk cheeses. Based on sequencing of the housekeeping gene *clpX*, most *Escherichia* isolates were confirmed as members of fecal commensal clades of *E. coli*. All cheese samples tested negative for *Salmonella*, *Staph. aureus*, and Shiga toxin-producing *E. coli*. *Listeria* spp. were found

in 12 cheese samples, including 5 samples positive for *L. monocytogenes*. Although no association was found between coliform and *Listeria* spp. detection, *Listeria* spp. were significantly more likely to be detected in cheese with the washed type of rind. Our data provide information on specific risk factors for pathogen detection in cheese, which will facilitate development of risk-based strategies to control microbial food safety hazards in cheese, and suggest that generic coliform testing cannot be used to assess the safety of natural cheese.

Key words: cheese, coliform, pathogen, fecal contamination, pasteurization

INTRODUCTION

The coliform bacteria are a nontaxonomic classification that by definition describes a group of gram-negative, facultative anaerobic rod-shaped bacteria that are able to ferment lactose with production of acid and gas within 48 h at 32 to 35°C (Davidson et al., 2004). This simple concept of coliforms was developed more than 100 yr ago to test water for fecal contamination. The coliform test, because of its convenience, was quickly adopted by dairy and other branches of the food industry. Today, generic coliform testing is still used in the U.S. dairy industry to indicate unsanitary conditions in which product was manufactured, including postprocessing contamination and, much less likely, the possibility of pasteurization failure. Because coliforms, and gram-negative bacteria in general, are inactivated by pasteurization, using a coliform test in fluid milk production provides some value even though coliforms are responsible for only a part of the spoilage caused by gram-negative bacteria (Martin et al., 2011). Although the generic coliform test is still used in cheese production to indicate unsanitary manufacturing conditions, its usefulness in this product is increasingly being questioned (Strongin, 2015).

Two general misconceptions exist with regard to use of coliforms as indicators, which contribute to possible misinterpretations of coliform detection in cheese. The first is that coliforms exclusively represent bacteria

Received February 29, 2016.

Accepted May 2, 2016.

¹Corresponding author: mw16@cornell.edu

coming from a fecal environment. Leclerc and colleagues (2001) separated coliforms into 3 groups, including (1) psychrotolerant environmental coliforms, (2) thermotolerant fecal coliforms, and (3) ubiquitous coliforms that also include some thermotolerant coliforms. Genera such as *Serratia*, *Hafnia*, *Rahnella*, *Buttiauxella*, and *Leclercia* represent environmental coliforms and were shown recently to include at least some strains able to grow in milk at refrigeration temperatures (Masiello et al., 2016). Typical representatives of ubiquitous coliform genera are *Enterobacter* and *Citrobacter*. Both genera include several species that can be found in many known environments, including milk and cheese (Leclerc et al., 2001; Coton et al., 2012; Quigley et al., 2013; Masiello et al., 2016). Fecal coliforms represent only a small proportion of the coliform group, and several different reports suggest that *Escherichia coli* is the only coliform to exclusively represent the fecal environment (Edberg et al., 2000; Stevens et al., 2003; Paruch and Maehlum, 2012). Even though *E. coli* is characterized as being associated with fecal sources, specific bacterial lineages that are not associated with fecal sources have also been identified within the genus *Escherichia*; representatives of these non-fecal-associated lineages are often almost indistinguishable from *E. coli* (Walk et al., 2009; Luo et al., 2011; Oh et al., 2012). Walk et al. (2009) analyzed 22 housekeeping genes and identified 5 new phylogenetic clades of *Escherichia*, of which clades III, IV, and V were isolated from freshwater beaches, suggesting adaptation to the environment outside the intestinal tract of a warm-blooded host.

The second misconception about coliforms is that they can be used as index organisms. By definition, an index organism is an organism whose presence relates to the possible occurrence of ecologically similar pathogens (Mossel et al., 1995; Buchanan, 2000; Kornacki, 2011). Coliforms can be found in the majority of known environments and consequently cannot represent a specific ecological niche of any pathogen. Expectations of coliforms to yield information about the presence of pathogens is again connected to coliforms being perceived as exclusive representatives of the fecal environment, which is further known as a common source of foodborne pathogens.

With regard to water quality, the relevance of coliforms as indicators of fecal contamination and presence of pathogens has already been questioned several times in the past (Stevens et al., 2003; Wu et al., 2011). A similar comprehensive evaluation of coliforms and pathogens in cheese is needed to evaluate the relevance of coliforms as indicator organisms in this food product. Therefore, the objectives of this study were (1) to evaluate cheese available on the market for presence of coliforms and key pathogens, and (2) to characterize

the coliforms present to assess their likely sources and public health relevance.

MATERIALS AND METHODS

Collecting and Handling Cheese Samples

Cheese samples were collected from market sources throughout New York State during 2014 and 2015, following a convenient and stratified sampling plan; stratification was performed to ensure representation of raw and pasteurized milk cheeses as well as representation of different cheese categories manufactured from cow, sheep, and goat milks. The 273 cheese samples were selected to capture the diversity of cheese present on the market without the attempt to capture a market basket that represents consumption quantities of different cheese categories. Among these samples, 213 represented natural cheese products that were sampled only once during the study. The remaining samples included instances in which the same cheese product was sampled more than once from a given processor representing either 2 ($n = 21$) or 3 ($n = 6$) different production dates. Among the 273 cheese samples, 88 and 185 represented raw milk cheese and pasteurized milk cheese, respectively. Cheese samples were manufactured from cow ($n = 125$), goat ($n = 75$), sheep ($n = 62$), and mixed milks ($n = 11$). Samples were selected to represent different cheese categories and rind types to ensure inclusion of cheeses representing a wide range and different combinations of pH and water activity. The majority of cheese samples were purchased in grocery shops and supermarkets located in New York State as well as directly from cheese producers, specialty shops, and wholesale distributors. Cheeses sampled were manufactured in the United States ($n = 137$), as well as 13 other countries ($n = 136$), including Canada, New Zealand, Israel, and European Union countries (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2016-11112>). Approximately 50% of the US cheese samples ($n = 68$) were manufactured in New York State.

The cheese samples were either prepackaged or packaged in food-grade packing material before being transported in coolers to Cornell University (Ithaca, NY). Cheese samples were held at 4°C until the start of testing. This period was never longer than 7 d for whole cheese wheels and prepackaged cheese samples and never longer than 24 h for cut and packaged cheese samples. Each cheese sample was homogenized by hand in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI), and four 25-g aliquots of each cheese sample were weighed into separate sterile Whirl-Pak bags. Cheese aliquots were individually used to determine the concentration of coliforms, as well as presence of *Salmonella*, *Staphy-*

lococcus aureus, Shiga toxin-producing *E. coli* (**STEC**), *Listeria monocytogenes*, and other *Listeria* species. The remaining cheese was used to measure water activity and pH.

Determining Water Activity and pH of Cheese Samples

Water activity of all cheese samples was measured using AquaLab 3TE (AquaLab, Pullman, WA) according to the manufacturer's instructions. The pH was measured directly in cheese samples using Mettler-Toledo FE20 pH-meter with InLab413 SG pH-probe (Mettler-Toledo Group, Schwerzenbach, Switzerland). Each sample was measured at 2 different locations of the cheese homogenate, and a mean value was recorded. When measurement of pH differed by more than 0.5, 5 additional measurements at different locations were made to calculate the mean value.

Testing Cheese Samples for Coliforms

Cheese samples were tested for coliforms according to *Standard Methods for the Examination of Dairy Products* (Davidson et al., 2004) using *E. coli*/coliform count (PEC) plate method (Petrifilm plate; 3M, Maplewood, MN). Briefly, a 25-g aliquot of cheese sample was mixed with 225 mL of Butterfield's Phosphate Buffer (BD Difco, Franklin Lakes, NJ) in a sterile Whirl-Pak filter bag and homogenized using Stomacher 400 Circulator (Seaward Ltd., West Sussex, UK) for 2 min at 260 rpm. The homogenate was strained through the filter into a sterile bottle and the pH adjusted to between 6.60 and 7.20. One milliliter of each cheese homogenate was plated on 6 Petrifilm plates, 2 plates per incubation temperature (6°C/14 d, 32°C/24 h, 42°C/24 h). For each cheese sample positive for coliforms, 1 to 12 coliform isolates were selected across all Petrifilm plates containing positive colonies (max. of 4 colonies/incubation temperature) and streaked for purity on brain heart infusion (**BHI**) agar (BD Difco), which was subsequently incubated at 32°C for 24 h. If needed, isolates were additionally tested by inoculation into brilliant green bile broth (**BGGB**) tubes with inverted Durham tubes (BD Difco), followed by incubation at 32°C for 48 h; growth with formation of gas confirmed the isolates as coliforms.

Identification of Coliforms by Sequencing Part of 16S rDNA. All coliform bacteria isolated from cheese samples were identified by sequencing part of the 16S rDNA as previously described (Huck et al., 2008). An individual colony of each given isolate was first streaked onto a BHI agar plate, followed by overnight incubation at 32°C to obtain individual colonies.

A single colony was transferred into a 0.2-mL PCR tube using a sterile toothpick, followed by lysis by heating in a 1200-W microwave for 3 min. The obtained lysate was suspended in 50 µL of nuclease-free water and 2 µL of a given lysate was used to perform a 25-µL PCR, amplifying a 763-nucleotide (nt) 16S rDNA fragment. The PCR product size was verified using gel electrophoresis, and products were purified using the ExoSAP method (Dugan et al., 2002). Bidirectional Sanger sequencing of the PCR product was performed by the Life Sciences Core Laboratory Center (Cornell University, Ithaca, NY). A 616-nt fragment of 16S rDNA was analyzed to initially identify the isolates to the genus or species level, using the Ribosomal Database Project (**RDP**) classifier (Cole et al., 2005). Identification was assigned based on a seqmatch score (S_ab) of 1.000 for species-level identification and 0.94 or higher for genus-level identification.

