EPIDEMIOLOGY OF *LISTERIA MONOCYTOGENES* ON A NEW YORK STATE DAIRY FARM

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2011

EPIDEMIOLOGY OF LISTERIA MONOCYTOGENES ON A NEW YORK STATE DAIRY FARM

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Cornell University 2011

Listeria monocytogenes is a zoonotic pathogen that causes listeriosis in humans. Dairy cattle are a healthy reservoir of *L. monocytogenes* and the presence of the pathogen on dairy farms has been frequently described.

The present study had as main objective to study the molecular epidemiology of *L. monocytogenes* on a New York State dairy farm by using data collected between February 2004 and June 2008, and Pulsed-field gel electrophoresis (PFGE) typing of *L. monocytogenes* isolates.

Fecal samples were collected every 6 months from all lactating cows. Approximately 20 environmental samples were obtained every 3 months. Bulk tank milk samples and in-line milk filter samples were obtained weekly. Samples from milking equipment and the milking parlor were obtained in May 2007, and in January and February 2008. A high prevalence of *L. monocytogenes* in bulk tank milk (25.4%) and in milk filters (66.6%) was observed, contrasting with a low prevalence of the pathogen in fecal (6 %) and environmental (7%) samples. The presence of a *L. monocytogenes*-containing biofilm in milking equipment was suggested as a potential source of bulk tank milk contamination. Furthermore, the presence of three predominant and persistent *L. monocytogenes* strains (PFGE types T, D, and F accounting for 28.6%, 22.6%, and 14.9% of *L. monocytogenes* isolates, respectively) was observed in the milking line. Predominant and persistent strains showed high adherence ability in an in-vitro

biofilm assay. Our results suggest that the milking system was exposed to several *L. monocytogenes* types from different sources. Only 3 PFGE types, however, were successful in persisting within the milking system, suggesting that some strains are more suitable to that particular ecological environment.

Finally, the risk of listeriosis due to consumption of raw milk sold by permitted dealers and due to consumption of raw milk on farms was estimated. Overall, the annual number of listeriosis cases due to raw milk consumption was predicted to be low by our model. A reduction in the number of cases per year in all populations was observed when a raw milk testing program was in place, especially when routine testing and recalling of milk was conducted.

BIOGRAPHICAL SKETCH

Alejandra Latorre Soto was born on October 8th, 1977 in Talca-Chile to Eva Mariana Soto Sepulveda and Jaime Osvaldo Latorre Guerra. Alejandra grew up in Talca, the city where she attended elementary and high school and in 1996 she moved to Chillan-Chile to pursue her studies in Veterinary Medicine in the Universidad de Concepcion. Alejandra graduated with the first place of her class of 80 and obtained her Bachelor Degree in Veterinary Science in 2000, the year that her daughter Javiera Trinidad was born. The same year Alejandra also obtained a Fellowship from the Universidad de Concepcion to pursue her Master studies in Food Hygiene and Technology. In 2001, Alejandra obtained her degree of Doctor in Veterinary Medicine and in 2003 she started a position as a Faculty of Veterinary Public Health in the College of Veterinary Medicine of the Universidad de Concepcion. As a Faculty member, she was devoted to teaching, extension, and research with particular interest in zoonotic pathogens until 2006. In 2005, Alejandra obtained a Fulbright Grant to pursue a PhD program in Animal Sciences at Cornell University, United States. In 2006, Alejandra also obtained the Chilean National Scholarship for Graduate Studies overseas. That year she started her PhD program at the Quality Milk Production Services, Cornell University, under the guidance of Professor Ynte Schukken. In October 2010, Alejandra returned to Chile to resume her position as a Faculty at the Universidad de Concepcion.

To my daughter Javiera

ACKNOWLEDGMENTS

I thank very much Dr. Ynte Schukken for being my mentor and a father during the past four years. I feel honored and blessed to have had not only a brilliant scientist as my advisor but also a model of kindness, humbleness, and patience.

I will be eternally grateful to Dr. Jo Ann Van Kessel for her guidance, support, and friendship during the course of my PhD research. I also thank her for welcome me and sharing her vast knowledge and for opening the doors of her home.

I thank Dr. Kathryn Boor for her insights, kindness, time, and care. I will never forget that despite her very busy schedule, since the time I was a prospective graduate student she has kindly received me, and she has always had time for me.

I am very grateful to Dr. Yrjo Gröhn for his always constructive and challenging remarks that deeply inspired me to follow the path of mathematic modeling.

I thank very much Dr. Jeff Karns for his tremendous support and critical insight of the procedures used in this dissertation. I also thank him for his friendship and for teaching me the route map of the American cuisine.

I am deeply grateful to my friend and colleague Dr. Abani Pradhan for his unconditional friendship, patience, time, and selfless teaching. It has been a joy both working and learning from him.

I thank Sharinne Sukhnanand for her friendship and for sharing with me her knowledge on laboratory procedures and techniques. I really enjoyed learning from and with her during our many laboratory hours, as well as I enjoy the privilege of being her friend.

I thank very much Dr. Mike Zurakowski for all his hard work and contributions to this study. I also thank him for his friendship and for being a model of strength and positivity that always inspired me to keep going regardless the hurdles of life.

I thank very much Dr. Kenneth Simpson, Dr. Holger Sondermann, and Dr. Martin Wiedmann for all their support, insights, and contributions to this work. I thank very much Dr. Anthony Hay and Dr. William Ghiorse for their valuable inputs and helpful discussions about biofilms. I thank Dr. Bhushan Jayarao for his kindness and for providing us with the bulk tank milk data generated in his laboratory for the discussion chapter of this dissertation.

I thank Dr. Daniel Rice and Kurt Mangione for his participation and contributions during the development of our risk assessment model.

I am deeply grateful and indebted to Dr. Alejandro Santa-Maria and to Dr. Fernando Gonzalez at my home University for their unconditional support during my PhD studies.

I thank very much Vikki Thomas for taking such a good care of us during all these years. I thank her very much for being there during the good times, but especially during the hard times. Thanks for being a friend I could always could count on.

I also thank Tollie Stuprich for her care and for being an endless source of advise about the American world.

I thank Dr. Linda Tikofsky and her lovely family for their friendship, for opening their arms and hearts, and for always making me feel at home.

I also thank Dr. Frank Welcome for his kindness, willingness to help me every time I needed him, and for being a patient and amazing source of knowledge.

I am grateful to Andrea Walsh, Natasha Belomestnykh, Suzanne Klaessig, and Dr. Carlos Santisteban for their excellent technical assistance and help, but especially for their friendship and support. I feel honored and happy to have had the chance of working with all of them.

I thank very much to Belinda Gross, Joan Taber, Jean Newmann, Kerry Case, and Rose Prince (R.I.P.) for their friendship and care during these years. You also made

me feel at home…and although Rose is not with us anymore, I keep her memory in a special part of my heart.

I thank my friend Dustin Wright for being present every time I needed him. I am grateful for his friendship and care during the past two years.

I am deeply grateful to my best friend Rodrigo Retamal for his time-distance-proof friendship. I thank him for being present in one of the toughest times of my life and for taking such a good care of me despite the distance.

And last, but not least I want to thank my beloved family. I deeply thank my fiancé Marcos Munoz for his unconditional love and care. For sharing this path with me and for being also a friend I could count on.

I thank my father Jaime Latorre for his unconditional support and for sending us so much love through the distance. Despite all the miles between us, I have always felt him right next to me during all these years. I thank my mother Eva Soto for her caring and unconditional love, patience, and wisdom. I thank my brother Jaime Latorre for cheering me up every time I needed.

I thank very much my godmother Hermosina Rojas for all her love, care, and support. I thank her for having the courage of joining me in this journey, in spite of all the difficulties. Thanks for being a second mother for my daughter and I.

I thank my daughter Javiera for being my light, my strength, my inspiration, and a reason for trying to be a better person every day. I deeply thank her for her patience and understanding.

I thank God for the blessing of being here and sharing my path with such a wonderful people during the past four years.

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is a gram-positive bacterium that has been reported to be widespread in nature (Welshimer and Donker-Voet, 1971; Ivanek et al, 2006) and frequently found in foods (Swaminatan, 2001). *Listeria monocytogenes* causes listeriosis in both animals and humans. The route of infection is primarily foodborne, although other routes such as intrauterine transmission or infection of neonates during delivery have also been suggested (Farber and Peterkin, 1991).

The number of reported human listeriosis cases in the United States ranged from 759 to 896 between 2005 and 2009 (Centers for Disease Control and prevention **CDC**, 2010). Healthy individuals are usually not affected or symptoms due to foodborne listeriosis are milder than the manifestations of septicemic illness (Riedo et al., 1994; Miettinen et al., 1999; Sim et al., 2002). However, in pregnant women, fetuses, elderly, and immuno-compromised people the manifestations of the disease can be severe, leading to stillbirth, neurological ailments, or even death (Linnan et al., 1988; Lyytikanen et al., 2000; McDonald et al., 2005; Mead et al., 2006). In addition, high hospitalization rates and mortality have been associated with listeriosis (Mead et al., 1999; CDC, 2010).

Although *L. monocytogenes* in foods has been primarily associated with ready-to-eat products (United States Department of Agriculture **USDA**/ Food Safety and Inspection Service **FSIS**, 2003), dairy products have been linked to several listeriosis outbreaks

(Fleming et al, 1985; Linnan et al, 1988; Dalton et al, 1998; Lyytikanen et al., 2000; CDC, 2001; MacDonald et al, 2005; CDC, 2008).

Listeria monocytogenes is ubiquitous on dairy farms where cattle are a healthy reservoir (Nightingale et al., 2004) and may shed the pathogen in their feces (Husu, 1990; Nightingale et al., 2004; Ho et al., 2007) contributing thus to the persistence and dispersion of the pathogen on the farm. The presence of *L. monocytogenes* in bulk tank milk has been frequently reported (Jayarao and Henning, 2001; Van Kessel et al., 2004; Jayarao et al., 2006; Latorre et al., 2009; Mohammed et al., 2009). Because unprocessed raw milk is the starting point of the milk production to consumption chain, prevention of milk contamination at the farm level is crucial as dairy farms may represent a link between the presence of the pathogen in animal production systems and human disease (Borucki et al., 2004). Assessing the prevalence, tracking sources, and detecting reservoirs of *L. monocytogenes* on dairy farms is crucial to implement control measures aimed to prevent contamination of milk.

The overall goal of this research was to study the molecular epidemiology of *L. monocytogenes* on a single New York State dairy farm by using pulsed-field gel electrophoresis typing and analysis of prevalence data collected over a 4-year period.

The study farm was one of the three farms in the Northeastern United States participating in the Regional Dairy Quality Management Alliance Project since 2004. At the beginning of the study, this farm was identified as having sporadic incidence of *L. monocytogenes* in samples collected from the environment as well as individual fecal samples. In addition, *L. monocytogenes* was never isolated from weekly bulk tank milk samples taken from February 2004 until November 2005. Starting in

November 2005, *L. monocytogenes* was isolated from bulk tank milk on a regular basis.

In chapter 2 we describe the prevalence of *L. monocytogenes* in fecal, environmental, in-line milk filters, bulk tank milk, and milking equipment samples collected during a 4-year period from the study farm. By using pulsed-field gel electrophoresis (**PFGE**) to characterize selected *L. monocytogenes* isolates, our goal was to identify diversity among *L. monocytogenes* strains, persistence, and potential sources of bulk tank milk contamination.

Our working hypothesis in chapter 3 was that milking equipment may be a source of *L. monocytogenes* on a dairy farm. The objective of this chapter was to assess the presence of a *L. monocytogenes*-containing biofilm in milking equipment as a potential source of bulk tank milk contamination on the study farm. By using molecular techniques and scanning electron microscopy we further investigated the role of milking equipment as a reservoir of *L. monocytogenes*.

In the fourth chapter we hypothesized that some *L. monocytogenes* strains on the farm may be out-competing other strains and that some strains may have a better ability to establish persistent presence in the milking system. The main objective of chapter 4 was to study the molecular epidemiology of *L. monocytogenes* on the study farm by expanding PFGE typing of isolates to all samples from which the pathogen was isolated. In addition, we aimed to assess the biofilm forming ability of representative *L. monocytogenes* strains by using an *in-vitro* biofilm assay.

Even though the presence of *L. monocytogenes* in unprocessed raw milk has been frequently reported, there is an increase of the number of farms that have been enrolled as permitted raw milk dealers in New York State during the past few years (Dr. Daniel Rice, personal communication), likely as a consequence of an increasing number of raw milk advocates. Our hypothesis for the 5th chapter of this dissertation was that *L. monocytogenes* in raw milk is a risk factor for listeriosis in certain groups of consumers. The objective of chapter 5 was to estimate the probability of illness among raw milk consumers due to the presence of *L. monocytogenes* in raw milk. Different scenarios, such as consumption of raw milk obtained from permitted raw milk dealers, raw milk consumed on dairy farms by farm personnel, and consumption of raw milk from farms with a high prevalence of the pathogen in milk samples are investigated.