Phylogenetic Analysis of Coliform Isolates. To confirm genus identifications for all isolates, a 16S rDNA-based parsimony phylogenetic tree was constructed using PAUP (version 4, Sinauer Associates Inc., Sunderland, MA); this tree included the sequences obtained in this study as well as all type strains of the family *Enterobacteriaceae* that were included in the RDP database. Isolates were confirmed as a specific genus if they clustered with a type strain in a phylogenetic tree.

Molecular Subtyping of *Escherichia* Isolates by Sequencing Part of *clpX*. All coliform isolates identified as representatives of the genus *Escherichia* were further subtyped by sequencing part of *clpX*, a gene that is part of the extended multilocus sequence typing scheme for *E. coli* (Qi et al., 2004). The general procedure was identical to the one described for sequencing part of 16S rDNA of coliform isolates, with the use of PCR and sequencing conditions described by Walk et al. (2009). The sequenced *clpX* fragment was aligned against a reference sequence (*E. coli* K12), trimmed to alignment length (523 nt), and used to generate a phylogenetic tree using 38 reference sequences previously used to distinguish between environmental and fecal commensal clades of *Escherichia* spp. (Walk et al., 2009; Luo et al., 2011; Oh et al., 2012).

Testing Cheese for *Listeria* spp./*Listeria monocytogenes*

Cheese samples were tested for presence of *L. monocytogenes* and other *Listeria* spp. using a modification of the standard method described in the US Food and Drug Administration *Bacteriological Analytical Manual* (**US FDA-BAM**; Hitchins and Jinneman, 2013). Briefly, a 25-g aliquot of cheese was homogenized in 225

mL of buffered listeria enrichment broth (BD Difco). Listeria selective enrichment supplement (Oxoid, Basingstoke, UK) was added after an initial 4-h incubation at 30°C, and incubation continued for 48 h overall. After 24- and 48-h incubation, 50 µL of the enrichment culture was streaked onto individual *Listeria monocytogenes* Plating Medium (**LMPM**; R&F Products Inc., Downers Grove, IL) and modified Oxford agar (**MOX**; BD Difco). The LMPM and MOX plates were incubated at 35°C and 30°C, respectively, and examined for presence of typical colonies after 48 h.

One isolate for each different type of characteristic colony phenotype on LMPM and MOX was streaked for purity on BHI agar (BD Difco) and confirmed by sequencing part of *sigB*. The general procedure was identical to that described for sequencing part of 16S rDNA of coliform isolates, with the use of previously described PCR and sequencing conditions (Nightingale et al., 2007). The obtained sequence of a 660-nt *sigB* fragment was compared with the Food Safety Laboratory (**FSL**) internal reference database to determine allelic type and identify the *Listeria* isolates to the species level as previously described (Sauders et al., 2012).

Testing Cheese for *Salmonella*

Cheese samples were tested for presence of *Salmonella* using a modification of the standard method described in US FDA-BAM (Wallace et al., 2012). Briefly, a primary enrichment was prepared by homogenizing a 25-g aliquot of cheese sample in 225 mL of lactose broth. The primary enrichment was initially incubated for 1 h at room temperature, followed by an additional 24 h at 35°C. After incubation, 1 mL and 0.1 mL of primary enrichment culture were transferred into 9.0 mL of tetrathionate medium (Oxoid) and Rappaport-Vassiliadis medium (BD Difco), respectively. The 2 secondary enrichments were incubated in a shaking water bath at 42°C for 24 h, and after incubation, 50-µL aliquots of each enrichment were streaked onto xylose lysine deoxycholate agar (**XLD**; Neogen, Lansing, MI) and CHROMagar *Salmonella* (CHROMagar, Paris, France). Both XLD and CHROMagar *Salmonella* were examined for typical colonies after 24 h of incubation at 35°C.

Testing Cheese for *Staphylococcus aureus*

Cheese samples were tested for presence of *Staph. aureus* using the direct plate count method described in US FDA-BAM (Reginald and Lancette, 2001). The cheese homogenate used for the enumeration of coliforms was used (before adjustment of pH) to test for presence of *Staph. aureus*. Three aliquots of 333 µL were spread-plated onto 3 individual plates of Baird-

Parker (**BP**) agar medium supplemented with egg-yolk tellurite (**EYT**; Oxoid), followed by incubation at 35°C for 48 h. When cheese manufactured from raw milk is being tested, the background flora of CNS can make it difficult to examine the plates for typical colonies. When this was the case, dilutions of the original cheese homogenate were also plated to obtain BP-EYT plates that could be examined. Typical colonies from BP-EYT were subcultured on to BP agar medium supplemented with rabbit plasma fibrinogen (**RPF**; Oxoid). The BP-RPF plates were examined for typical colonies after 48 h of incubation at 35°C. Typical colonies on BP-RPF were streaked for purity on BHI agar and identified by sequencing a fragment of the 16S rDNA. The general procedure was identical to the one described for sequencing part of the 16S rDNA of coliform isolates.

Testing Cheese for *STEC*

Cheese samples were tested for presence of *STEC* using a modification of the standard method described in US FDA-BAM (Feng et al., 2011). The final multiplex PCR for Shiga-like toxin I (*sltI*) and II (*sltII*) genes was performed according to (Hu et al., 1999). Briefly, a 25-g aliquot of cheese was homogenized by hand in 225 mL of modified buffered peptone water with addition of pyruvate. After initial incubation at 37°C for 5 h, the antibiotic supplement containing acriflavin, cefsulodin, and vancomycin was added and the incubation continued at 42°C for an additional 24 h. A 1-mL aliquot of the enrichment culture was used to prepare a thermal cell lysate (100°C/10 min) that was subsequently used to perform the multiplex PCR for *sltI* and *sltII*. A positive PCR result for any of the 2 *slt* genes was followed up by isolating and purifying 100 to 200 colonies from enrichment culture by plating on Levine's eosin-methylene blue agar (BD Difco) and MacConkey agar with added sorbitol (**SMAC**; BD Difco). Lysates from these purified colonies were tested with the multiplex PCR for both *sltI* and *sltII*. Enrichment cultures of all cheese samples were used to streak 50 µL onto SMAC agar plates; if available, 2 to 4 colonies were isolated and purified from these plates. All isolates from SMAC agar plates were confirmed as coliforms by inoculation into BGBB tubes; isolates confirmed as coliforms were retained and further characterized by sequencing part of 16S rDNA.

Isolate Information

Isolates were stored at -80°C in BHI broth (BD Difco) with 15% glycerol as part of the culture collection maintained by FSL, Department of Food Science, Cornell University (Ithaca, NY). Detailed information

on all isolates is available through Food Microbe Tracker (<http://www.foodmicrobetracker.com>). Requests for isolates should be addressed to the corresponding author (M. Wiedmann).

Data Analysis

The data were collected and managed in Excel (version 2007, Microsoft Corp., Redmond, WA). Coliform count data were log-transformed before analysis, and an additional binary data set was created based on it to represent presence (>10 cfu/g) or absence (≤ 10 cfu/g) of coliforms in cheese. Statistical analyses were performed using milk type, heat treatment of milk, rind type, pH, and water activity as variables of cheese characteristics. Cheese samples manufactured from mixed (cow, sheep, goat) milk ($n = 11$) were excluded from the analysis when milk type was included as a variable in the analysis. All statistical analyses were performed using R statistical software together with the R stats package (version 3.0.1; R Project, Vienna, Austria). Multivariable logistic regression analyses were performed to assess the effect of cheese characteristics on presence and absence of coliforms, *Escherichia*, and *Listeria* spp. in cheese. Multivariable linear regression analysis was performed to assess the effect of cheese characteristics on concentration of coliforms in cheese. Significance of individual variables in the logistic and linear model was evaluated by Type II ANOVA. Associations of different coliform genera with cheese manufactured from raw or pasteurized milk were tested using Fisher's exact tests performed on individual contingency tables; a Holm-Bonferroni correction for multiple comparisons was used. Fisher's exact test was also used to test for association between coliform detection and *Listeria* spp. detection in cheese. A Student's *t*-test was performed to test whether coliform concentration differed between cheese samples that tested positive and negative for presence of *Escherichia* spp.; only samples positive for coliforms (>10 cfu/g) were included in this analysis.

RESULTS

This study represents a comprehensive study on coliforms in cheese and determines the prevalence of 4 major pathogens of concern in this food product (*L. monocytogenes*, *Salmonella*, *Staph. aureus*, and STEC). Cheese samples tested represented pH values from 3.5 to 7.7 and water activity values from 0.880 to 0.996. Among the 273 cheese samples tested, 27% (75/273) had coliforms present in concentrations >10 cfu/g; an additional 16% (43/273) of cheese samples were con-

taminated with lower levels of coliforms (<10 cfu/g, but ≥ 1 cfu/25 g). For the purpose of this paper the concentrations >10 cfu/g will be referred to as positive coliform test, unless stated otherwise. In addition, 4% (12/273) of samples were positive for *Listeria* spp., including 5 samples positive for *L. monocytogenes*.

Positive Coliform Test Results Were Associated with Pasteurization Status, pH, Water Activity, and Selected Cheese Characteristics

Cheese manufactured from cow, goat, and sheep milks had 27, 33, and 18% prevalence of coliforms (at >10 cfu/g), respectively (Table 1). Raw milk cheese samples showed a higher coliform prevalence (42%) compared with pasteurized milk cheese samples (21%). Coliform prevalence by cheese type ranged from no positives among 19 brined feta-style cheeses (representing 18 pasteurized and 1 raw milk cheese) to 48% positives among soft cheeses. All samples of fresh cheese were manufactured from pasteurized milk, and 16% were positive for coliforms. The hard, semihard, and soft cheeses tested included 135 cheese samples with propagated surface microflora (washed, bloomy, and natural rind). Cheese samples with and without the propagated surface microflora had 41% (55/135) and 14% (20/138) prevalence of coliforms, respectively.

The lower limit of water activity in cheese in which coliforms were still detected (at >10 cfu/g) was 0.932. Although 2 cheese samples in which coliforms were detected had a pH of <5.0 (pH 4.76 and 4.66), all other cheese samples positive for coliforms had pH > 5.0 (Figures 1 and 2). Multivariable logistic regression analysis (Table 2; Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2016-11112>) showed that pasteurization of milk was the most significant factor associated with coliform detection ($P < 0.0001$), followed by cheese pH ($P < 0.001$) and water activity ($P < 0.01$). The predicted odds of a positive coliform test were 4.6 times higher for a raw milk cheese than a pasteurized milk cheese. With every increase in cheese pH by 1 unit, the predicted odds of positive coliform test increased by a factor of 2.3. With every 0.01-unit increase in water activity, the predicted odds of positive coliform test increased by a factor 1.4. Both milk type and rind type were also significantly associated ($P < 0.05$) with detection of coliforms in cheese (>10 cfu/g). For example, cheese manufactured from goat milk had 3.2 times higher predicted odds of a positive coliform test compared with cheese manufactured from cow milk. The predicted odds of positive coliform test in washed rind cheese were 4.0 times higher compared with cheese without rind (Table 2).

Table 1. Distribution of cheese samples positive for coliforms (>10 cfu/g) according to milk type and cheese category

Milk type	No. of samples positive for coliforms/no. of samples tested within cheese category (%) ¹						
	Hard	Semihard	Soft	Blue	Brined	Fresh	All ²
Cow							
Raw	8/17	9/19	7/10	1/11	— ³	—	25/57 (44)
Pasteurized	0/9	1/16	4/19	1/9	0/4	3/11	9/68 (13)
Total	8/26 (31)	10/35 (29)	11/29 (38)	2/20 (10)	0/4 (0)	3/11 (3)	34/125 (27)
Goat							
Raw	1/2	6/7	1/1	—	0/1	—	8/11 (73)
Pasteurized	2/8	4/14	9/22	2/6	0/4	0/10	17/64 (27)
Total	3/10 (30)	10/21 (48)	10/23 (43)	2/6 (67)	0/5 (0)	0/10 (0)	25/75 (33)
Sheep							
Raw	1/10	0/2	1/1	1/6	—	—	3/19 (16)
Pasteurized	1/10	1/14	5/6	0/1	0/8	1/4	8/43 (19)
Total	2/20 (10)	1/16 (6)	6/7 (86)	1/7 (14)	0/8 (0)	1/4 (25)	11/62 (18)
Mixed							
Raw	1/1	—	—	—	—	—	1/1 (100)
Pasteurized	0/1	0/1	4/5	0/1	0/2	—	4/10 (40)
Total	1/2 (50)	0/1 (0)	4/5 (80)	0/1 (0)	0/2 (0)	—	5/11 (45)
All ⁴							
Raw	11/30 (37)	15/28 (54)	9/12 (75)	2/17 (12)	0/1 (0)	—	37/88 (42)
Pasteurized	3/28 (11)	6/45 (13)	22/52 (42)	3/17 (18)	0/18 (0)	4/25 (16)	38/185 (21)
Total	14/58 (24)	21/73 (29)	31/64 (48)	5/34 (15)	0/19 (0)	4/25 (16)	75/273 (27)

¹Percentage of cheese samples positive for coliforms within the individual cheese group.

²A sum of cheese samples of all cheese categories within the animal category, separated by raw and pasteurized milk.

³No cheese samples tested within the group.

⁴A sum of cheese samples within each cheese category, made of all milk types, separated by raw and pasteurized milk.

Water Activity Was the Only Significant Factor Affecting Coliform Levels in Cheese

Statistical analysis using linear regression (Supplementary Tables S3 and S4; <http://dx.doi.org/10.3168/jds.2016-11112>) showed that water activity was the single significant factor associated with the concentration of coliforms in cheese ($P < 0.01$). The linear regression model suggests an average increase in coliform concentration by 0.541 log cfu/g for every 0.01-unit increase in water activity.

The Coliform Genera *Escherichia*, *Enterobacter*, and *Hafnia* Are More Common in Raw Milk than in Pasteurized Milk Cheeses

The 588 coliform isolates obtained from cheese samples were classified into 13 different genera within the family *Enterobacteriaceae* (*Buttiauxella*, *Cedecea*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Lelliottia*, *Rahnella*, *Raoultella*, *Serratia*; Table 3). The genus most frequently isolated from all tested cheeses was *Hafnia*, followed by *Raoultella*, *Serratia*, and *Escherichia*. The genera *Cedecea*, *Kluyvera*, *Klebsiella*, *Lelliottia*, *Rahnella*, *Buttiauxella*, and *Leclercia* were each isolated from less than 5% of cheese samples. In raw milk cheese, *Escherichia*, *Enterobacter*, and *Hafnia* were the most frequently isolated coliform

genera; these 3 genera were significantly more common among the coliform isolates from raw milk cheeses compared with those from pasteurized milk cheeses. *Escherichia* was mostly isolated together with other coliform genera; from 5, 11, and 4 cheese samples (with coliforms >10 cfu/g), we respectively isolated 1, 2, and 3 additional coliform genera besides *Escherichia*. We identified 3 cheese samples in which *Escherichia* was the only genus isolated. From 29% of cheese samples positive for coliforms (at >10 cfu/g), we isolated only 1 coliform genus per sample, whereas from 24, 31, 15, and 1% of cheese samples, we isolated 2, 3, 4 and 5 different coliform genera, respectively.

Logistic regression was performed to specifically identify factors associated with detection of the genus *Escherichia*. This logistic regression analysis showed that pasteurization of milk used for cheese was the most significant factor associated with reduced prevalence of the genus *Escherichia* ($P < 0.0001$; Supplementary Tables S5 and S6; <http://dx.doi.org/10.3168/jds.2016-11112>). The predicted odds of finding *Escherichia* species in cheese were 13.9 times higher if raw instead of pasteurized milk was used to manufacture the cheese. We also detected a significant association ($P = 0.04$) between detection of *Escherichia* spp. and rind type. Even though *Escherichia* spp. were found in almost one-third of cheese samples positive for coliforms (at >10 cfu/g), the detection of this genus was not associ-

ated with higher concentration of coliforms ($P > 0.1$; Student's t -test).

The Majority of *Escherichia* Isolates from Cheese Represent Fecal Commensals

Escherichia spp. were isolated from 23 cheese samples positive for coliforms (Table 3). These isolates were separated into 19 different sequence types (ST), based on partial *clpX* sequences (*clpX* ST). Three *clpX* ST represented the majority of *Escherichia* isolates ($n = 94$), and these isolates were obtained from 17 different

cheese samples. The remaining 16 *clpX* ST represented one-third ($n = 47$) of the 141 *Escherichia* isolates; each of these ST was isolated from 3 or fewer cheese samples. All 19 *clpX* ST were compared with *clpX* ST of known *Escherichia* strains by building a maximum-likelihood phylogenetic tree. The 3 *clpX* ST most commonly found in cheese clustered together with strains of *E. coli* known as human fecal commensals. One *clpX* ST, representing 2 cheese samples, clustered together with recently identified environmental clades III and IV. The remaining 15 *clpX* ST clustered nonspecifically between enteric and commensal strains of *Escherichia* (Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2016-11112>).

Figure 1. Concentration of coliforms in cheese with different (a) pH and (b) water activity values. Each symbol represents a cheese sample positive for coliforms. Cheese samples made from raw and pasteurized milk are represented by open and closed symbols, respectively. Box-and-whisker plots represent the distribution of pH and water activity of cheese samples with negative coliform test (CNR = raw milk cheese; CNP = pasteurized milk cheese). The vertical line within each box indicates the median, the boundaries of the box indicate first and third quartile, the whiskers indicate the entire range of values excluding the outliers, and the dot represents an outlier.

Listeria monocytogenes Was the only Pathogen Detected in Tested Cheese Samples

None of the 273 cheese samples tested positive for *Salmonella*, *Staph. aureus*, or STEC. *Listeria* spp. were detected in 12 cheese samples, of which 5 samples tested positive for *L. monocytogenes* (Table 4). Only 5 cheese samples positive for presence of *Listeria* spp. were also positive for presence of coliforms (>10 cfu/g); among them, only 1 cheese sample was positive for both *L. monocytogenes* and coliforms. Cheese samples positive for *Listeria* spp. included raw and pasteurized milk cheeses, manufactured from cow, sheep, and goat milks. Blue cheese as well as a range of cheese categories (hard, semihard, soft) and rind types (natural, washed, bloomy) tested positive for *Listeria* spp. The pH and water activity values determined in cheese samples positive for *Listeria* spp. ranged from 5.25 to 7.02 and from 0.939 to 0.981, respectively (Figure 2). The predicted odds of detecting *Listeria* spp. in washed rind cheese were more than 31 times higher than in cheese without treated rind ($P = 0.0043$; Supplementary Table S7; <http://dx.doi.org/10.3168/jds.2016-11112>). Cheese rind and specifically cheese with washed type of rind was found to be significantly associated with detection of *Listeria* spp. ($P < 0.01$; Supplementary Table S8; <http://dx.doi.org/10.3168/jds.2016-11112>). Out of 12 cheese samples positive for *Listeria* spp., 7 tested negative for coliforms (<10 cfu/g), including 4 cheese samples positive for *L. monocytogenes* (Table 4). In 5 samples from 1 producer, we identified *Listeria* spp. with 4 different *sigB* allelic types, of which 2 represented *L. monocytogenes*. These cheese samples were manufactured more than a year apart, and coliforms were present in high concentrations the first year ($n = 2$) but negative the second year ($n = 3$). Even though coliforms were absent the second year, *Listeria* spp. with matching *sigB* allelic types were identified in both years. Similar results were obtained with 1 imported

Figure 2. Presence and absence of coliforms in cheese with different combinations of pH and water activity. All cheese samples fall within the solid line; all cheese samples positive for coliforms fall within the dotted line. Triangles and circles represent cheese samples positive for *Listeria monocytogenes* and other *Listeria* spp., respectively. Open and closed symbols represent cheese samples negative and positive for coliforms, respectively. Top and right side histograms represent number of cheese samples analyzed within individual pH and water activity range, respectively. The number of cheese samples positive for coliforms are represented by the dashed part of each column.

cheese product sampled at 2 production dates (9 mo apart). An isolate of *L. innocua* (AT 108) was found together with coliforms (6.0×10^5 cfu/g) at the second sampling, but at the first sampling, we isolated AT 108 without detection of coliforms. We identified 3 cheese samples that were contaminated with both *Listeria* and *Escherichia* spp. All 3 cheese samples were representatives of washed rind cheese; 2 were pasteurized goat milk cheeses contaminated with *L. innocua* and *L. ivanovii*, respectively, and 1 was raw milk cow cheese contaminated with both *L. monocytogenes* and *L. seeligeri*.