In chapter 6, the main findings of this research are discussed and compared with previously published work. We also discuss the results of our work and their implications for future research. Finally, we propose science-based guidelines aimed to the control of *L. monocytogenes* on dairy farms and, ultimately, to the prevention of the appearance of *L. monocytogenes* in bulk tank milk.

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CHAPTER 2

MOLECULAR ECOLOGY OF *LISTERIA MONOCYTOGENES*: EVIDENCE FOR A RESERVOIR IN MILKING EQUIPMENT ON A DAIRY FARM*

ABSTRACT

A longitudinal study aimed to detect *Listeria monocytogenes* on a New York State dairy farm was conducted between February 2004 and July 2007. Fecal samples were collected every 6 months from all lactating cows. Approximately 20 environmental samples were obtained every 3 months. Bulk tank milk samples and in-line milk filter samples were obtained weekly. Samples from milking equipment and the milking parlor environment were obtained in May 2007. Fifty-one of 715 fecal samples (7.1%) and 22 of 303 environmental samples (7.3%) were positive for *L. monocytogenes*. A total of 73 of 108 in-line milk filter samples (67.6%) and 34 of 172 bulk tank milk samples (19.7%) were positive for *L. monocytogenes*. *Listeria* monocytogenes was isolated from 6 of 40 (15%) sampling sites in the milking parlor and milking equipment. In-line milk filter samples had a greater proportion of *L. monocytogenes* than did bulk tank milk samples ($P < 0.05$) and samples from other sources ($P < 0.05$). The proportion of *L. monocytogenes*-positive samples was greater among bulk tank

* Latorre, A. A., J. S. Van Kessel, J. S. Karns, M. J. Zurakowski, A. K. Pradhan, R. N. Zadoks, K. J. Boor, and Y. H. Schukken. 2009. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. J. Appl. Environ. Microbiol. 75: 1315–1323.

milk samples than among fecal or environmental samples ($P < 0.05$). Analysis of 60 isolates by pulsed-field gel electrophoresis (**PFGE**) yielded 23 PFGE types after digestion with AscI and ApaI endonucleases. Three PFGE types of *L. monocytogenes* were repeatedly found in longitudinally collected samples from bulk tank milk and in-line milk filters.

INTRODUCTION

Listeria monocytogenes can cause listeriosis in humans. This illness, despite being underreported, is an important public health concern in the United States (Mead et al., 1999) and worldwide. According to provisional incidence data provided by the Centers for Disease Control and Prevention **(CDC)**, 762 cases of listeriosis were reported in the United States in 2007. In previous years (2003 to 2006), the number of reported annual listeriosis cases in the United States ranged between 696 and 896 cases per year (CDC, 2008).

Exposure to food-borne *L. monocytogenes* may cause fever, muscle aches, and gastroenteritis (Riedo et al., 1994), but does not usually cause septicemic illness in healthy nonpregnant individuals (Riedo et al., 1994; Dalton et al., 1997). Elderly and immunocompromised people, however, are susceptible to listeriosis (Fleming et al., 1985; Linnan et al., 1988), and they may develop more severe symptoms (Fleming et al., 1985). Listeriosis in pregnant women may cause abortion (Linnan et al., 1988; Riedo et al., 1994) or neonatal death (Linnan et al., 1988).

Dairy products have been identified as the source of several human listeriosis outbreaks (Fleming et al., 1985; Linnan et al., 1988; Dalton et al., 1997; Carrique-Mas et a., 2003). *Listeria* is ubiquitous on dairy farms (Nightingale et al., 2004), and it has been isolated from cows' feces, feed (Borucki et al., 2005; Nightingale et al., 2004), and milk (Jayarao and Henning, 2001; Van Kessel et al., 2004). In ruminants, *L. monocytogenes* infections may be asymptomatic or clinical. Clinical cases typically present with encephalitis and uterine infections, often resulting in abortion (Nightingale et al., 2004; Wagner et al., 2005). Both clinically infected and healthy animals have been reported to excrete *L. monocytogenes* in their feces (Husu, 1990), which could eventually cause contamination of the bulk tank milk or milk-processing premises (Wagner et al., 2005).

On-farm epidemiologic research provides science-based information to improve farming and management practices. The Regional Dairy Quality Management Alliance **(RDQMA)** launched a combined United States Department of Agriculture **(USDA)**-RDQMA pilot project in January 2004 to scientifically validate intervention strategies in support of recommended best management practices among northeast dairy farms. The primary goal of the project was to track dynamics of infectious microorganisms on well-characterized dairy farms. Target species included *Salmonella* spp. (Van Kessel et al., 2007; Chapagain et al., 2008; Van Kessel et al., 2008), *Mycobacterium avium* subsp. *paratuberculosis* (Gollnick et al., 2007; Mitchell et al., 2008), and *L. monocytogenes*.

The objectives of this study were to describe the presence of *L. monocytogenes* on a dairy farm over time and to perform molecular subtyping by pulsed-field gel electrophoresis **(PFGE)** on *L. monocytogenes* isolates obtained from bulk tank milk,

milk filters, milking equipment, feces, and the environmental samples to identify diversity among *L. monocytogenes* strains, persistence, and potential sources of bulk tank milk contamination.

MATERIALS AND METHODS

Farm description

This study was conducted on a New York State dairy farm between February 2004 and July 2007. This dairy farm is considered typical among the better-managed New York State dairy farms in terms of size, management, and milk production. The selection criteria for inclusion of the study farm were the size of the herd, participation in the New York State Cattle Health Assurance Program **(NYSCHAP)** (http://nyschap.vet.cornell.edu), and Dairy Herd Improvement Association **(DHIA)** (http://www.dhia.org/) membership. The availability of a record system, proper identification of the animals, and willingness of the producer to participate were also taken into consideration (Pradhan et al., 2009).

The study farm had an average of 330 milking cows. Of these, 300 were housed in a free-stall barn, and approximately 30 were housed in a tie-stall facility. The average milk production was 12,700 kg/cow/year. The herd had monthly veterinary check-ups, annual NYSCHAP evaluation, and monthly DHIA milk testing. No listeriosis cases in the cows have been reported. The milk (approximately 9,071 kg per day) was transported every 48 h to the milk processing plant, where it was pasteurized before distribution.

Sampling

Environmental and fecal samples were collected between February 2004 and April 2007 for detection of pathogens, including *L. monocytogenes*. Fecal samples were collected every 6 months from all lactating cows. Fecal samples were obtained directly from the rectum of each cow, using a separate clean plastic sleeve for each sample. The plastic sleeves were inverted, and the content was aseptically transferred into sterile plastic vials (conical 50-ml propylene screw top; VWR International, Inc., West Chester, PA).

In addition, approximately 20 environmental samples were obtained every 3 months. These environmental samples included feed as presented to the animals, source water used to fill water troughs, and drinking water from water troughs in the pens housing the lactating cows, dry cows, and heifers. Samples were collected from the same locations on each of the samplings. For this purpose, a detailed sampling map was constructed at the initial visit and used at subsequent samplings. Manure composite samples from the walkways, calf area, dry cow pen, precalving pen, and sick cow pen were also collected at all samplings. Samples from specific potential "hot spots" for *Listeria*, such as bedding or, when present, standing water, birds, bird droppings, feral-animal feces, and insects, were also obtained.

Liquid samples were collected into sterile 500-ml bottles. Feed material samples and other solid samples were placed in sterile Whirl-Pak bags (NASCO, Ft. Atkinson, WI). Manure composite samples were obtained from different areas in each pen, using a clean plastic sleeve. The plastic-sleeve content was homogenized, and an aliquot was aseptically transferred into 50-ml plastic vials. Bird dropping samples were obtained by scraping stall dividers and were collected in 50-ml sample vials. Flies were caught

in calf hutches, in the lactating cows' pen, and in an outdoor site at least 30 m from the animal-holding facilities. For this purpose, QuikStrike (Wellmark International, Schaumburg, IL) fly abatement strips (for house flies) and previously sterilized sweep nets (for stable flies) were used.

In-line milk filter samples were obtained in October 2004 (two samples) and January 2005 (one sample). Starting from April 2005, in-line milk filter samples were obtained on a weekly basis, until July 2007. Milk filter samples were aseptically transferred into a clean sealable plastic bag for transport. Bulk tank milk samples (100 ml) were aseptically collected on a weekly basis from February 2004 to July 2007.

An additional sampling was carried out in May 2007 to assess the presence of *L. monocytogenes* in the milking machine and milking parlor environment. Forty sampling sites were selected based on areas prone to *Listeria* contamination or on a particular interest to assess the presence of this pathogen in a given sampling site. Sampling sites from the milking equipment included teat cup liners, milk meters, milk pipelines, elbow fittings, and the milk tank outlet. Milk pump surfaces, the motor, and the floor in the storage area for miscellaneous supplies were also sampled. Floors and floor drains, areas previously described as sources of *L. monocytogenes* in food plant environments (Tompkin et al., 1999), were also included. Samples were collected using a Bacti-Sponge kit (Hardy Diagnostics, Santa Maria, CA) moistened with 10 ml of neutralizing buffer (Difco; BD Diagnostics, Sparks, MD) (Thimothe et al., 2004; Ho et al., 2007). For the milking equipment, a sponge was used to wipe the inner surface of the selected site. Sterile cotton swabs were used to sample the milk tank outlet and every other milk meter after the routine washing cycle was complete. Samples from drains were aseptically obtained by rubbing the sponge on the exposed

surface and inner portions within reach. For surface sampling, individual sponges were used to wipe an area of approximately 0.6 by 0.6 m (Thimothe et al., 2004) on floors and pumps. Sponges were placed in the sterile bags containing neutralizing buffer, and cotton swabs were placed in sterile tubes containing 3 ml of neutralizing buffer.

All samples were packed in coolers with ice packs and transported overnight to the USDA-Beltsville Agricultural Research Center for *L. monocytogenes* detection.

Bacterial analysis

Approximately 25 g of feces or other sampling material, such as composite samples or bedding, was weighed into a filtered stomacher bag (GSI Creos Corporation, Japan), diluted with 50 g of 1% buffered peptone water (BD Diagnostics, Sparks, MD), and pummeled in an automatic bag mixer (Bag- Mixer Interscience Laboratories, Inc., Weymouth, MA) for 2 min. For enrichment of *Listeria* spp., 5 ml of filtrate was added to 5 ml of double-strength modified *Listeria* enrichment broth (**MLEB**; BD Diagnostics, Sparks, MD) to yield 1 × MLEB. For feed samples, larger aliquots (40 to 60 g) were used, and when the samples were low in moisture content, larger volumes of buffered peptone water were used for extraction. For samplings performed between February 2004 and October 2005, every fifth fecal sample was tested for the presence of *L. monocytogenes*. All fecal samples collected in May 2006 and every third fecal sample in April 2007 were tested for the presence of *L. monocytogenes*.

Milk (250 µl) was plated in triplicate directly onto modified Oxford medium **(MOX)** agar (Difco Laboratories, Detroit, MI) as described by Van Kessel et al. (2004). For specific enrichment of *Listeria* spp., 5 ml of milk was added to 5 ml of double-strength MLEB.

In-line milk filters were cut into small (30 to 50 cm^2) pieces and placed in a filtered stomacher bag, diluted (2 to 1 [wt/wt]) with 1% buffered peptone water, and pummeled in an automatic bag mixer for 2 min. The bag was removed from the mixer, filter pieces were repositioned to the bottom of the bag, and the bag was repummeled for two additional minutes. For enrichment of *Listeria* spp., 5 ml of filtrate was added to 5 ml of double-strength MLEB. The extract from the milk filters (250 µl) was also plated directly onto MOX plates.

Water (250 µl) was plated in triplicate directly onto MOX agar, using an Autoplate 4000 spiral plater (Spiral Biotech, Gaithersburg, MD). Plates were incubated at 37°C and scored for presumptive *Listeria* colonies (black colonies with esculin hydrolysis) at 24 h and 48 h. For enrichment of *Listeria*, water samples (100 ml) were filtered through sterile 0.45-µm cellulose filters (47 mm; Osmonics, Inc., Westborough, MA) with suction, and the filter was placed in 10 ml MLEB.