DISCUSSION

Coliforms are frequently used to predict the hygienic conditions in which dairy products are manufactured. Considering the effect that these predictions can have on the dairy industry, coliforms are very poorly defined as a group. Information is also lacking about the taxonomic diversity of coliforms in the dairy environment. Several studies have analyzed the microbial population of raw milk and reported the presence of coliforms (Jayarao and Wang, 1999; D'Amico and Donnelly, 2010; Jackson et al., 2012; Quigley et al., 2013),

Table 2. Association between cheese characteristics and presence of coliforms (>10 cfu/g) in 273 cheese samples

Variable	Estimate ¹	Odds ratio ²	SE ³	Z statistic ⁴	P-value ⁵
Water activity	0.314	1.4	0.120	2.615	0.0089**
pH	0.829	2.3	0.258	3.217	0.0013**
Heat treatment					
Raw milk	1.515	4.6	0.398	3.805	0.0001***
Milk type					
Goat	1.149	3.2	0.427	2.689	0.0072**
Sheep	0.758	2.1	0.481	1.578	0.1145
Rind type					
Natural	0.275	1.3	0.514	0.535	0.5927
Washed	1.392	4.0	0.442	3.147	0.0016**
Bloomy	0.874	2.4	0.492	1.778	0.0754

¹Parameter estimate of explanatory variable in logistic mixed effect model.

²For continuous variables, the odds ratios are with respect to a unit change (1 unit for pH; 0.01 units for water activity); for categorical variables, odds ratios are with respect to the reference level ("pasteurized milk" for heat treatment; "cow" for milk type; "without rind" for rind type).

³Standard error of effect estimation in logistic mixed effect model.

⁴Z statistic for effect test.

⁵P-value for effect test.

** $P < 0.01$; *** $P < 0.001$.

but studies on final dairy products including raw milk cheeses are still largely missing. In the current study, we focused on analyzing the coliform populations in different cheeses and evaluated the association of coliform detection with the detection of 4 major pathogens (*L.*

monocytogenes, *Salmonella*, *Staph. aureus*, and STEC). Our data suggest that more than a quarter of the cheese present on the New York State market contains coliforms above the state limit (10 cfu/g). Dealing with this large amount of cheese that does not comply with

Table 3. Distribution of coliform genera in cheese manufactured from raw and pasteurized milk

Genus	Class ¹	No. of positive samples (% of tested samples) ²		
		All cheese ³	Raw milk cheese ⁴	Pasteurized milk cheese ⁵
<i>Hafnia</i>	E	31 (11)	18 (20)*	13 (7)
<i>Raoultella</i>	E	26 (10)	9 (10)	17 (9)
<i>Serratia</i>	E ⁶	25 (9)	8 (9)	17 (9)
<i>Escherichia</i>	F ⁷	23 (8)	19 (22)***	4 (2)
<i>Citrobacter</i>	U	17 (6)	10 (11)	7 (4)
<i>Enterobacter</i>	U ⁷	16 (6)	13 (15)***	3 (2)
<i>Cedecea</i>	U ⁷	11 (4)	4 (5)	7 (4)
<i>Kluyvera</i>	E ⁶	8 (3)	1 (1)	7 (4)
<i>Klebsiella</i>	U ⁷	6 (2)	3 (3)	3 (2)
<i>Lelliottia</i>	U ⁶	5 (2)	0 (0)	5 (3)
<i>Rahnella</i>	E ⁶	4 (1)	1 (1)	3 (2)
Other ⁸	E ⁶	4 (1)	0 (0)	4 (2)

¹Coliform genera were classified into groups based on Leclerc et al. (2001) and Brady et al. (2013): E = environmental coliforms; F = fecal coliforms; U = ubiquitous coliforms.

²A sample was positive for coliforms when concentration determined on 3M *E. coli*/Coliform Count Plates (3M, Abbottford, MN) was >10 cfu/g.

³Percentage of total (273) cheese samples tested; total >100% because several cheese samples were positive for multiple genera.

⁴Percentage of all (88) tested raw milk cheese samples; total >100% because several cheese samples were positive for multiple genera.

⁵Percentage of all (185) tested pasteurized milk cheese samples; total >100% because several cheese samples were positive for multiple genera.

⁶Genus was never isolated at 42°C.

⁷Genus was never isolated at 6°C.

⁸*Buttiauxella* and *Leclercia*.

* $P < 0.05$ and *** $P < 0.001$: Genus significantly associated with raw milk cheese.

Table 4. Presence of *Listeria* species in cheese according to milk type and cheese category

Milk type ¹		Cheese category	Rind	Species ²	Allelic type ³	Concentration of coliforms (cfu/g) ⁴
Cow (A, C, G)	Raw	Hard	Washed	<i>L. monocytogenes</i>	64	1.0 × 10 ⁴ (E)
				<i>L. seeligeri</i>	7	
Cow (A, C, H)	Raw	Hard	Washed	<i>L. monocytogenes</i>	58	<10
Cow (A, D, G)	Raw	Semihard	Washed	<i>L. seeligeri</i>	7	6.5 × 10 ⁵
Cow (A, D, H)	Raw	Semihard	Washed	<i>L. monocytogenes</i>	64	<10
				<i>L. monocytogenes</i>	58	
				<i>L. monocytogenes</i>	64	
Cow (A, H)	Raw	Soft	Washed	<i>L. monocytogenes</i>	64	<10
				<i>L. innocua</i>	26	
Cow	Raw	Blue	NA ⁵	<i>L. innocua</i>	56	<10
Goat	Pasteurized	Semihard	Washed	<i>L. innocua</i>	164	1.3 × 10 ² (E)
Goat	Pasteurized	Semihard	Washed	<i>L. ivanovii</i>	165	5.6 × 10 ⁶ (E)
Goat (B, F, G)	Pasteurized	Soft	Bloomy	<i>L. innocua</i>	108	<10
Goat (B, F, H)	Pasteurized	Soft	Bloomy	<i>L. innocua</i>	108	6.0 × 10 ⁵
Sheep	Raw	Hard	Natural	<i>L. innocua</i>	22	<10
Sheep	Pasteurized	Semihard	Natural	<i>L. monocytogenes</i>	57	<10

¹Cheese samples with a matching letter A or B were manufactured by the same natural cheese producer; cheese samples with a matching letter C, D, and F are the same natural cheese product; cheese samples with G or H were sampled at the beginning or at the end of 9- to 12-month period, respectively.

²Species of *Listeria* identified by comparing the 660-nt sequence of *sigB* to the internal reference database.

³*sigB* allelic type.

⁴Presence of *Escherichia* spp. indicated by (E).

⁵Not applicable.

the state regulation represents a large burden for both cheese producers and regulatory officers and calls for a re-evaluation of how the generic coliform test is interpreted.

Milk Pasteurization, Low pH, Low Water Activity, and Other Cheese Characteristics Significantly Contribute to Lower Prevalence of Coliforms in Cheese

Pasteurization of milk used for cheese making was the most significant factor predicting the detection of coliforms in cheese; raw milk cheese was 4.6-fold more likely to be positive for coliforms. By comparison, D'Amico and colleagues (D'Amico et al., 2008; D'Amico and Donnelly, 2010) reported that between 32 and 39% of raw milk used for making cheese in Vermont (United States) contained coliforms at >10 cfu/mL. Other US studies reported even higher percentages of raw milk with coliforms above this concentration, ranging from 56% (Jayarao and Wang, 1999) to 80% (Pantoja et al., 2009) and 87% in a more recent study (Jackson et al., 2012). These results, together with results from our study, strongly suggest that raw milk is a very important source of coliforms in cheese made from unpasteurized milk.

We detected coliforms (>10 cfu/g) in 42% of tested raw milk cheese samples, but similar studies performed in the United States (Brooks et al., 2012) and Japan (Esho et al., 2013) found that prevalences in raw milk cheese were 12 and 20%, respectively. Besides the

geographic location, the difference between the results could also result from a number of other factors, including cheese categories selected for analysis and incubation temperature used when testing for coliforms [e.g., Esho et al. (2013) used 37°C, but Brooks et al. (2012) used 35°C]. All 3 studies show lower prevalence in cheese than previously reported for raw milk (Jayarao and Wang, 1999; Pantoja et al., 2009; Jackson et al., 2012), which suggests that the general cheese-making procedure and ripening process reduce the prevalence of coliforms. Other authors have reached a similar conclusion (Tham et al., 1990; Mullan, 2000; Sheehan, 2011), specifically suggesting that quick initial acidification is the most important factor in reducing coliform loads and preventing defects such as early blowing in cheese.

Even though coliforms are considered thermolabile and do not survive pasteurization, we found that 21% of pasteurized milk cheeses tested positive for coliforms (>10 cfu/g). *Escherichia* and *Enterobacter* were identified as the least prevalent genera in pasteurized milk cheese but were among the most prevalent genera in raw milk cheese. Both *Escherichia* and *Enterobacter* have been reported to be among the genera commonly isolated from raw milk (Jayarao and Wang, 1999; Jackson et al., 2012). This finding suggests that coliforms in pasteurized milk cheese are less likely to originate from raw milk, with the more likely source being postpasteurization contamination.

Cheese rind type was identified as a factor significantly associated with detection of coliforms. Specifically, washed rind cheese showed a 4 times higher risk

of coliform detection than cheese without treated rind. In a previous study on Belgian washed rind cheese made from raw and pasteurized milk, numerically higher levels of *Enterobacteriaceae* were found on the rind compared with the core, regardless of the pasteurization status (Deltenserie et al., 2014). Production of washed rind cheese is typically associated with frequent manual handling during ripening. In addition, the wash solutions used in the process can be very diverse and introduce different components that are associated with higher microbial contamination (i.e., herbs and spices).

Cheese pH was another factor that was significantly associated with detection of coliforms in cheese. Of the 47 tested cheese samples with pH <5.0, only 2 were positive for coliforms. This specific observation is consistent with previous studies; Manolopoulou and colleagues (2003) reported detecting die-off of *E. coli* and other coliforms in feta-style cheese with pH <5.0, and other studies demonstrated good growth of 10 different coliform strains (representing *Hafnia*, *Serratia*, *Enterobacter*, and *Escherichia*) in cheese with pH between 5.2 and 5.3 (Morales et al., 2003). One of the coliform-positive cheese samples with pH <5.0 was a soft bloomy rind cheese, and the other sample (pH 4.76) represented a fresh cheese, which may have been too young to allow for substantial coliform die-off. Previous studies have shown that die-off of coliforms and *E. coli*, even at pH <5.0 may take several days (Feresu and Nyati, 1990; Manolopoulou et al., 2003). Our findings thus suggest that even low-pH cheeses may occasionally test positive for coliforms.

Our data showed that water activity was significantly associated with detection of coliforms. None of the 20 cheese samples with water activity <0.932 were positive for coliforms. Coliforms are mainly representatives of bacterial family *Enterobacteriaceae* and, in a recent report, water activity of 0.94 was determined to be the minimum at which members of this family can still grow (Baylis et al., 2011). By comparison, one previous study found that 4 of 17 cheese samples with water activity >0.93 were positive for coliforms, but only 1 of 23 cheese samples with a lower water activity was positive for coliforms (this semihard cheese had a water activity of 0.88; Brooks et al., 2012). Another study, in which water activity in cheese was not determined, also reported a numerically higher prevalence of coliforms in the soft cheese category compared with the hard and semihard categories (Esho et al., 2013). Higher counts of coliforms and problems with the early blowing defect are known to be more prevalent in rennet-coagulated soft and semihard cheese in which both water activity and pH are higher (Sheehan, 2011). Our results indicate that prevalence of coliforms in cheese at concentrations

>10 cfu/g is significantly reduced when water activity in cheese is below the limit of 0.93.

The milk type used to make cheese was also significantly associated with detection of coliforms; specifically, cheese manufactured from goat milk showed a higher risk of coliform detection. In previous work on the presence of coliforms in raw milk, researchers determined that the prevalence of coliforms does not significantly differ in raw cow, sheep, or goat milk (D'Amico et al., 2008; D'Amico and Donnelly, 2010). Other intrinsic factors in goat milk, goat cheese, or both (not including pH or water activity) might allow a higher prevalence of coliforms in the final product. A second possibility is that procedures involved in making goat cheese (not including pasteurization and treatment of cheese rind) may be associated with more frequent contamination with coliforms; for example, goat cheese producers may be more likely to represent small facilities with reduced resources related to food safety.

Water Activity Is the Only Factor that Determines the Concentration at which Coliforms Are Present

Although several intrinsic and extrinsic factors were identified as being significant for detecting the presence or absence of coliforms in cheese (i.e., pH, water activity, pasteurization, milk type, rind type), water activity was the only factor significantly associated with the final concentration of coliforms in cheese. Given that we found the highest concentrations of coliforms in pasteurized milk cheese, growth in cheese seems to be a likely reason for higher concentrations. Morales et al. (2003) demonstrated that different strains of *Hafnia*, *Serratia*, *Enterobacter*, and *Escherichia* can grow by more than 4 log cfu/g in 1 d when inoculated into fresh cheese with a high water activity and pH of 5.2. Another study also found a positive correlation between water activity and the presence of generic *E. coli* in raw milk cheese (Almeida et al., 2007). These results suggest that if coliforms are able to establish their presence in cheese with pH >5.0 and water activity >0.93, they will be able to grow, and a more rapid growth is expected in cheeses with higher water activity.

Coliforms Identified in Cheese Include Genera that Benefit Cheese Quality and *Escherichia* Isolates from Fecal Commensal Clades

In cheese samples tested during this study, we identified *Hafnia*, *Raoultella*, and *Serratia* as the most prevalent coliform genera. In a previous metagenomic study, *Hafnia* and *Serratia* were identified as common members of rind population and, in some cases, even the dominant ones (Wolfe et al., 2014). Both coliform gen-

era were identified as having active proteolytic enzymes that degrade caseins and influence textural as well as sensory properties of cheese (Morales et al., 2003). *Hafnia alvei* is a very common species in cheese and it was found to contribute to accumulation of volatile aroma compounds, while having no negative effect on safety from excessive accumulation of biogenic amines (Abriouel et al., 2008; Delbès-Paus et al., 2012; Irlinger et al., 2012a). Besides *Hafnia* and *Serratia*, other coliform genera identified in our study, such as *Raoultella*, *Escherichia*, *Citrobacter*, *Enterobacter*, *Kluyvera*, and *Klebsiella*, were isolated from cheese in other studies (Chaves-López et al., 2006; Cotton et al., 2012; Irlinger et al., 2015). The coliform genera that were identified in our study but not reported previously represent genera that were found in less than 5% of cheese samples. These genera are mainly represented by environmental coliform genera *Rahnella*, *Buttiauxella*, and *Leclercia*, as well as recently identified species of *Lelliottia* (Brady et al., 2013). These genera are commonly found in different water sources as well as in fish, mollusks, and insects (Leclerc et al., 2001; Brady et al., 2014). Among *Rahnella* isolates, we also identified a newly classified environmental species, *Rahnella inusitata* (Brady et al., 2014). This species was previously isolated from a sample of pasteurized milk and was included in our standard bacterial isolate set of dairy spoilage (Trmčić et al., 2015). The strain included in the collection is able to cause a ropy defect in fluid milk most likely through the production of exopolysaccharides. Production of exopolysaccharides is often identified as a positive contributor to texture and other sensory properties of cheese and other dairy products (Trancoso-Reyes et al., 2014; Wu et al., 2014; Oberg et al., 2015). Our results suggest that many of the coliforms present in cheese may contribute to the final quality of cheese and do not represent indicators for unhygienic conditions in which cheese was manufactured.

Of 23 cheese samples with coliforms >10 cfu/g and detected *Escherichia*, 17 were determined to contain strains identified as most similar to known fecal commensal *E. coli* clades. *Escherichia coli* is considered a unique representative of the intestinal environment and a good indicator of fecal contamination (Edberg et al., 2000; Paruch and Maehlum, 2012). These results suggest that the majority of *Escherichia* isolates detected in cheese samples represent an actual direct or indirect fecal contamination of cheese.

Raw Milk Cheese Has a Higher Prevalence of Coliforms that Indicate Fecal Contamination

For raw milk cheeses, we found that the genera *Escherichia*, *Hafnia*, and *Enterobacter* were the most com-

mon coliforms; all 3 of these genera were significantly more common in raw milk cheeses compared with pasteurized milk cheeses. Although *Hafnia* may have positive effects on the quality of raw milk cheese, the isolation of *Escherichia* suggests fecal contamination. Similarly, these 3 genera were reported to be found at high frequency among coliforms isolated from raw milk (Jayarao and Wang, 1999; Quigley et al., 2013). Even though some studies have found no differences in the prevalence of coliforms or generic *E. coli* in pasteurized and raw milk cheese (Coveney et al., 1994; Little et al., 2008), given other studies that report high prevalence of *Escherichia* in raw milk and its absence in pasteurized milk (Jayarao and Wang, 1999; Jackson et al., 2012; Masiello et al., 2016), the most likely source of this genus in raw milk cheese is the raw milk itself.

We detected *Escherichia* in 19 of the raw milk cheese samples with coliform concentrations > 10 cfu/g; these *Escherichia*-positive samples represent 22% of raw milk cheese samples tested. Among these 19 raw milk cheeses, 15 yielded *Escherichia* isolates that were classified as fecal commensal clades. Until recently, the US FDA was using a 3-class sampling plan ($n = 5$, $c = 2$, $m = 10$ MPN/g, $M = 100$ MPN/g) to test for nontoxigenic *E. coli* and evaluate the sanitary conditions in which cheese was made. This plan involved determining most probable number (MPN) of *E. coli* in 5 subsamples (n) of the same product, and if it was found at levels greater than 10 MPN/g (m) in more than 2 (c) subsamples or greater than 100 MPN/g (M) in 1 or more samples, the product was considered to be made in unsanitary conditions and therefore adulterated (Correll, 2014; U.S. FDA, Center for Food Safety and Applied Nutrition, 2010). The US FDA, in its letter to the American Cheese Society, reported that out of 885 samples of raw milk cheese, 5% were found to not meet the criteria of the 3-class sampling plan for nontoxigenic *E. coli* (Correll, 2014). The results from the US FDA are difficult to compare with our results because of several methodological differences. For example, the 2 methods differ in the amount of product tested. Also, we isolated and characterized single colonies obtained by direct plating (without enrichment), but the MPN procedure includes, by definition, a growth step in liquid medium (i.e., an enrichment). Hence, differential growth of *Escherichia* and competing microorganisms (e.g., other coliforms) can affect the likelihood of *Escherichia* detection. The potential for competition is supported by our observation that we isolated *Escherichia* as well as 1, 2, and 3 other coliform genera from 5, 11, and 4 cheese samples, respectively; only 3 cheese samples were identified in which *Escherichia* was the only genus isolated. Hence, *Escherichia* could be outcompeted in some of the MPN tests by other coliforms.