For all samples, enrichment tubes were incubated at 37°C for 48 h, and broth (10 µl) was streaked onto MOX agar. Cycloheximide-supplemented MOX (50 µg/ml) was used for fecal and milk filter samples to inhibit fungal growth. Plates were incubated at 37°C and scored at 24 and 48 h for presumptive *Listeria* colonies. Isolated, presumptive *Listeria* colonies were transferred from MOX or cycloheximide-supplemented MOX plates onto MOX, **PALCAM** (polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol; BD Diagnostics), Trypticase soy agar with 0.6% yeast extract, and a chromogenic plating medium, BCM *Listeria* (Biosynth International, Inc., Naperville, IL). Colonies that exhibited the *Listeria* phenotype (as described above on MOX; gray-green colonies with esculin hydrolysis on PALCAM)

were preserved for future analysis. Colony biomass was transferred from the PALCAM plates to 1.5 ml tryptic soy broth, incubated at 37°C, and stored at - 80°C as previously described (Van Kessel et al., 2004). Hemolytic activity of select presumptive *L. monocytogenes* isolates (blue colonies on BCM *Listeria* medium) and the Christie, Atkins, Munch-Peterson **(CAMP)** tests were performed as described by Van Kessel et al. (2004).

Pulsed-field gel electrophoresis

In total, 60 *L. monocytogenes* isolates obtained from different sources at the farm were analyzed by PFGE. Only one *L. monocytogenes* isolate per sample was used for PFGE typing.

First, 36 *L. monocytogenes* isolates were selected to represent the time period between June 2004 and July 2007. Specifically, multiple *L. monocytogenes* isolates isolated in the same month but from different sources were selected. One *L. monocytogenes* fecal isolate from each of the samplings carried out in October 2005, May 2006, and April 2007 was included for PFGE analysis. Furthermore, the first *L. monocytogenes* isolates from the milk filter and from bulk tank milk were included, as well as all *L. monocytogenes* isolates found in milking equipment. A purposive selection of other isolates was used. With the exception of *L. monocytogenes* isolates obtained in June 2004 and May 2005, isolates used for PFGE typing were collected at intervals of 4 months or less for this set of 36 isolates. In addition, 24 *L. monocytogenes* isolates were randomly selected among all previously nonselected *L. monocytogenes*-positive fecal and environmental samples obtained between February 2004 and April 2007. The selection of *L. monocytogenes* isolates was done in proportion to the number of *L.*

monocytogenes- positive fecal/environmental samples, available for a particular sampling date.

The standardized CDC PulseNet protocol (http://www.cdc.gov/pulsenet/protocols /pulsenet_listeria_protocol%20.pdf) with modifications was used to do PFGE analysis of *L. monocytogenes* isolates. Bacterial cell suspensions for agarose plug preparation were made using an optical density of 1.50 to 1.59 at a wavelength of 610 nm (SmartSpecPlus spectrophotometer; Bio-Rad Laboratories, Hercules CA). Lysis of agarose plugs was done in a shaking incubator (Labnet 311 DS; Edison, NJ) for 5 h at 54°C and at 170 rpm. The washes were done using sterile distilled water and Tris-EDTA buffer (pH 8.0) in a shaking incubator at 54°C and 70 rpm. DNA digestion using AscI (New England BioLabs, Inc., Ipswich, MA) was carried out at 37°C for at least 5.5 h. The digestion with ApaI (New England BioLabs) was carried out overnight at 30°C using 131 µl of sterile distilled water, 15 µl of NE buffer 4, and 4 µl (50 U/µl) of ApaI endonuclease. *Salmonella enterica* serotype Braenderup (H9812) was used as the reference standard, after digestion with restriction enzyme XbaI (Roche, Indianapolis, IN, or New England BioLabs). The DNA digestion with XbaI from Roche Laboratories was carried out as described in the PulseNet protocol. When using XbaI from New England BioLabs, the DNA digestion was done at 37°C using 132.5 µl of sterile distilled water, 15 µl of NE buffer 2, and 2.5 µl (20 U/µl) of XbaI endonuclease.

The 1% SeaKem Gold agarose (Lonza, Rockland, ME) gel used for DNA separation was run using a contour-clamped homogeneous electric field mapper XA system (Bio-Rad Laboratories). Images were obtained with a Bio-Rad Gel Doc XR system, using the software Quantity One 4.4.1 (Bio-Rad Laboratories), after staining with ethidium

bromide (EMD Chemicals, Inc., Gibbstown, NJ). Band patterns were analyzed by two independent observers, using visual inspection. The criteria described by Tenover et al. (1995) were used to assign PFGE types/subtypes to *L. monocytogenes* isolates. Comparison of the PFGE patterns was also done using BioNumerics 3.5 software (Applied Maths, Saint-Matins-Latem, Belgium), as described by Fugett et al. (2007).

A secondary identification label (Quality Milk Production identification [**QMP ID**]) was assigned to each of the isolates, and general and source information is available in Pathogen Tracker 2.0 at http://www.pathogentracker.net.

Statistical analysis

Data for the presence of *Listeria* spp. and *L. monocytogenes* were analyzed using statistical software JMP 7.0 (SAS Institute Inc., Cary, NC). Differences among source categories were evaluated using the chi-square test of independence and Fisher's exact test. A significance level (α) of 0.05 was used.

RESULTS

Fecal samples

A total of 2,272 fecal samples were obtained in eight samplings. Of these, 715 samples were analyzed for the presence of *Listeria* spp. and *L. monocytogenes*. One hundred seventy-nine samples (25.0%) were *Listeria* species positive, and 51 (7.1%) were positive for *L. monocytogenes*. A summary of the sampling regimen and the results for fecal samples is presented in Table 2.1.

Environmental samples

A total of 303 environmental samples were obtained in 14 samplings. Of the 303 samples, 87 (28.7%) were positive for *Listeria* spp., and 22 (7.3%) were positive for *L. monocytogenes*. The total number of samples obtained at each sampling and the number and percentage of *Listeria* species and *L. monocytogenes*-positive samples are summarized in Table 2.1.

Environmental samples were classified according to their source. The total number of samples obtained from each of the sampled materials is summarized in Table 2. The numbers and percentages of samples positive for *Listeria* species and *L. monocytogenes* found in each of the sampled materials are also shown in Table 2.2.

During each of the 13 samplings, approximately one source water sample, five drinking water samples, four feed samples, and 10 manure composite samples were obtained. *Listeria* was never isolated from the source water that was used to supply drinking water troughs. The proportion of *L. monocytogenes*-positive samples was significantly greater for drinking water samples obtained from the water troughs than for feed and manure composite samples ($P < 0.05$). No significant differences between the proportions of *L. monocytogenes* in feed and manure composite samples were found. Temporal variation in the percentages of *L. monocytogenes*-positive samples among drinking water, feed, and manure composite samples is shown in Figure 2.1. The prevalence of *L. monocytogenes* in drinking water was generally highest from February through April.

Table 2.1. Number and percentage of *Listeria* spp.[1] and *L. monocytogenes* isolated from environmental and fecal samples over a 3-year period in a single dairy herd

	Environmental					Fecal					
Sampling Date	Samples Collected	*Listeria* spp.		*Listeria. monocytogenes*		Samples Collected	Samples Analyzed		*Listeria* spp.		*Listeria monocytogenes*	
2/17/2004	10	3	(30%)	3	(30%)	308	72	(23.4%)	18	(25%)	3	(4.2%)
6/17/2004	34	6	(17.6%)	1	(2.9%)	-	-	-	-	-	-	-
6/29/2004	3	3	(100%)	1	(33.3%)	-	-	-	-	-	-	-
10/5/2004	23	2	(8.7%)	0	(0%)	316	65	(20.6%)	0	(0%)	0	(0%)
1/11/2005	19	6	(31.6%)	1	(5.3%)	12	12	(100%)	0	(0%)	0	(0%)
4/12/2005	24	10	(41.7%)	2	(8.3%)	335	66	(19.7%)	14	(21.2%)	7	(10.6%)
7/11/2005	29	3	(10.3%)	0	(0%)	-	-	-	-	-	-	-
10/3/2005	26	5	(19.2%)	0	(0%)	308	63	(20.5%)	5	(7.9%)	1	(1.6%)
2/20/2006	21	10	(47.6%)	0	(0%)	-	-	-	-	-	-	-
5/1/2006	22	7	(31.8%)	2	(9.1%)	327	327	(100%)	67	(20.5%)	12	(3.7%)
7/10/2006	25	1	(4%)	1	(4%)	-	-	-	-	-	-	-
10/9/2006	23	3	(13%)	0	(0%)	333	0	(0%)	N/A	N/A	N/A	N/A
1/8/2007	22	9	(40.9%)	4	(18.2%)	-	-	-	-	-	-	-
4/16/2007	22	19	(86.4%)	7	(31.8%)	333	110	(33%)	75	(68.2%)	28	(25.5%)
Total	303	87	(28.7%)	22	(7.3%)	2272	715	(31.5%)	179	(25%)	51	(7.1%)

[1] Including *L. monocytogenes.*

[2] 1/11/2005: Not a bi-annual fecal sampling

[3] N/A= Not applicable

Table 2.2 Number and percentage of *Listeria* spp.[1] and *L. monocytogenes* positive environmental samples obtained from different sampling materials over a 3-year period in a single dairy herd

Sampled Material	Number of samples	*Listeria* spp.		*L .monocytogenes*	
Bedding	15	4	(26.7%)	1	(6.7%)
Birds/Bird Droppings	11	2	(18.2%)	0	(0%)
Manure Composite	123	31	(25.2%)	3	(2.4%)
Other Composites[2]	3	2	(66.7%)	1	(33.3%)
Feed	55	4	(7.3%)	1	(1.8%)
Flies	16	8	(50%)	2	(12.5%)
Other insects	5	2	(40%)	0	(0%)
Source Water	13	0	(0%)	0	(0%)
Drinking Water for Animals	47	29	(61.7%)	14	(29.8%)
Water (Other)[3]	13	5	(38.5%)	0	(0%)
Other	2	0	(0%)	0	(0%)
Total	303	87	(28.7%)	22	(7.3%)

[1] Including *L. monocytogenes.*

[2]Waste around a water trough and a feed bunk, composite of feral animal's feces found in one of the silage bunkers (this last tested positive for *L. monocytogenes*)

[3] Standing water, runoff silage water, mud puddles.

In-line milk filter samples

A total of 108 in-line milk filter samples were obtained over the study period. Seventy-nine (73.1%) of these were positive for *Listeria* spp. and 73 (67.6%) for *L. monocytogenes*. *Listeria monocytogenes* was first isolated from an in-line filter in May 2005. In-line filters were negative for *L. monocytogenes* in the 16 subsequent weekly samplings until September 2005. Starting in September 2005, *L. monocytogenes* was regularly isolated from the in-line filters (Figure 2.2).

The percentage of *L. monocytogenes*-positive filters on a monthly basis varied between 20% and 100% during this period, except for samples obtained in October 2006 (0%).

Bulk tank milk samples

A total of 172 milk samples were obtained from the bulk tank between February 2004 and July 2007. Of these samples, 40 (23%) were positive for *Listeria* spp., and 34 (19.7%) were positive for *L. monocytogenes*. *Listeria monocytogenes* was not isolated from any of the bulk tank milk samples analyzed from the beginning of the study until November 2005. A non-*monocytogenes Listeria* species was isolated once from a bulk tank milk sample in May 2005. However, *L. monocytogenes* started to appear regularly in milk samples obtained from the milk tank after November 2005 (Figure 2.2).

Figure 2.1. Temporal variation in the number and percentage of *L. monocytogenes*-positive drinking water, feed, and manure composite samples obtained in a dairy farm over 3 years.