***Listeria monocytogenes* Is the Main Pathogen of Concern in Cheese**

All 273 cheese samples tested were negative for *Salmonella*, *Staph. aureus*, and STEC. Similar results in cheese were also reported by others for both *Salmonella* and STEC (Quinto and Cepeda, 1997; Little et al., 2008; Brooks et al., 2012; Esho et al., 2013). In the US FDA study, none of 885 raw milk cheese samples tested positive for *E. coli* O157:H7, but 2 cheese samples tested positive for *Salmonella* (Correll, 2014). These results suggest that the prevalence of *Salmonella* and STEC are low in cheese and that a larger sample size is needed to estimate their prevalence and to identify risk factors for these pathogens being present in cheese. Even though these results suggest that the prevalence of these pathogens in cheese is low, outbreaks involving both *Salmonella* and STEC from cheese do occur (Kousta et al., 2010; Gill and Oudit, 2015) and should not be neglected. Although several studies have reported the common presence of *Staph. aureus* in cheese (Coveney et al., 1994; Kousta et al., 2010; Rosengren et al., 2010; Brooks et al., 2012), we did not isolate *Staph. aureus* from any of the cheese samples tested. Because CNS are common within raw milk cheese microflora and are known to contribute to the ripening process (Irlinger et al., 2012b; Dugat-Bony et al., 2015), we cannot exclude the possibility that low contamination levels with *Staph. aureus* were not detected because of competition by CNS.

We identified *Listeria* spp. in more than 4% of tested cheese samples; 1.8% of samples were positive for *L. monocytogenes*. These results are consistent with previous studies that reported between 1.3 and 4.8% *L. monocytogenes* prevalence in cheese (Mena et al., 2004; Manfreda et al., 2005; Colak et al., 2007; Cabedo et al., 2008). By comparison, 2 studies, which tested 126 and 151 cheese samples, reported no *L. monocytogenes*-positive samples (Rosengren et al., 2010; Esho et al., 2013), but other studies reported *L. monocytogenes* prevalences of >40% (Loncarevic et al., 1995; Pintado et al., 2005; Filiouis et al., 2009); these high prevalences were obtained for samples of soft and semi-soft cheese manufactured from raw milk. The same 2 cheese categories (made from both raw and pasteurized milk) were tested by Rudol and Scherer (2001), who reported 15.8 and 6.4% prevalence of *Listeria* spp. and *L. monocytogenes*, respectively. Another study identified *L. monocytogenes* as the only pathogen that can survive the ripening process in semihard Swiss-type cheese (Bachmann and Spahr, 1995). This conclusion is consistent with our findings as well as with reports from the US FDA (Correll, 2014) and other studies (Colak et al., 2007; Cabedo et al., 2008) that report *L.*

monocytogenes as a more prevalent pathogen in cheese than *Salmonella* or STEC.

Listeria monocytogenes, as well as other *Listeria* spp. of the same *sigB* allelic type, were identified in cheese samples manufactured by the same cheese producer at different production dates. Consistent with these observations, others have shown that different *Listeria* species can persist in food and dairy processing environments over time (Ho et al., 2007; Dalmasso and Jordan, 2012; Almeida et al., 2013; Schoder et al., 2013) and can lead to repeated contamination of products produced in a given facility.

Detection of *Listeria* spp. in Cheese Was Not Found to Be Associated with Detection of Coliforms

A positive coliform test (>10 cfu/g) provides no additional information about the presence of *Listeria* spp. in cheese. When comparing presence of *Listeria* spp. in all tested cheese samples and in cheese samples that tested positive for coliforms, only a minimal numerical increase in prevalence was observed (from 4.4 to 6.7%), and the prevalence decreased in a comparison of the data for *L. monocytogenes* (from 1.8 to 1.3%). In accordance with these observations, no statistical association was found between detection of coliforms and *Listeria* spp. in cheese. A statistically significant association was found between detection of *Listeria* spp. and cheese rind, particularly washed type of cheese rind. Other studies also identified smear or washed rind cheese to be associated with higher detection of *L. monocytogenes* and other *Listeria* spp. (Loncarevic et al., 1995; Rudol and Scherer, 2001). Additionally, outbreaks of *L. monocytogenes* have been linked to this cheese category in the past (Rudol and Scherer, 2001; Gaulin et al., 2012; Schoder et al., 2013). Growth models of *L. monocytogenes* on the surface of smear-ripened cheese identified surface pH as being associated with higher growth, but the same association could not be found for surface water activity (Schvartzman et al., 2014). Effect of surface pH or water activity on detection of *Listeria* spp. on cheese could not be determined during our study because our measurements determined the average values in each cheese sample. Although the exact mechanism is still to be determined, our results suggest that washed rind cheese presents the highest risk of contamination with different *Listeria* species.

CONCLUSIONS

We evaluated the cheese available on the market in New York State for the presence of coliforms and key pathogens linked to dairy products, and we concluded that coliform testing should no longer be used to assess

the safety of cheese. Generic coliform testing in cheese could be replaced by (1) testing for *E. coli*, a potentially better indicator of fecal contamination; or (2) testing for *L. monocytogenes* or other pathogen that might present a risk in a given cheese category. Pathogen testing may provide an improved ability to detect actual public health hazards though because our data show that *E. coli*-negative samples may still test positive for *L. monocytogenes*. Our data furthermore provide information on cheese categories and characteristics that may be associated with a higher likelihood of *E. coli* or *Listeria* spp. contamination, facilitating development of risk-based strategies to control microbial food safety hazards in cheese.

ACKNOWLEDGMENTS

This project was supported by the New York State Milk Promotion Advisory Board contract #C200796 through the New York State Department of Agriculture and Markets (Albany, NY). We thank all cheese producers and distributors who participated in this project by providing us with an array of different cheese samples, as well as Cornell University Cooperative Extension team for making the necessary connections. We also thank Carmen Moraru (Department of Food Science, Cornell University, Ithaca, NY) for enabling us to measure water activity of cheese samples, Shelley Clark (Department of Food Science, Cornell University) for her help with the pulsed-field gel electrophoresis, and MPS student Tsung-Han (Kent) Hsieh (Department of Food Science, Cornell University) for his enthusiastic help in the laboratory.

REFERENCES

- Abriouel, H., A. Martin-Platero, M. Maqueda, E. Valdivia, and M. Martinez-Bueno. 2008. Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 127:200–208. <http://dx.doi.org/10.1016/j.ijfoodmicro.2008.07.004>.
- Almeida, G., A. Figueiredo, M. Rola, R. M. Barros, P. Gibbs, T. Hogg, and P. Teixeira. 2007. Microbiological characterization of randomly selected Portuguese raw milk cheeses with reference to food safety. *J. Food Prot.* 70:1710–1716.
- Almeida, G., R. Magalhaes, L. Carneiro, I. Santos, J. Silva, V. Ferreira, T. Hogg, and P. Teixeira. 2013. Foci of contamination of *Listeria monocytogenes* in different cheese processing plants. *Int. J. Food Microbiol.* 167:303–309. <http://dx.doi.org/10.1016/j.ijfoodmicro.2013.09.006>.
- Bachmann, H. P., and U. Spahr. 1995. The fate of potentially pathogenic bacteria in Swiss hard and semihard cheeses made from raw milk. *J. Dairy Sci.* 78:476–483. [http://dx.doi.org/10.3168/jds.S0022-0302\(95\)76657-7](http://dx.doi.org/10.3168/jds.S0022-0302(95)76657-7).
- Baylis, C., M. Uyttendaele, H. Joosten, and A. Davies. 2011. The *Enterobacteriaceae* and their Significance to the Food Industry. Pages 1–52 in ILSI Europe Report Series. International Life Sciences Institute, Washington, DC.
- Brady, C., I. Cleenwerck, S. Venter, T. Coutinho, and P. De Vos. 2013. Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): Proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Syst. Appl. Microbiol.* 36:309–319. <http://dx.doi.org/10.1016/j.syapm.2013.03.005>.
- Brady, C., G. Hunter, S. Kirk, D. Arnold, and S. Denman. 2014. *Rahnella victoriana* sp. nov., *Rahnella bruchi* sp. nov., *Rahnella woolbedingensis* sp. nov., classification of *Rahnella* genomospecies 2 and 3 as *Rahnella variigena* sp. nov. and *Rahnella inusitata* sp. nov., respectively and emended description of the genus *Rahnella*. *Syst. Appl. Microbiol.* 37:545–552. <http://dx.doi.org/10.1016/j.syapm.2014.09.001>.
- Brooks, J. C., B. Martinez, J. Stratton, A. Bianchini, R. Krokstrom, and R. Hutkins. 2012. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food Microbiol.* 31:154–158. <http://dx.doi.org/10.1016/j.fm.2012.03.013>.
- Buchanan, R. L. 2000. Acquisition of microbiological data to enhance food safety. *J. Food Prot.* 63:832–838.
- Cabedo, L., L. Picart i Barrot, and A. Teixido i Canelles. 2008. Prevalence of *Listeria monocytogenes* and *Salmonella* in ready-to-eat food in Catalonia, Spain. *J. Food Prot.* 71:855–859.
- Chaves-López, C., M. De Angelis, M. Martuscelli, A. Serio, A. Paparella, and G. Suzzi. 2006. Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *J. Appl. Microbiol.* 101:353–360. <http://dx.doi.org/10.1111/j.1365-2672.2006.02941.x>.
- Colak, H., H. Hampikyan, E. B. Bingol, and B. Ulusoy. 2007. Prevalence of *L. monocytogenes* and *Salmonella* spp. in Tulum cheese. *Food Contr.* 18:576–579. <http://dx.doi.org/10.1016/j.foodcont.2006.02.004>.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): Sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294–D296. <http://dx.doi.org/10.1093/nar/gki038>.
- Correll, W. A. 2014. Letter to American Cheese Society (ACS) with an Update on What Has Been Done with Respect to Non-toxicogenic *Escherichia coli* (*E. coli*) in Raw Milk Cheese. Public Health Service, Food and Drug Administration, College Park, MD.
- Coton, M., C. Delbès-Paus, F. Irlinger, N. Desmasures, A. Le Fleche, V. Stahl, M.-C. Montel, and E. Coton. 2012. Diversity and assessment of potential risk factors of gram-negative isolates associated with French cheeses. *Food Microbiol.* 29:88–98. <http://dx.doi.org/10.1016/j.fm.2011.08.020>.
- Coveney, H. M., G. F. Fitzgerald, and C. Daly. 1994. A study of the microbiological status of Irish farmhouse cheeses with emphasis on selected pathogenic and spoilage microorganisms. *J. Appl. Bacteriol.* 77:621–630. <http://dx.doi.org/10.1111/j.1365-2672.1994.tb02810.x>.
- D'Amico, D. J., and C. W. Donnelly. 2010. Microbiological quality of raw milk used for small-scale artisan cheese production in Vermont: Effect of farm characteristics and practices. *J. Dairy Sci.* 93:134–147. <http://dx.doi.org/10.3168/jds.2009-2426>.
- D'Amico, D. J., E. Groves, and C. W. Donnelly. 2008. Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production. *J. Food Prot.* 71:1580–1589.
- Dalmasso, M., and K. Jordan. 2012. Process environment sampling can help to reduce the occurrence of *Listeria monocytogenes* in food processing facilities. *Ir. J. Agric. Food Res.* 52:93–100.
- Davidson, P. M., L. A. Roth, and S. A. Gambrel-Lenarz. 2004. Coliform and other indicator bacteria. Pages 187–226 in *Standard*