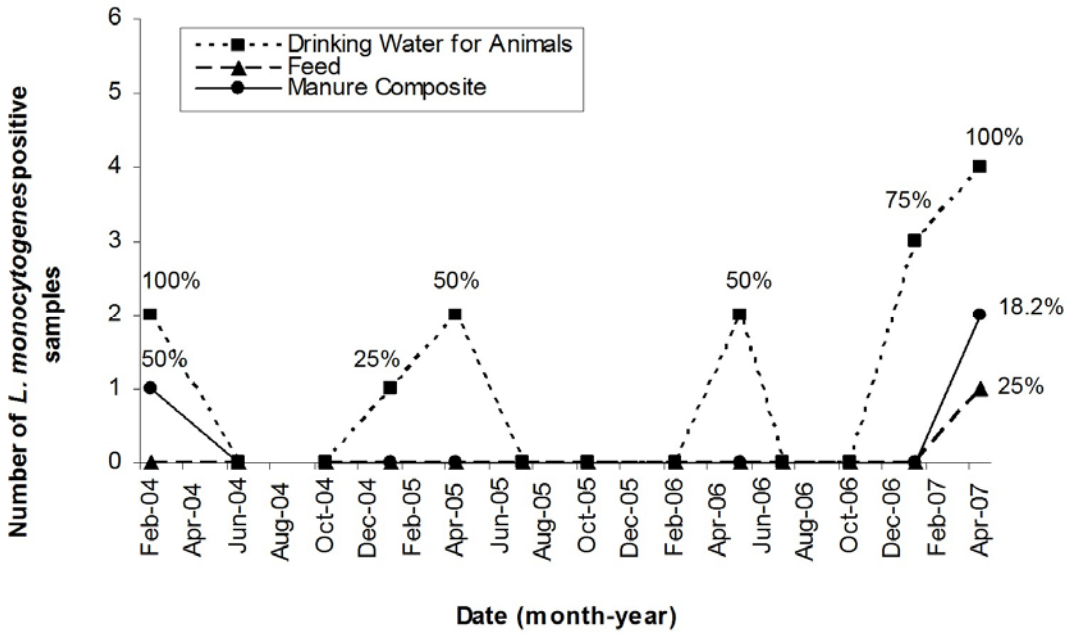
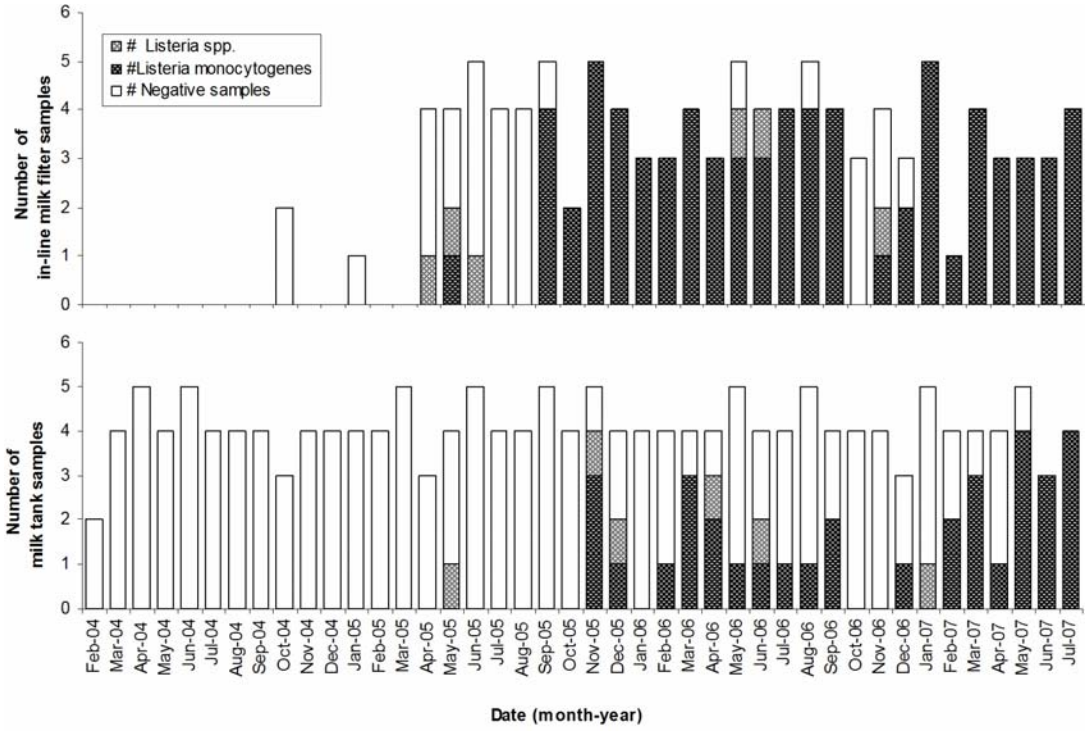

Drinking Water for Animals
Feed
Manure Composite
Number of L. monocytogenes positive samples
Date (month-year)
0
1
2
3
4
5
6
Feb-04
Apr-04
Jun-04
Aug-04
Oct-04
Dec-04
Feb-05
Apr-05
Jun-05
Aug-05
Oct-05
Dec-05
Feb-06
Apr-06
Jun-06
Aug-06
Oct-06
Dec-06
Feb-07
Apr-07
100%
50%
25%
50%
50%
75%
100%
18.2%
25%

Figure 2.2. Number of *Listeria* species- and *L. monocytogenes*-positive samples obtained from in-line milk filters and bulk tank milk during the study period.


Listeria spp.
#Listeria monocytogenes
Negative samples
Number of in-line milk filter samples
Number of milk tank samples
0
1
2
3
4
5
6
Feb-04
Mar-04
Apr-04
May-04
Jun-04
Jul-04
Aug-04
Sep-04
Oct-04
Nov-04
Dec-04
Jan-05
Feb-05
Mar-05
Apr-05
May-05
Jun-05
Jul-05
Aug-05
Sep-05
Oct-05
Nov-05
Dec-05
Jan-06
Feb-06
Mar-06
Apr-06
May-06
Jun-06
Jul-06
Aug-06
Sep-06
Oct-06
Nov-06
Dec-06
Jan-07
Feb-07
Mar-07
Apr-07
May-07
Jun-07
Jul-07
Date (month-year)

Milking parlor and milking equipment samples

Samples from 40 sites were analyzed for *Listeria*. *Listeria* species were found in 14 (35%) of these sites, and *L. monocytogenes* was found in 6 (15%) of these sites. Two samples obtained from the floor (parlor pit and storage area) were positive for *L. monocytogenes*. *Listeria monocytogenes* was also detected in one rubber liner and two milk meters, and one positive sample was obtained from the bulk tank outlet.

In-line milk filters had a significantly greater proportion of *L. monocytogenes*-positive samples than did fecal, environmental, milking parlor, and bulk tank milk samples ($P< 0.005$). Bulk tank milk had a greater proportion of *L. monocytogenes*-positive samples than did fecal and environmental samples ($P< 0.05$).

Pulsed-field gel electrophoresis

Sixty of 186 *L. monocytogenes* isolates obtained betweenFebruary 2004 and July 2007 were typed by PFGE. Thirteen PFGE types and eight subtypes were distinguished with the restriction endonuclease AscI through visual inspection. Fourteen PFGE types and seven subtypes were found by visual inspection when using the restriction endonuclease ApaI. Analysis of the combined AscI and ApaI restriction profiles by automated cluster analysis using the Dice coefficient (tolerance of 1.5%) and unweighted-pair group method with arithmetic averages (**UPGMA**) showed 23 PFGE types, using a similarity score value of 100% as the cutoff.

Cluster analysis of the combined AscI and ApaI restriction digest profiles showed that PFGE types F, T, and D were predominant from September 2005 through February 2006, from March 2006 to May 2007, and from May 2007 through July 2007,

respectively. Types F, T, and D accounted for 13.3%, 25%, and 15% of *L. monocytogenes* isolates subjected to PFGE typing, respectively.

The PFGE type F was first detected in an in-line milk filter sample from September 2005 and, subsequently, in additional milk filter samples collected in September (several sampling dates), October and December 2005, and February 2006. PFGE type F was also isolated from a bulk tank milk sample in November 2005. This sample was the first bulk tank milk sample from which *L. monocytogenes* was isolated.

PFGE type T was first detected in a feral-animal feces composite in June 2004. Type T was not detected in 2005 on this set of isolates. PFGE type T was subsequently found in in-line milk filter samples obtained in March, July, September, and December 2006 and in February and April 2007 (Figure 2.3). Furthermore, PFGE type T was found in bulk tank milk samples in May, July, September, and December 2006, and February and May 2007 (Figure 2.3). In May 2007, type T was also obtained from a milk meter and from the milk tank outlet (Figure 2.4). PFGE type S was closely related to PFGE type T. It was found in a manure composite sample obtained in February 2004 and in water samples in February 2004 and January 2007.

PFGE type D was first detected in a bulk tank milk sample in May 2007 (approximately 2 weeks before the sampling of the milking equipment) and subsequently in one of the milk meters (May 2007) and in in-line milk filter and bulk tank milk samples obtained between May and July 2007. PFGE type E, which was closely related to PFGE type D, was found in a sample obtained from a rubber liner.

AscI and ApaI restriction digest profiles of the 60 *L. monocytogenes* isolates are shown in Figure 2.4. Clustering of these *L. monocytogenes* isolates and the PFGE types assigned by visual inspection and by automated cluster analysis are also presented.

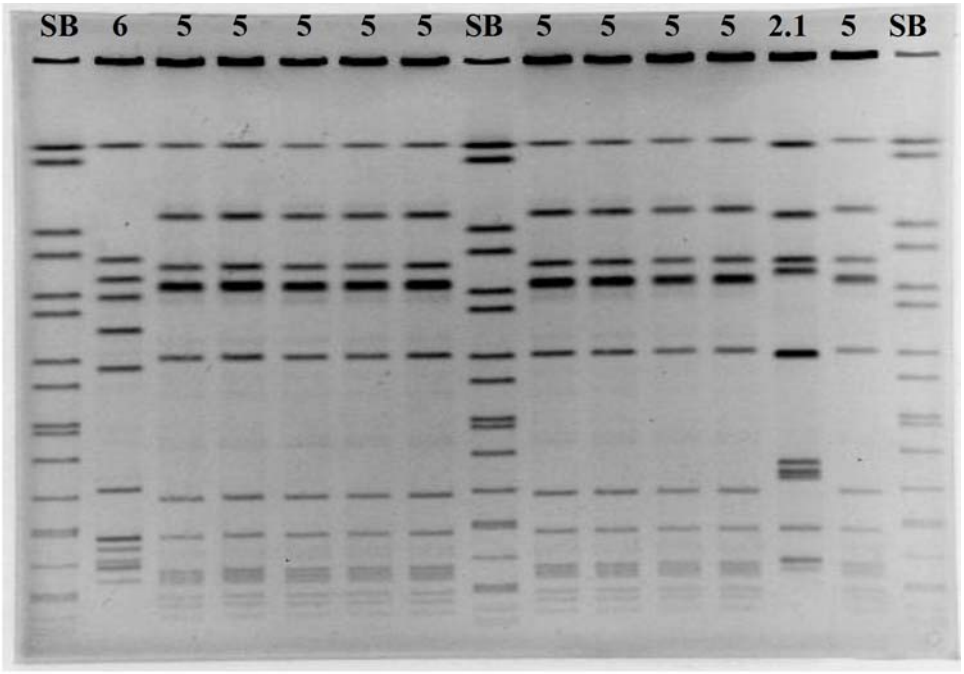
DISCUSSION

Detection of *L. monocytogenes* in bulk tank milk has been previously reported. In a regional survey of 131 dairy herds in South Dakota and Minnesota, *L. monocytogenes* was isolated from 4.6% of the bulk tank milk samples (Jayarao and Henning, 2001). In a national survey, *L. monocytogenes* was isolated from 6.5% of collected bulk tank milk samples (Van Kessel et al., 2004). Even on this high-prevalence farm where *L. monocytogenes* was on average isolated in one of five weekly samples (19.8%), a single point time survey might have missed *L. monocytogenes* in raw milk. Hence, repeated sampling over time is a more reliable method to gauge the potential presence of *L. monocytogenes*.

The proportion of *L. monocytogenes*-positive in-line filter samples (67.6%) was even greater than the proportion of *L. monocytogenes*-positive bulk tank milk samples (19.8%), suggesting that testing in-line filters could be a more sensitive means to detect pathogens than bulk tank milk samples. In-line milk filters have previously been used in a survey of New York State dairy farms (Hassan et al., 2000). In this survey of 404 farms, *L. monocytogenes* was isolated from 12.6% of the filters. Higher sensitivity of detection in in-line milk filters than that in bulk tank milk has also been reported for *Salmonella* spp. (Van Kessel et al., 2008).

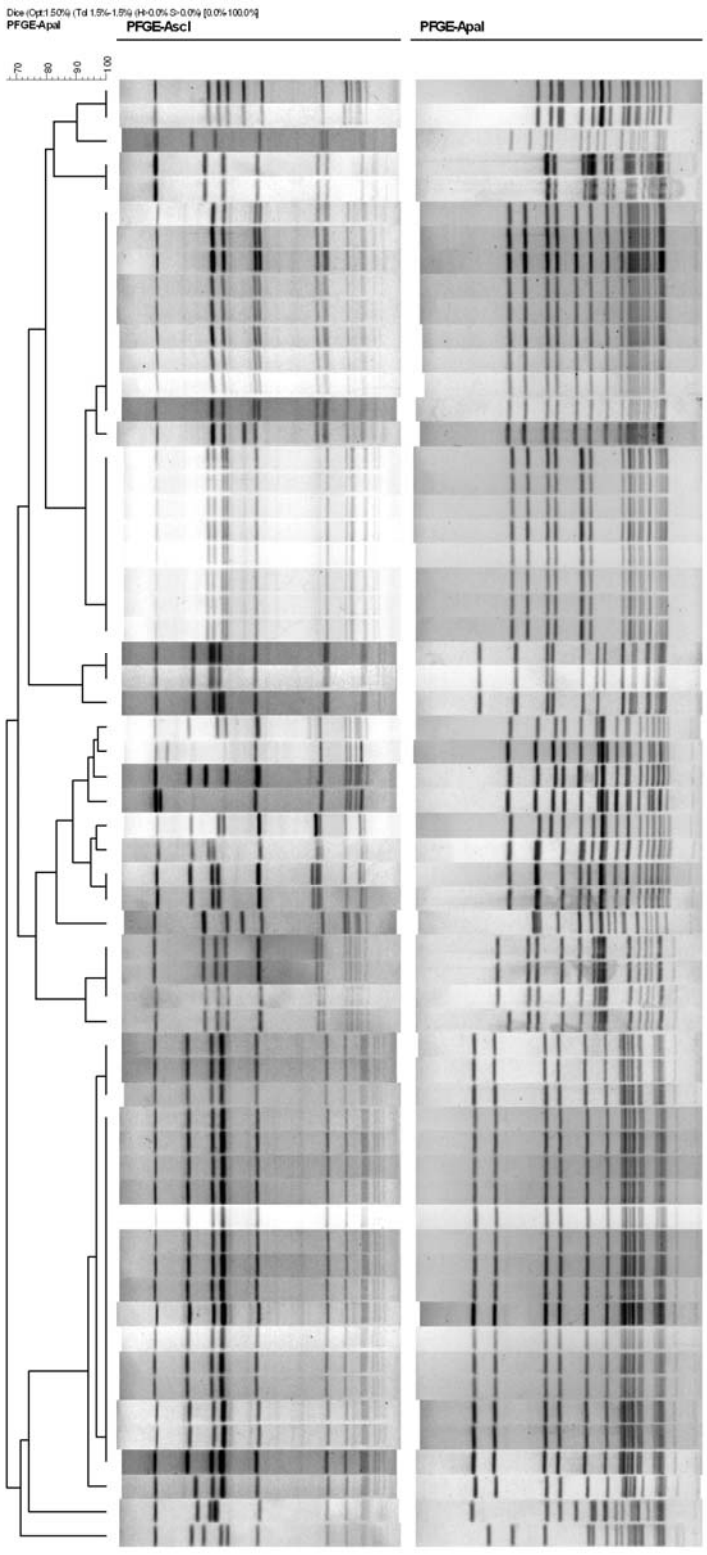
Figure 2.3. Examples of *L. monocytogenes* PFGE types/subtypes among isolates obtained between June 2004 and April 2007, using restriction endonuclease AscI. The first row (top panel) indicates the (sub)type assigned to PFGE patterns based on the criteria from Tenover et al. (32). First and second rows (bottom panel) indicate the source (feces [F], bulk tank milk [BM], and milk filter [MF]) and sample identification (QMP ID, L1), respectively. Samples are in chronological order from left to right, with sampling date (month/day) and year shown on the third and fourth rows (bottom panel), respectively.

Lanes 1, 8, and 15 contain *Salmonella enterica* serotype Braenderup (SB; standard).



SB	**F**	**BM**	**BM**	**MF**	**BM**	**MF**	**SB**	**BM**	**MF**	**BM**	**MF**	**F**	**MF**	**SB**
QMP L1	003	005	006	015	007	016		008	017	025	024	023	022	
	5/1	5/16	7/6	7/11	9/12	9/19		12/12	12/12	2/14	2/20	4/16	4/26	
	2006	2006	2006	2006	2006	2006		2006	2006	2007	2007	2007	2007	

Figure 2.4. Automated cluster analysis of the 60 *L. monocytogenes* isolates selected for PFGE typing, after digestion with restriction endonucleases AscI and ApaI. PFGE types assigned by visual inspection (AscI-ApaI), and PFGE types assigned by automated cluster analysis (A.C.A.) of the combined AscI and ApaI restriction digest profiles are shown to the right of the dendrogram.



		AscI	ApaI	A.C.A.
QMPL1-003	Fecal 5/1/06	6	6	A
QMPL1-136	Fecal 4/16/07	6	6	A
QMPL1-080	Water 1/11/05	9	9	B
QMPL1-109	Water 4/16/07	13	13	C
QMPL1-121	Fecal 4/16/07	13	13	C
QMPL1-021	Bulk Milk 5/2/07	7	7	D
QMPL1-026	Filter 5/22/07	7	7	D
QMPL1-027	Milk Meter#2 5/16/07	7	7	D
QMPL1-045	Bulk Milk 5/30/07	7	7	D
QMPL1-047	Filter 6/6/07	7	7	D
QMPL1-052	Bulk Milk 6/27/07	7	7	D
QMPL1-053	Filter 7/5/07	7	7	D
QMPL1-059	Bulk Milk 7/18/07	7	7	D
QMPL1-081	Water 4/12/05	7	7	D
QMPL1-020	Teat Cup 5/16/07	7.1	7	E
QMPL1-004	Bulk Milk 11/9/05	3	3	F
QMPL1-010	Filter 10/6/05	3	3	F
QMPL1-011	Filter 12/7/05	3	3	F
QMPL1-012	Filter 12/21/05	3	3	F
QMPL1-013	Filter 2/21/06	3	3	F
QMPL1-030	Filter 9/8/05	3	3	F
QMPL1-031	Filter 9/14/05	3	3	F
QMPL1-032	Filter 9/20/05	3	3	F
QMPL1-083	Fecal 4/12/05	10	10	G
QMPL1-101	Fecal 5/1/06	10	10	G
QMPL1-085	Fecal 4/12/05	10.1	10.1	H
QMPL1-001	Flies 6/29/04	1	1	I
QMPL1-002	Fecal 10/3/05	4	4	J
QMPL1-091	Water 5/1/06	1.1	1.1	K
QMPL1-097	Fecal 5/1/06	4.1	4.1	L
QMPL1-009	Filter 5/10/05	2	2	M
QMPL1-134	Fecal 4/16/07	2.2	2.1	N
QMPL1-023	Fecal 4/16/07	2.1	2.2	O
QMPL1-114	Fecal 4/16/07	2.1	2.2	O
QMPL1-073	Fecal 2/17/04	8	8	P
QMPL1-110	Water 4/16/07	6.1	14	Q
QMPL1-111	Pit 4/16/07	6.1	14	Q
QMPL1-122	Fecal 4/16/07	6.1	14	Q
QMPL1-120	Fecal 4/16/07	6.1	14.1	R
QMPL1-074	Water 2/17/04	5	5	S
QMPL1-075	Composite 2/17/04	5	5	S
QMPL1-105	Water 1/8/07	5	5	S
QMPL1-005	Bulk Milk 5/16/06	5	5	T
QMPL1-006	Bulk Milk 7/6/06	5	5	T
QMPL1-007	Bulk Milk 9/12/06	5	5	T
QMPL1-008	Bulk Milk 12/12/06	5	5	T
QMPL1-014	Filter 3/29/06	5	5	T
QMPL1-015	Filter 7/11/06	5	5	T
QMPL1-016	Filter 9/19/06	5	5	T
QMPL1-017	Filter 12/12/06	5	5	T
QMPL1-018	Milk Meter#1 5/16/07	5	5	T
QMPL1-022	Filter 4/26/07	5	5	T
QMPL1-024	Filter 2/20/07	5	5	T
QMPL1-025	Bulk Milk 2/14/07	5	5	T
QMPL1-028	Bulk Milk 5/15/07	5	5	T
QMPL1-029	Bulk Tank Outlet 5/16/07	5	5	T
QMPL1-078	Composite 6/17/04	5	5	T
QMPL1-098	Fecal 5/1/06	5.1	5.1	U
QMPL1-103	Water 1/8/07	12	12	V
QMPL1-102	Flies 7/17/06	11	11	W

The milk produced on the study farm is pasteurized before its distribution to consumers, and the presence of *Listeria* in bulk tank milk is therefore unlikely to pose a human health hazard. Pasteurized milk from retail stores in the United States (Frye and Donnelly, 2005) and in England and Wales (Greenwood et al., 1991), however, tested positive for *L. monocytogenes* in 0.018% and 1.1% of samples, respectively. Outbreaks of human listeriosis have been attributed to the consumption of pasteurized milk (Fleming et al., 1985; Dalton et al, 1997) or dairy products manufactured with improperly pasteurized milk (Linnan et al., 1988). Raw milk contaminated with *Listeria* could be a source of contamination for a milk processing plant (Waak et al., 2002). Hence, prevention of *Listeria* contamination is important for milk that is consumed raw, as well as for milk that will be pasteurized before consumption.

In the current study, weekly samplings for in-line milk filters were not part of the original sampling protocol and started in April 2005; therefore, it is not certain whether *L. monocytogenes* was endemic before this time or only sporadically present. The three in-line milk filter samples obtained in October 2004 and January 2005 were negative for *L. monocytogenes*, so it would appear that the contamination was established after January 2005. Starting in September 2005, *L. monocytogenes* was isolated from the in-line milk filter samples on a regular basis. Standard plate counts of bulk tank milk were unusually high during that month (peak, 593,000 CFU/ml) (unpublished data). High standard plate counts have been associated with deficiencies in the cleaning of the milking equipment, because the presence of milk residues may provide ideal conditions for bacterial growth (Murphy and Boor, 2000).

Excretion of *L. monocytogenes* in milk has been reported for cows suffering from mastitis (Fedio et al., 1990; Rawool et al., 2007; Winter et al., 2004). In our study,

milk from all clinical mastitis cases from the dairy farm was cultured as part of the routine examination of mastitis cases, and *Listeria* was never isolated from these samples. We did not specifically culture samples from cows with subclinical mastitis; however, the mammary gland would not be a specific target for *L. monocytogenes* in cattle (Fedio et al., 1990), and isolation of the organism from nonclinical milk samples is extremely rare (QMPS, unpublished data). Thus, milk from individual cows is unlikely to have been an important source of *L. monocytogenes* in the bulk tank of the study farm.

Milk and milk filters were positive for *L. monocytogenes* more frequently than expected, based on the low incidence of the pathogen in fecal samples (Van Kessel et al., 2008). In our study, 7.1% of fecal samples were positive for *L. monocytogenes*, with a range from 0 to 25.5% at any given sampling time. When 15 *L. monocytogenes* isolates obtained from fecal samples were characterized by PFGE, 12 PFGE types were observed, demonstrating a high level of heterogeneity among fecal isolates. If the presence of *L. monocytogenes* in bulk tank milk was due to fecal contamination, we would have expected to find heterogeneity among *L. monocytogenes* isolates (Borucki et al., 2005; Ho et al., 2007) in the bulk tank milk as well. However, only three *L. monocytogenes* PFGE types, each persisting over time, were observed in milk. Furthermore, the PFGE types of fecal isolates were different from those observed in bulk tank milk and milk filters. By visual inspection, the PFGE types N/O (three fecal samples obtained in April 2007) and U (one fecal sample obtained in May 2006) were "closely related" but not identical (Tenover et al., 1995) to PFGE types M (one isolate from a milk filter in May 2005) and T (15 isolates from milk and milk filter in March 2006 to May 2007). It is possible that some strains present in feces were not detected with our study design (Döpfer et al., 2008).

In addition to feces, a variety of environmental sources harbored *L. monocytogenes*, and some strains found in bulk tank milk were previously isolated from other sources on the farm. Our data suggest that the presence of *L. monocytogenes* in the milk system was initially caused by fecal or environmental contamination and that specific strains could have subsequently established themselves in the milking system as a biofilm. *Listeria monocytogenes* has the ability to form biofilms (Harvey et al., 2007; Takhistov and George, 2004) on stainless steel surfaces and other materials (Beresford et al., 2001) that can be present in dairy operations. Bacterial cells can detach from biofilms (Hall-Stoodley et al., 2004), and this could explain the presence of the same *L. monocytogenes* PFGE types in bulk tank milk and filters for prolonged periods of time. Persistent *L. monocytogenes* strains can be defined as those strains in a particular dairy premise that are repeatedly found over time in bulk tank milk samples (Borucki et al., 2003). This definition agrees with our finding. In previous studies, persistent strains have shown a better ability to form biofilms than transient strains (Borucki et al., 2003; Norwood and Gilomour, 1999). Although the biofilm-forming ability of the persistent PFGE types from our study has not yet been assessed, formation of biofilm could potentially explain our observations.