- Methods for the Examination of Dairy Products. 17th ed. H. M. Wehr and J. F. Frank, ed. American Public Health Association, Washington, DC.
- Delbès-Paus, C., S. Pochet, S. Helinck, P. Veisseire, C. Bord, A. Lebecque, M. Coton, N. Desmasures, E. Coton, F. Irlinger, and M.-C. Montel. 2012. Impact of gram-negative bacteria in interaction with a complex microbial consortium on biogenic amine content and sensory characteristics of an uncooked pressed cheese. *Food Microbiol.* 30:74–82. <http://dx.doi.org/10.1016/j.fm.2011.12.008>.
- Delcenserie, V., B. Taminiau, L. Delhalle, C. Nezer, P. Doyen, S. Crevecoeur, D. Roussey, N. Korsak, and G. Daube. 2014. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. *J. Dairy Sci.* 97:6046–6056. <http://dx.doi.org/10.3168/jds.2014-8225>.
- Dugan, K. A., H. S. Lawrence, D. R. Hares, C. L. Fisher, and B. Budowle. 2002. An improved method for post-PCR purification for mtDNA sequence analysis. *J. Forensic Sci.* 47:811–818.
- Dugat-Bony, E., C. Straub, A. Teissandier, D. Onesime, V. Loux, C. Monnet, F. Irlinger, S. Landaud, M. N. Leclercq-Perlat, P. Bento, S. Fraud, J. F. Gibrat, J. Aubert, F. Fer, E. Guedon, N. Pons, S. Kennedy, J. M. Beckerich, D. Swennen, and P. Bonnarne. 2015. Overview of a surface-ripened cheese community functioning by meta-omics analyses. *PLoS One* 10:e0124360 <http://dx.doi.org/10.1371/journal.pone.0124360>.
- Edberg, S. C., E. W. Rice, R. J. Karlin, and M. J. Allen. 2000. *Escherichia coli*: The best biological drinking water indicator for public health protection. *Symp. Ser. Soc. Appl. Microbiol.* (29):106S–116S.
- Esho, F. K., B. Enkhtuya, A. Kusumoto, and K. Kawamoto. 2013. Microbial assessment and prevalence of foodborne pathogens in natural cheeses in Japan. *Biomed Res. Int.* 2013:205801. <http://dx.doi.org/10.1155/2013/205801>.
- Feng, P., S. D. Weagant, and K. Jinneman. 2011. Diarrheagenic *Escherichia coli*. Chapter 4A in *Bacteriological Analytical Manual (BAM)*. <http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070080.htm>.
- Feresu, S., and H. Nyati. 1990. Fate of pathogenic and non-pathogenic *Escherichia coli* strains in two fermented milk products. *J. Appl. Bacteriol.* 69:814–821.
- Filiouis, G., A. Johansson, J. Frey, and V. Perreten. 2009. Prevalence, genetic diversity and antimicrobial susceptibility of *Listeria monocytogenes* isolated from open-air food markets in Greece. *Food Contr.* 20:314–317. <http://dx.doi.org/10.1016/j.foodcont.2008.05.018>.
- Gaulin, C., D. Ramsay, and S. Bekal. 2012. Widespread listeriosis outbreak attributable to pasteurized cheese, which led to extensive cross-contamination affecting cheese retailers, Quebec, Canada, 2008. *J. Food Prot.* 75:71–78. <http://dx.doi.org/10.4315/0362-028X.JFP-11-236>.
- Gill, A., and D. Oudit. 2015. Enumeration of *Escherichia coli* O157 in outbreak-associated Gouda cheese made with raw milk. *J. Food Prot.* 78:1733–1737. <http://dx.doi.org/10.4315/0362-028X.JFP-15-036>.
- Hitchins, A. D., and K. Jinneman. 2013. Detection and enumeration of *Listeria monocytogenes* in foods. Chapter 10 in *Bacteriological Analytical Manual (BAM)*. Accessed Feb. 13, 2014. <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm>.
- Ho, A. J., V. R. Lappi, and M. Wiedmann. 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. *J. Dairy Sci.* 90:2517–2524. <http://dx.doi.org/10.3168/jds.2006-392>.
- Hu, Y., Q. Zhang, and J. C. Meitzler. 1999. Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. *J. Appl. Microbiol.* 87:867–876.
- Huck, J. R., M. Sonnen, and K. J. Boor. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *J. Dairy Sci.* 91:1218–1228. <http://dx.doi.org/10.3168/jds.2007-0697>.
- Irlinger, F., S. A. Yung, A.-S. Sarthou, C. Delbès-Paus, M.-C. Montel, E. Coton, M. Coton, and S. Helinck. 2012a. Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *Int. J. Food Microbiol.* 153(3):332–338. <http://dx.doi.org/10.1016/j.ijfoodmicro.2011.11.022>.
- Irlinger, F., V. Loux, S. Layec, S. Helinck, and E. Dugat-Bony. 2015. Cheese rind microbial communities: Diversity, composition and origin. *FEMS Microbiol. Lett.* 362:1–11. <http://dx.doi.org/10.1093/femsle/fnu015>.
- Irlinger, F., V. Loux, P. Bento, J. F. Gibrat, C. Straub, P. Bonnarne, S. Landaud, and C. Monnet. 2012b. Genome sequence of *Staphylococcus equorum* ssp. *equorum* Mu2, isolated from a French smear-ripened cheese. *J. Bacteriol.* 194:5141–5142. <http://dx.doi.org/10.1128/JB.01038-12>.
- Jackson, E. E., E. S. Erten, N. Maddi, T. E. Graham, J. W. Larkin, R. J. Blodgett, J. E. Schlessner, and R. M. Reddy. 2012. Detection and enumeration of four foodborne pathogens in raw commingled silo milk in the United States. *J. Food Prot.* 75:1382–1393. <http://dx.doi.org/10.4315/0362-028X.JFP-11-548>.
- Jayarao, B. M., and L. Wang. 1999. A study on the prevalence of gram-negative bacteria in bulk tank milk. *J. Dairy Sci.* 82:2620–2624. [http://dx.doi.org/10.3168/jds.S0022-0302\(99\)75518-9](http://dx.doi.org/10.3168/jds.S0022-0302(99)75518-9).
- Kornacki, J. L. 2011. Indicator organism assays: Chaos, confusion and criteria. *Food Safety Mag.* 17:26–28.
- Kousta, M., M. Mataragas, P. Skandamis, and E. H. Drosinos. 2010. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Food Contr.* 21:805–815. <http://dx.doi.org/10.1016/j.foodcont.2009.11.015>.
- Leclerc, H., D. A. Mossel, S. C. Edberg, and C. B. Struijk. 2001. Advances in the bacteriology of the coliform group: Their suitability as markers of microbial water safety. *Annu. Rev. Microbiol.* 55:201–234. <http://dx.doi.org/10.1146/annurev.micro.55.1.201>.
- Little, C. L., J. R. Rhoades, S. K. Sagoo, J. Harris, M. Greenwood, V. Mithani, K. Grant, and J. McLauchlin. 2008. Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in the UK. *Food Microbiol.* 25:304–312. <http://dx.doi.org/10.1016/j.fm.2007.10.007>.
- Loncarevic, S., M. L. Danielsson-Tham, and W. Tham. 1995. Occurrence of *Listeria monocytogenes* in soft and semi-soft cheeses in retail outlets in Sweden. *Int. J. Food Microbiol.* 26:245–250.
- Luo, C., S. T. Walk, D. M. Gordon, M. Feldgarden, J. M. Tiedje, and K. T. Konstantinidis. 2011. Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. *Proc. Natl. Acad. Sci. USA* 108:7200–7205. <http://dx.doi.org/10.1073/pnas.1015622108>.
- Manfreda, G., A. De Cesare, S. Stella, M. Cozzi, and C. Cantoni. 2005. Occurrence and ribotypes of *Listeria monocytogenes* in Gorgonzola cheeses. *Int. J. Food Microbiol.* 102:287–293. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.11.045>.
- Manolopoulou, E., P. Sarantinopoulos, E. Zoidou, A. Aktypis, E. Moschopoulou, I. G. Kandarakis, and E. M. Anifantakis. 2003. Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *Int. J. Food Microbiol.* 82:153–161.
- Martin, N. H., M. L. Ranieri, S. C. Murphy, R. D. Ralve, M. Wiedmann, and K. J. Boor. 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. *J. Dairy Sci.* 94:1211–1222. <http://dx.doi.org/10.3168/jds.2010-3915>.
- Masiello, S. N., N. H. Martin, A. Trmčić, M. Wiedmann, and K. J. Boor. 2016. Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk. *J. Dairy Sci.* 99:130–140. <http://dx.doi.org/10.3168/jds.2015-9728>.
- Mena, C., G. Almeida, L. Carneiro, P. Teixeira, T. Hogg, and P. A. Gibbs. 2004. Incidence of *Listeria monocytogenes* in different food products commercialized in Portugal. *Food Microbiol.* 21:213–216. [http://dx.doi.org/10.1016/S0740-0020\(03\)00057-1](http://dx.doi.org/10.1016/S0740-0020(03)00057-1).
- Morales, P., E. Fernandez-Garcia, and M. Nunez. 2003. Caseinolysis in cheese by *Enterobacteriaceae* strains of dairy origin. *Lett. Appl. Microbiol.* 37:410–414.
- Mossel, D. A. A., J. E. L. Corry, C. B. Struijk, and R. M. Baird, ed. 1995. *Essentials of the Microbiology of Foods. A Textbook for Advanced Studies*. J. Wiley, Chichester, UK.