In this study, the low number of manure composite samples positive for *L. monocytogenes* suggests low levels of fecal shedding in *L. monocytogenes*-positive animals. Samples of silage and other feeds were negative, except in April 2007, when one feed sample (25%) was positive for *L. monocytogenes*. On the same sampling date, the highest percentages of *L. monocytogenes*-positive fecal (25.5%) and drinking water (100%) samples were also reported. Fecal contamination of the feed cannot be ruled out, since *L. monocytogenes*-positive samples were obtained from feedstuffs that were fed to animals ("feed as presented to the animals"). Because all source water

samples were negative throughout the study, fecal contamination could be the likely source of *L. monocytogenes* in water. Results of PFGE typing of *L. monocytogenes* isolates obtained from both fecal and water samples also suggest feces as the source of water contamination.

Pulsed-field gel electrophoresis results were concordant for most of the 60 *L. monocytogenes* isolates regardless of which restriction endonuclease (AscI and ApaI) was used. A few discrepancies were observed between the analysis using visual inspection and automated cluster analysis. For example, PFGE type S, which was observed in three environmental samples obtained in February 2004 (drinking water and manure composite samples) and January 2007 (drinking water), was considered indistinguishable from PFGE type T by visual inspection, whereas types S and T were closely related but distinguishable based on computer- assisted analysis. PFGE types I/K and J/L were considered a main type and a subtype, respectively, when analyzing digestion profiles by visual inspection, whereas they were considered to be distinct profiles based on computer-assisted data analysis. The differences in interpretation of banding patterns based on visual or automated comparison are subtle and do not affect the interpretation of the study results.

In conclusion, our study on this farm shows high heterogeneity of *L. monocytogenes* isolates in a variety of on-farm sources and predominant homogeneity of the *L. monocytogenes* population in in-line milk filters and bulk tank milk, implying a potential presence of *L. monocytogenes* biofilm in the milking equipment.

Even though we report a suggested presence of *L. monocytogenes* biofilm in the milking system of just one dairy farm, the relatively high prevalence of this organism

in bulk tank milk surveys combined with the documented ability of *L. monocytogenes* to form biofilms on stainless steel (Norwood and Gilmour, 1999; Beresford et al., 2001) would suggest that this is not an isolated finding. Further research to quantify the importance of biofilms in milk harvesting equipment and methods to prevent buildup of such biofilms is needed.

To our knowledge, this is the first report to indicate the potential presence of *L. monocytogenes*-containing biofilms in dairy farm milk harvesting equipment. Measures to prevent *L. monocytogenes* contamination and persistence on dairy operations, as well as the communication of the risk attributed to the consumption of contaminated raw milk or dairy products made with nonpasteurized milk, are encouraged.

ACKNOWLEDGMENTS

Financial support for this work was provided by the USDA Agricultural Research Service (agreement no. 58-1265-3-156) for the Regional Dairy Quality Management Alliance.

We thank the participating producer, who kindly allowed us to do this research on his dairy farm facility. We acknowledge the valuable help of the Quality Milk Production Services and USDA-ARS teams during samplings, laboratory testing, and all previous logistics.

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CHAPTER 3

BIOFILM IN MILKING EQUIPMENT ON A DAIRY FARM AS A POTENTIAL SOURCE OF BULK TANK MILK CONTAMINATION WITH *LISTERIA MONOCYTOGENES**

ABSTRACT

The objective of this study was to assess the presence of a *Listeria monocytogenes*-containing biofilm in milking equipment as a potential source of bulk tank milk contamination on a dairy farm where milk contamination had been previously documented. Samples were collected from milking equipment and milking parlor premises on 4 occasions and analyzed for the presence of *L. monocytogenes*. Pulsed-field gel electrophoresis (**PFGE**) typing was conducted on *L. monocytogenes* isolates from the milking equipment, parlor and storage room floors, bulk tank milk, and in-line milk filters. Pieces from milk meters and rubber liners were obtained to visually assess the presence of a biofilm using scanning electron microscopy. A total of 6 (15%), 4 (25%), and 1 (6%) samples were culture-positive for *L. monocytogenes* in the first, second, and third sample collection, respectively.

*Latorre , A. A. , J. S. Van Kessel , J. S. Karns , M. J. Zurakowski , A. K. Pradhan , K. J. Boor , B. M. Jayarao , B. A. Houser , C. S. Daugherty , and Y. H. Schukken. 2010. Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. J. Dairy Sci. 93: 2792-2802.

Two samples were *L. monocytogenes hly* PCR-positive but were culture-negative in the fourth sample collection. Combined AscI and ApaI restriction analysis yielded 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment, parlor, bulk tank milk, and milk filters. A predominant and persistent PFGE type (PFGE type T) was observed among these *L. monocytogenes* isolates (9/15 isolates). Scanning electron microscopy of samples from the bottom cover of 2 milk meters showed the presence of individual and clusters of bacteria, mainly associated with surface scratches. The presence of a bacterial biofilm was observed on the bottom covers of the 2 milk meters. Prevention of the establishment of biofilms in milking equipment is a crucial step in fulfilling the requirement of safe, high-quality milk.

INTRODUCTION

The safety of milk is an important attribute for consumers of milk and dairy products. Milk pasteurization safeguards consumers from many potential foodborne hazards in milk and milk products. Despite the pasteurization process, the quality and safety of raw milk are important in reducing the risk of foodborne diseases associated with milk because raw milk is the starting point of the milk production-consumption chain.

Dairy products contaminated with *Listeria monocytogenes* have been responsible for human listeriosis outbreaks (Dalton et al., 1997; Centers for Disease Control and Prevention, 2008). The total number of listeriosis cases reported in the United States was 808 and 656 in 2007 and 2008, respectively (Centers for Disease Control and Prevention, 2009). The serious consequences of listeriosis, such as a septicemic form

of the illness in elderly and immunocompromised people, and abortion in pregnant women or death of their newborn, constitute a serious threat to public health.

The presence of biofilms has been well documented in the food industry (Carpentier and Cerf, 1993) and these biofilms are a potential source of bacterial contamination. *Listeria monocytogenes* has the potential to form biofilms on materials such as stainless steel (Norwood and Gilmour, 1999; Beresford et al., 2001), rubber, or plastic (Beresford et al., 2001), and these materials are frequently found in milk handling equipment, milk lines, or milk tanks. The ability of *L. monocytogenes* to form biofilms (Harvey et al., 2007) may contribute to its persistence in food processing plants (Thimothe et al., 2004).

Previous biofilm studies have primarily been conducted in processing plants and little is known about the presence of biofilms on dairy farms. The presence of *Listeria*-containing biofilms in milking equipment has not yet been reported. However, previous work suggested biofilms in the milking equipment as a possible source of persistent *L. monocytogenes* contamination of bulk tank milk **(BTM)** (Latorre et al., 2009). This previous study demonstrated the frequent presence of *L. monocytogenes* in BTM and in in-line milk filter samples collected from a single farm between 2004 and 2007. Subsequent samplings indicated that *L. monocytogenes* was still frequently isolated from BTM and in-line milk filter samples collected until the end of the present study (March 2008; data not shown). The objective of this study was to assess the presence of *L. monocytogenes*-containing biofilm in milking equipment as a potential source of BTM contamination on a dairy farm.

MATERIALS AND METHODS

Study Farm

The study was conducted on a single 330-cow dairy farm in New York State (Latorre et al., 2009; Pradhan et al., 2009). The farm has an average milk production of approximately 9,071 kg of milk/d and the milk is transported daily to a milk processing plant and subsequently pasteurized. The cows are milked 3 times/d at an interval of approximately 8 h in a double 8 herringbone milking parlor. Plastic (polysulfone) milk meters were installed in May 2005. Rubber liners were replaced every 2 wk (approximately every 928 milkings).

Routine washes of the milking machine and milk line were carried out after each milking using the following protocol: 1) prerinse cycle with water, 2) wash cycle using a cleaning product with potassium hydroxide, polyphosphates, and sodium hypochlorite as active ingredients, and 3) acid wash cycle using a clean-in-place **(CIP)** acid cleaner (phosphoric and sulfuric acids as active ingredients). The milking equipment was sanitized with a sodium hypochlorite solution immediately before every milking. Milk tank washes were carried out every 24 h using the same CIP protocol described above. Time and temperatures of the pipeline and bulk tank washes were monitored and recorded by Milk- Guard (Dairy Check Inc., Ontario, Canada).

Weekly tests to monitor SCC, SPC, and preliminary incubation count **(PIC)** in BTM samples were conducted from February 2004 until April 2008. The analysis of BTM samples was performed as described by Jayarao et al. (2004). A DeLaval cell counter (DeLaval International AB, Tumba, Sweden) was used for SCC determination.

Sample Collection

Sponge-swab samples from the inner surface of milking equipment and the parlor environment were collected on 4 occasions using BactiSponge kits (Hardy Diagnostics, Santa Maria, CA) as described previously (Thimothe et al., 2004; Latorre et al., 2009). For the first sample collection, 40 sampling sites were selected based on the criteria described previously (Latorre et al., 2009). Samples were obtained in May 2007 from the sites described in Table 1. Based on the results obtained in the first sample collection, in January 2008 swab samples were collected from all milk meters and all individual rubber liners (second collection; Table 1). Based on the results obtained in previous sample collections, a resampling of all milk meters was carried out in February 2008 (third collection; Table 1). Two sets of swab samples were obtained in March 2008 from the bowl of 2 milk meters (fourth collection). One set of sponges was used for culture of *L. monocytogenes* and the other was used for nonselective enrichment with brain heart infusion broth (**BHI**; BD Diagnostics, Sparks, MD) for further analysis by PCR (Table 1).

All samples were transported on ice overnight to the laboratory. A summary of sample sources, number of samples, and collection dates is presented in Table 3.1.

Laboratory Procedures

Listeria monocytogenes Analysis. *Listeria monocytogenes* analysis was conducted on all sponge samples that were collected (Table 3.1). For this, 20 mL of 1% buffered peptone water (BD Diagnostics) was added to the Whirl-Pak bags (included in BactiSponge kits; Hardy Diagnostics) containing the sampling sponge. The Whirl-Pak was put into a stomacher bag (GSI Creos Corporation, Tokyo, Japan) and pummeled 3

Table 3.1. Summary of sample sources, number of samples obtained, and date of sample collections to milking parlor room and milking equipment[1]

	Number of samples			
Sample source	May 2007	January 2008	February 2008	March 2008
Drains in parlor room	2	NC[2]	NC	NC
Floor in parlor pit	3	NC	NC	NC
Milk pipelines	7	NC	NC	NC
Teat cup rubber liners	4	64	NC	NC
Milk meters before washing	8	NC	NC	NC
Milk meters after washing	8	16	16	2[3]
Milk tank outlet	1	NC	NC	NC
Floor under milk tank	1	NC	NC	NC
Milk-pump surface	1	NC	NC	NC
Vacuum pump surface	1	NC	NC	NC
Floor in washing room	1	NC	NC	NC
Floor in storage area	1	NC	NC	NC
Drains in washing room	2	NC	NC	NC
Total	40	80	16	2

[1] All samples collected were cultured to assess the presence of *L. monocytogenes*.

[2] NC = No samples were collected.

[3] Milk meters that tested *Listeria* spp.- or *L. monocytogenes*-positive on the third sample collection. These milk meters were selected for further analysis by Scanning Electron Microscopy and *hly*-PCR.

or 4 times for 30 s in an automatic bag mixer (BagMixer Interscience Laboratories Inc., Weymouth, MA). Then, 5 mL of the extract was added to 5 mL of doublestrength modified *Listeria* enrichment broth (BD Diagnostics). Enrichments were incubated at 37°C for 48 h. Enriched broth (10 μL) was streaked onto modified Oxford agar plates (Difco Laboratories, Detroit, MI), incubated at 37°C, and read at 24 and 48 h for presumptive *Listeria* colonies as described previously (Van Kessel et al., 2004). Presumptive *Listeria* colonies were further analyzed as described by Van Kessel et al. (2004) and Latorre et al. (2009).

Pulsed-Field Gel Electrophoresis Typing. Pulsed-field gel electrophoresis typing was conducted on 1 *L. monocytogenes* isolate from each positive sample obtained from the milking equipment (n = 9), floors in the parlor (n = 1), and storage room (n = 1). Pulsed-field gel electrophoresis typing was also done on *L. monocytogenes* isolates from select BTM and milk filter samples, which were collected weekly as part of another ongoing study (data not shown).

The PFGE typing of *L. monocytogenes* isolates was done following the Centers for Disease Control and Prevention (2004) Pulsenet Protocol with modifications, as described previously (Latorre et al., 2009). Analysis of DNA band patterns of AscI and ApaI digestions was carried out as described by Latorre et al. (2009).

Scanning Electron Microscopy Analysis. In March 2008, pieces from milk meters and rubber liners were obtained to assess the presence of a biofilm using scanning electron microscopy **(SEM)**. Selection of the units 3-left and 8-left (numbers indicate the number assigned to each of the milking units tested; left and right indicate the position of the units in the parlor) was based on the presence of *L. monocytogenes* and

Listeria spp., respectively, in samples collected on the third sample collection. Immediately after the routine washing cycle was finished, the top and bottom cover of the selected milk meters as well as 3 rubber liners (from unit 3-left) were aseptically removed. Samples were individually placed in zip-lock bags, labeled, and immediately transported on ice to the laboratory for processing (transport time of approximately 3.5 h).

The covers of milk meters were aseptically cut in half using a junior hacksaw (Task Force, Mineola, NY) with steam-sterilized 15-cm hacksaw blades (Task Force).
After cutting, the internal surface of the pieces was stained using an aqueous solution of 0.1% Alcian blue 8GX (Sigma-Aldrich, St. Louis, MO; Rayner et al., 2004). Then, the samples were washed 3 times with sterile distilled water and immediately transported for additional cutting using a Bridgeport 39196 milling machine (Bridgeport Inc., Bridgeport, CT). For this purpose, a steam-sterilized 25-mm hole saw (Lenox, East Longmeadow, MA) was used. Samples (25 mm × 25 mm) were aseptically obtained from each cover of the milk meters (top and bottom) and placed into a sterile Petri dish.

Rubber liners were cut into pieces that would fit into 25 mm × 25 mm SEM specimen mounts using sterile surgical scissors. The liner pieces were stained with an aqueous solution of 0.1% Alcian blue 8GX, as described above, and placed into sterile Petri dishes.

Milk meter and liner parts were transported to the Cornell Integrated Microscopy Center (Ithaca, NY) for SEM. The parts were immediately put in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) and stored overnight at 4°C. Samples

were then washed 3 times in 0.1 M sodium cacodylate buffer (pH 7.0) at 4°C, placed in a 1% buffered solution of osmium tetroxide at 4°C, and held overnight. The samples were washed 3 times in water at 4°C and then gradually dehydrated in a graded series of 10, 30, and 50% ethanol (10 min at each step). Following the dehydration step, the samples were soaked in 2% uranyl acetate in 70% ethanol for 20 min. Dehydration was continued in 90% ethanol followed by 3 changes of 100% ethanol. The samples were then critical point dried in a Bal-Tec Critical Point dryer (model 030, Bal-Tec Inc., Brookline, NH), attached to a specimen support, and coated with a gold and palladium target using a Bal-Tec SCD sputter coater (model 050; Bozzola and Dee Russell, 1999). Samples were then viewed using a Hitachi S4500 scanning electron microscope using 3 KV (Hitachi High Technologies America Inc., Electron Microscope Division, Pleasanton, CA).

PCR Analysis. For nonselective enrichment, 20 mL of BHI was added to each of the sponges. The sponges were manually pummeled and 5 mL of the extract was put into 5 mL of double-strength BHI (in triplicate for each sponge) and incubated at 37°C for 48 h. After incubation, 1.5 mL of each of the enrichments was centrifuged and the pellet was removed, put in a Microbank vial (Pro-Lab Diagnostics, Austin, TX), and stored at −80°C until PCR analysis.

For lysate preparation, enrichments stored at −80°C were put in 5 mL of BHI broth and incubated overnight at 37°C. Bacterial lysates were prepared as described by Furrer et al. (1991) with minor modifications (M. Wiedmann, Cornell University, Ithaca, NY; personal communication). Briefly, overnight cultures were vortexed and 250 μL was removed and centrifuged for 10 min at 15,000 × g. The pellet was resuspended in 95 μL of 1× PCR buffer (Roche Diagnostics, Indianapolis, IN). Four

microliters of lysozyme (50 mg/mL) was added. After incubation, 1 µL of proteinase K (20 mg/mL) was added and suspensions were incubated in a heating block for 60 min at 58°C, followed by a final incubation step of 8 min at 95°C. Lysates were centrifuged for 1 min at 15,000 × g and stored at −20°C until analysis.

Each PCR reaction contained 10.25 µL of nucleasefree water, 12.50 µL of Go-Taq Green (Promega, Madison, WI), 0.125 µL of *hly* primers F (5′ TCC GCA AAA GAT GAA GTT C′3) and R (5′ ACT CCT GGT GTT TCT CGA TT′3) (Jothikumar et al., 2003), and 2 µL of the lysate template. *Listeria monocytogenes* FSL K2–017 (information available at www.pathogentracker.net) was used as a positive control. *Escherichia coli* ATCC 25922, BHI, and nuclease-free water were used as negative controls. Polymerase chain reaction conditions as described by Jothikumar et al. (2003) were used with the following modifications: initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. Electrophoresis conditions, gel stain and destain, and image capture were done according to standard procedures.

Data Analysis

For statistical analysis and graphics of SPC and PIC data, all bacterial counts reported as >1,000,000 cfu/mL were truncated at 1,000,000 cfu/mL. Statistical analysis and graph of SCC were carried out using the data as reported by the cell counter. Statistical analysis of SCC, SPC, and PIC data was done using the software JMP 7.0 (SAS Institute Inc., Cary, NC). Graphs were generated using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA).

RESULTS

BTM Quality Parameters

A total of 193 BTM samples were analyzed between February 2004 and April 2008 to assess SCC, whereas 196 samples were analyzed to assess the total number of aerobic bacteria and PIC. The geometric means of SCC, SPC, and PIC were 233,301 cells/mL, 5,109 cfu/mL, and 18,778 cfu/mL, respectively. The trends in SCC, SPC, and PIC from January 2004 to April 2008 are shown in Figure 3.1.

Assessment of the Presence of Listeria monocytogenes in Parlor and Milking Machines

In the first sampling (May 2007), *L. monocytogenes* was isolated from 2 out of 10 samples from the milk house environment. These positive samples corresponded to floors in the parlor pit and storage area. One sample collected from the bulk tank outlet was positive for *L. monocytogenes*, as was 1 set of rubber liners (i.e., 4 liners in 1 cluster). In the second sample collection (January 2008), 5 individual rubber liners (from 4 milking units) were positive for *Listeria* spp. but none of the rubber liners were positive for *L. monocytogenes*. A summary of the milk meters culture results for the 4 sample collections is presented in Table 3.2.

Pulsed-field gel electrophoresis typing

Combined AscI and ApaI restriction analysis showed 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment (9 isolates), floors in the parlor and storage room (2 isolates), BTM (2 isolates), and in-line milk filters (2 isolates). Pulsed-field gel electrophoresis banding patterns using AscI for 12 of these

Figure 3.1. Bulk tank SCC, SPC, and preliminary incubation counts (PIC) over a period of 4.25 yr (February 2004–April 2008).

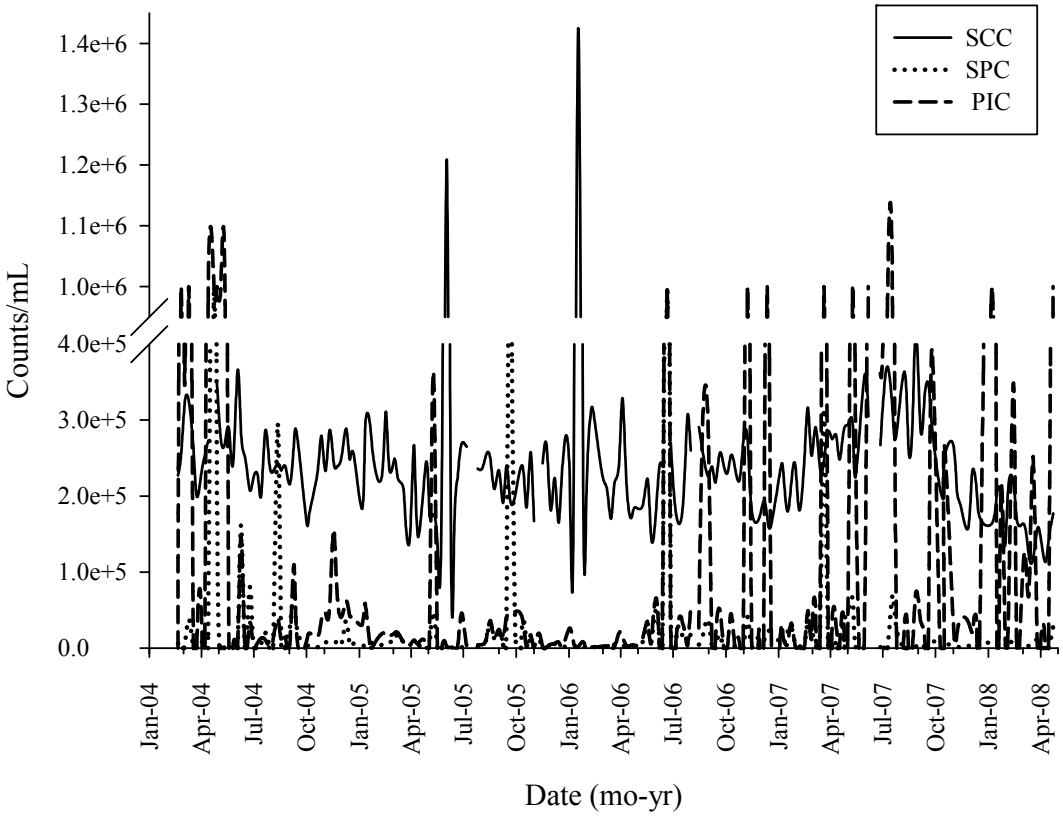

SCC
SPC
PIC
Counts/mL
1.4e+6
1.3e+6
1.2e+6
1.1e+6
1.0e+6
4.0e+5
3.0e+5
2.0e+5
1.0e+5
0.0
Jan-04
Apr-04
Jul-04
Oct-04
Jan-05
Apr-05
Jul-05
Oct-05
Jan-06
Apr-06
Jul-06
Oct-06
Jan-07
Apr-07
Jul-07
Oct-07
Jan-08
Apr-08
Date (mo-yr)

Table 3.2. Summary of milk meters **(MM)** culture results for *Listeria* spp. and *L. monocytogenes*

Description[1]	May 2007	January 2008	February 2008	March 2008
MM 1- Left	*Listeria* spp.[2]	*Listeria* spp.	-[3]	NC[4]
MM 2- Left	NC	*Listeria* spp.	-	NC
MM 3- Left	-	*L. monocytogenes*	*L. monocytogenes*	- +
MM 4- Left	NC	*L. monocytogenes*	-	NC
MM 5- Left	*L. monocytogenes*	*Listeria* spp.	-	NC
MM 6- Left	NC	*Listeria* spp.	-	NC
MM 7- Left	-	*Listeria* spp.	-	NC
MM 8- Left	NC	*L. monocytogenes*	*Listeria* spp.	- +
MM 1- Right	NC	*Listeria* spp.	-	NC
MM 2- Right	*L. monocytogenes*	*Listeria* spp.	-	NC
MM 3- Right	NC	*L. monocytogenes*	-	NC
MM 4- Right	-	*Listeria* spp.	-	NC
MM 5- Right	NC	*Listeria* spp.	-	NC
MM 6- Right	*Listeria* spp.	*Listeria* spp.	-	NC
MM 7- Right	NC	-	-	NC
MM 8- Right	-	-	-	NC

[1]Numbers from 1 to 8 indicate the number assigned to each of the milking units tested. Left and right indicate the position of the units in the parlor.

[2]Including *Listeria monocytogenes.*

[3]- = culture negative for *Listeria* spp.; + = *hly*-PCR positive for *Listeria monocytogenes*.

[4]NC = no samples were collected.

isolates are shown in Figure 3.2 and a dendrogram based on the combined AscI and ApaI digestion profiles for all isolates is shown in Figure 3.3.

Pulsed-field gel electrophoresis type T was observed in 2 *L. monocytogenes* isolates obtained from BTM and milk filter samples that were collected approximately 1 wk before the first sample collection (May 2007) from the milking parlor and milking equipment. The same PFGE type T was subsequently found in a BTM sample received in the laboratory 1 d before the sampling of the parlor, as well as in the bulk tank outlet and in one of the milk meters sampled on the first sample collection (milk meter 2-right). In addition, *L. monocytogenes* PFGE type T was isolated from 3 milk meters in the second sample collection (January 2008; milk meters 3-left, 4-left, and 3-right) and from a milk meter at the third sampling (February 2008; milk meter 3-left). The PFGE type U, closely related (approximately 96.5% similarity) to PFGE type T (Tenover et al., 1995), was isolated from a milk meter in January 2008 as well (milk meter 8-left). Closely related *L. monocytogenes* PFGE types D and E (92.5% similar to each other) were isolated from one of the milk meters (milk meter 5-left) and in a rubber liner analyzed on May 2007 (Figure 3.3). A PFGE type D was also isolated from a milk filter sample that was collected 1 wk after this sampling. Pulsed-field gel electrophoresis types X and Y were isolated from floor samples in the parlor and storage room during the first sample collection from parlor and milking equipment. The similarity between these PFGE types was only 76%, and types X and Y were remarkably different (approximately 57.5% similarity) from the other *L. monocytogenes* PFGE types observed in this study.