- Mullan, W. M. A. 2000. Causes and control of early gas production in cheddar cheese. *Int. J. Dairy Technol.* 53:63–68. <http://dx.doi.org/10.1111/j.1471-0307.2000.tb02660.x>.
- Nightingale, K., L. Bovell, A. Grajczyk, and M. Wiedmann. 2007. Combined *sigB* allelic typing and multiplex PCR provide improved discriminatory power and reliability for *Listeria monocytogenes* molecular serotyping. *J. Microbiol. Methods* 68:52–59. <http://dx.doi.org/10.1016/j.mimet.2006.06.005>.
- Oberg, E. N., C. J. Oberg, M. M. Motawee, S. Martini, and D. J. McMahon. 2015. Increasing stringiness of low-fat mozzarella string cheese using polysaccharides. *J. Dairy Sci.* 98:4243–4254. <http://dx.doi.org/10.3168/jds.2014-8733>.
- Oh, S., S. Buddenborg, D. R. Yoder-Himes, J. M. Tiedje, and K. T. Konstantinidis. 2012. Genomic diversity of *Escherichia* isolates from diverse habitats. *PLoS One* 7:e47005. <http://dx.doi.org/10.1371/journal.pone.0047005>.
- Pantoja, J. C., D. J. Reinemann, and P. L. Rugg. 2009. Associations among milk quality indicators in raw bulk milk. *J. Dairy Sci.* 92:4978–4987. <http://dx.doi.org/10.3168/jds.2009-2329>.
- Paruch, A. M., and T. Maehlum. 2012. Specific features of *Escherichia coli* that distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. *Ecol. Indic.* 23:140–142. <http://dx.doi.org/10.1016/j.ecolind.2012.03.026>.
- Pintado, C. M. B. S., A. Oliveira, M. E. Pampulha, and M. A. S. S. Ferreira. 2005. Prevalence and characterization of *Listeria monocytogenes* isolated from soft cheese. *Food Microbiol.* 22:79–85. <http://dx.doi.org/10.1016/j.fm.2004.04.004>.
- Qi, W., D. W. Lacher, A. C. Bumbaugh, K. E. Hyma, L. M. Ouellette, T. M. Large, C. L. Tarr, and T. S. Whittam. 2004. EcMLST: An online database for multi locus sequence typing of pathogenic *Escherichia coli*. Pages 520–521 in *Proc. 2004 IEEE Computational Systems Bioinformatics Conference (CSB 2004)*. IEEE Computer Society, Stanford, CA.
- 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. <http://dx.doi.org/10.1111/1574-6976.12030>.
- Quinto, E. J., and A. Cepeda. 1997. Incidence of toxigenic *Escherichia coli* in soft cheese made with raw or pasteurized milk. *Lett. Appl. Microbiol.* 24:291–295. <http://dx.doi.org/10.1046/j.1472-765X.1997.00072.x>.
- Reginald, W. B., and G. A. Lancette. 2001. *Staphylococcus aureus*. Chapter 12 in *Bacteriological Analytical Manual (BAM)*. Accessed Feb. 13, 2014. <http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm071429.htm>.
- Rosengren, Å., A. Fabricius, B. Guss, S. Sylvén, and R. Lindqvist. 2010. Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies. *Int. J. Food Microbiol.* 144:263–269. <http://dx.doi.org/10.1016/j.ijfoodmicro.2010.10.004>.
- Rudol, M., and S. Scherer. 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. *Int. J. Food Microbiol.* 63:91–98. [http://dx.doi.org/10.1016/S0168-1605\(00\)00413-X](http://dx.doi.org/10.1016/S0168-1605(00)00413-X).
- Saunders, B. D., J. Overvest, E. Fortes, K. Windham, Y. Schukken, A. Lembo, and M. Wiedmann. 2012. Diversity of *Listeria* species in urban and natural environments. *Appl. Environ. Microbiol.* 78:4420–4433. <http://dx.doi.org/10.1128/AEM.00282-12>.
- Schoder, D., P. Skandamis, and M. Wagner. 2013. Assessing in-house monitoring efficiency by tracing contamination rates in cheese lots recalled during an outbreak of listeriosis in Austria. *Int. J. Food Microbiol.* 167:353–358. <http://dx.doi.org/10.1016/j.ijfoodmicro.2013.09.020>.
- Schvartzman, M. S., U. Gonzalez-Barron, F. Butler, and K. Jordan. 2014. Modeling the growth of *Listeria monocytogenes* on the surface of smear- or mold-ripened cheese. *Front. Cell. Infect. Microbiol.* 4:90. <http://dx.doi.org/10.3389/fcimb.2014.00090>.
- Sheehan, J. J. 2011. Cheese: Avoidance of gas blowing. Pages 661–666 in *Encyclopedia of Dairy Sciences*. 2nd ed. J. W. Fuquay, P. F. Fox, and P. L. H. McSweeney, ed. Academic Press, San Diego, CA.
- Stevens, M., N. Ashbolt, and D. Cunliffe. 2003. Review of Coliforms as Microbial Indicators of Drinking Water Quality. Recommendations to Change the Use of Coliforms as Microbial Indicators of Drinking Water Quality. Australian Government, National Health and Medical Research Council. Biotext Pty Ltd., Canberra, Australia.
- Strongin, D. 2015. Some things matter. Cheese report. Accessed Jan. 20, 2016. <http://www.cheesereporter.com/Strongin/strongin-november6.2015.htm>.
- Tham, W. A., L. J. Hajdu, and M. L. Danielsson-Tham. 1990. Bacteriological quality of on-farm manufactured goat cheese. *Epidemiol. Infect.* 104:87–100.
- Trancoso-Reyes, N., N. Gutierrez-Mendez, D. R. Sepulveda, and L. R. Hernandez-Ochoa. 2014. Assessing the yield, microstructure, and texture properties of miniature Chihuahua-type cheese manufactured with a phospholipase A1 and exopolysaccharide-producing bacteria. *J. Dairy Sci.* 97:598–608. <http://dx.doi.org/10.3168/jds.2013-6624>.
- Trmčić, A., N. H. Martin, K. J. Boor, and M. Wiedmann. 2015. A standard bacterial isolate set for research on contemporary dairy spoilage. *J. Dairy Sci.* 98:5806–5817. <http://dx.doi.org/10.3168/jds.2015-9490>.
- US FDA, Center for Food Safety and Applied Nutrition. 2010. Guidance for FDA Staff: Compliance Policy Guide Sec. 527.300 Dairy Products—Microbial Contaminates and Alkaline Phosphatase Activity. Accessed Dec. 10, 2014. <http://www.fda.gov/downloads/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/UCM238465.pdf>.
- Walk, S. T., E. W. Alm, D. M. Gordon, J. L. Ram, G. A. Toranzos, J. M. Tiedje, and T. S. Whittam. 2009. Cryptic lineages of the genus *Escherichia*. *Appl. Environ. Microbiol.* 75:6534–6544. <http://dx.doi.org/10.1128/AEM.01262-09>.
- Wallace, H. A., A. Jacobson, and T. Hammack. 2012. *Salmonella*. Chapter 10 in *Bacteriological Analytical Manual (BAM)*. Accessed Feb. 13, 2014. <http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070149.htm>.
- Wolfe, B. E., J. E. Button, M. Santarelli, and R. J. Dutton. 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* 158:422–433. <http://dx.doi.org/10.1016/j.cell.2014.05.041>.
- Wu, J., S. C. Long, D. Das, and S. M. Dorner. 2011. Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. *J. Water Health* 9:265–278. <http://dx.doi.org/10.2166/wh.2011.117>.
- Wu, Q., H. M. Tun, F. C. Leung, and N. P. Shah. 2014. Genomic insights into high exopolysaccharide-producing dairy starter bacterium *Streptococcus thermophilus* ASCC 1275. *Sci. Rep.* 4:4974. <http://dx.doi.org/10.1038/srep04974>.