Scanning electron microscopy

Deep scratches in the inner surface of the milk meters were readily observed with no

Figure 3.2. *Listeria monocytogenes* pulsed-field gel electrophoresis types and subtypes among isolates obtained between May 2007 and February 2008 using restriction endonuclease AscI. The first and second rows on the bottom indicate the sample identification and source: bulk tank milk (BTM), bulk tank outlet (BTO), rubber liner (LN), milk meter (MM) with corresponding milking unit number and position in the parlor (R = right, L = left), floor in the parlor room (FPR), and floor in the storage room (FSR). Sampling date (month-day) and year are shown on the third and fourth (bottom) rows, respectively. Lanes 1, 8, and 15 contain the standard Salmonella enterica serotype Braenderup (SB). Pulsed-field gel electrophoresis profiles of *L. monocytogenes* isolates L1–027, L1–020, L1–018, L1–028, and L1–029 were described previously by Latorre et al. (2009).

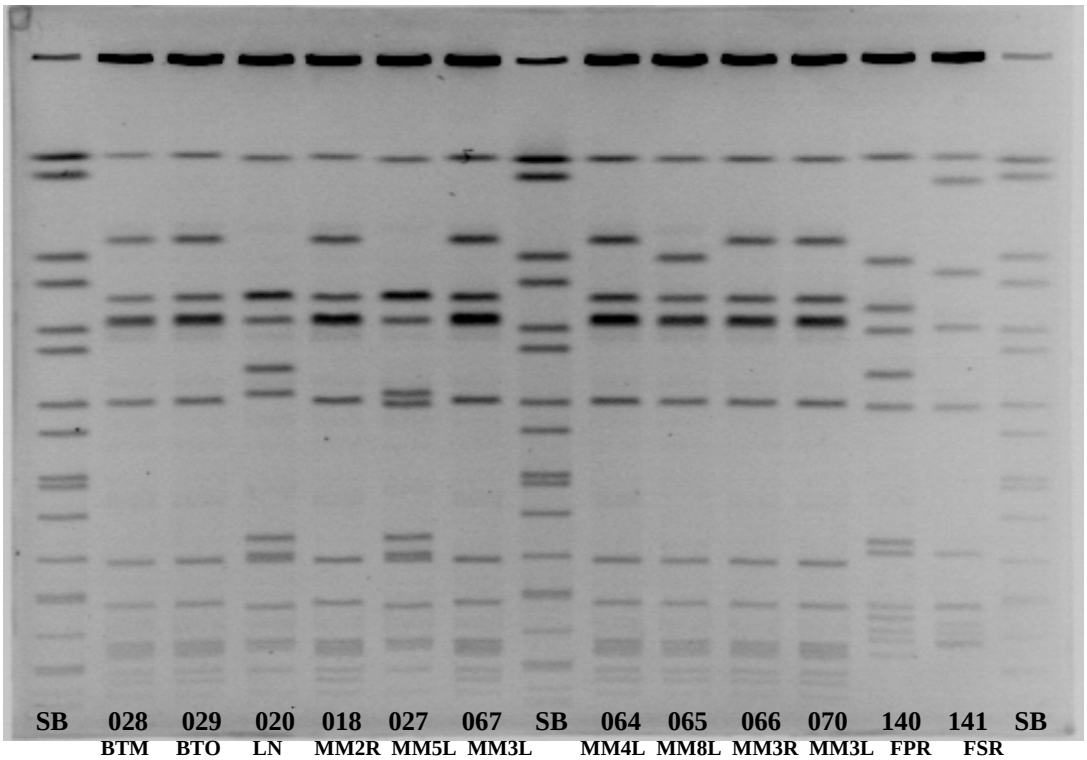
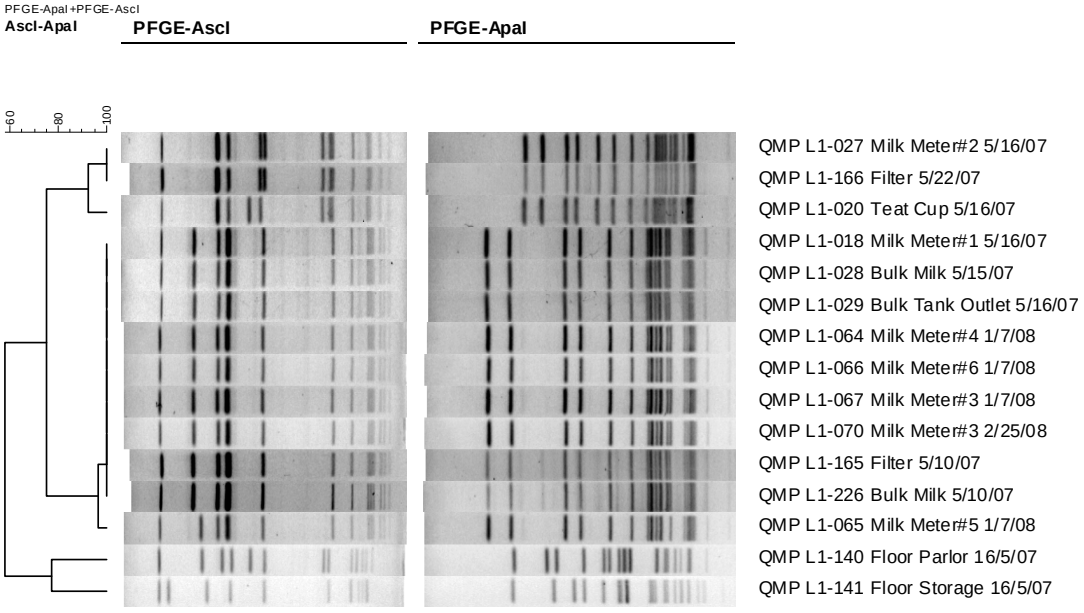

SB
028
029
020
018
027
067
SB
064
065
066
070
140
141
SB
BTM May-15 2007
BTO May-16 2007
LN May-16 2007
MM2R May-16 2007
MM5L May-16 2007
MM3L Jan-7 2008
MM4L Jan-7 2008
MM8L Jan-7 2008
MM3R Jan-7 2008
MM3L Feb-25 2008
FPR May-16 2007
FSR May-16 2007

Figure 3.3. Dendrogram of *Listeria monocytogenes* isolates obtained from bulk tank milk, milking equipment, and floors in the parlor and storage rooms. In the dendrogram, milk meter numbers 1, 2, 3, 4, 5, and 6 correspond to milk meters 2-right, 5-left, 3-left, 4-left, 8-left, and 3-right, respectively. The dendrogram is based on the combined analysis of the AscI and ApaI digestion profiles using a cutoff of 100%. Pulsed-field gel electrophoresis (PFGE) types assigned by automated cluster analysis (ACA) of the combined AscI and ApaI restriction digest profiles are shown at the right of the dendrogram. Pulsed-field gel electrophoresis profiles of *L. monocytogenes* isolates L1–027, L1–020, L1–018, L1–028, and L1–029 were described previously by Latorre et al. (2009). QMP = Quality Milk Production identification.


PFGE-ApaI +PFGE- AscI
AscI-ApaI
PFGE-AscI
PFGE-ApaI
60
80
100
QMP L1-027 Milk Meter#2 5/16/07
QMP L1-166 Filter 5/22/07
QMP L1-020 Teat Cup 5/16/07
QMP L1-018 Milk Meter#1 5/16/07
QMP L1-028 Bulk Milk 5/15/07
QMP L1-029 Bulk Tank Outlet 5/16/07
QMP L1-064 Milk Meter#4 1/7/08
QMP L1-066 Milk Meter#6 1/7/08
QMP L1-067 Milk Meter#3 1/7/08
QMP L1-070 Milk Meter#3 2/25/08
QMP L1-165 Filter 5/10/07
QMP L1-226 Bulk Milk 5/10/07
QMP L1-065 Milk Meter#5 1/7/08
QMP L1-140 Floor Parlor 16/5/07
QMP L1-141 Floor Storage 16/5/07

magnification when cutting the parts for SEM analysis. Scanning electron microscopy of a sample from the bottom cover of milk meter 3-left showed the presence of numerous bacteria, mainly associated with surface scratches (Figure 3.4). Bacteria were also observed on surface scratches on the bottom cover of milk meter 8-left, but in fewer number than on milk meter 3-left.

A bacterial biofilm was observed on the bottom cover of the 2 milk meters analyzed (Figure 3.5 and 3.6). Only a few bacterial cells were observed in the top cover of milk meters 3-left and 8-left. Scanning electron microscopy of a rubber liner sample showed the presence of cracks and of areas containing patches of a foreign material on the surface. No bacteria were observed in the microscopy fields of the sample that was analyzed.

PCR

The *hly* gene was detected in the BHI enrichments of the 2 sponge samples obtained from the bowl component of milk meters 3-left and 8-left. The 3 enrichments from milk meter 3-left were *hly* PCR-positive. For milk meter 8-left, 2 of 3 BHI enrichment replicates were *hly* positive.

Analysis of clean-in-place temperatures for milk pipelines and milk tank

The pipeline and milk tank washing cycle temperatures collected and stored by the MilkGuard equipment during an approximately 1.7-mo period (between September and October 2007) were evaluated. The recorded peak temperature of drainage water in the routine pipeline wash cycles never exceeded 53°C (approximately) during this 1.7-mo monitoring period. For milk tank washes, the recorded temperatures never went above 47°C (approximately) during the monitoring period.

Figure 3.4. Scanning electron microscopy image of scratches on the surface of the bottom cover of milk meter 3-left (scale: 1,000 nm). Arrows indicate the presence of bacteria associated with these scratches in the plastic material.

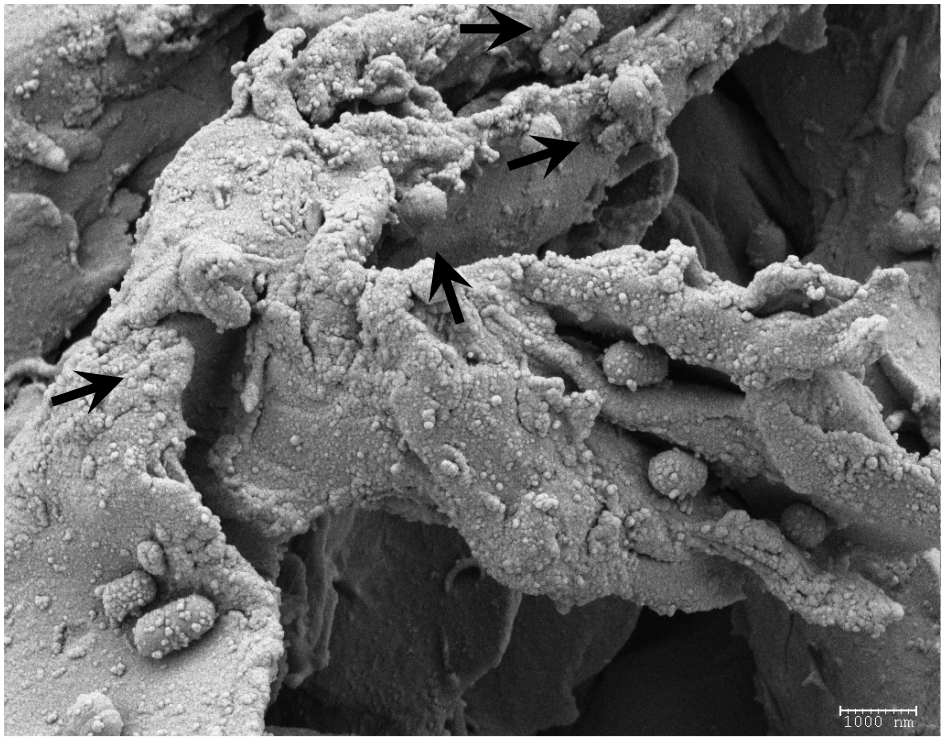

1000 nm

Figure 3.5. A) Scanning electron microscopy image of a biofilm on the surface of the bottom cover of milk meter 3-left (scale: 2,000 nm). Arrows show the presence of exopolymeric matrix. Panels B and C show 2 different areas of this biofilm that are magnified. B) Close-up of different types of bacteria in the biofilm (scale: 1,000 nm). C) Arrows show the presence of exopolymeric matrix anchoring the bacteria to the surface of the milk meter (scale: 1,000 nm).

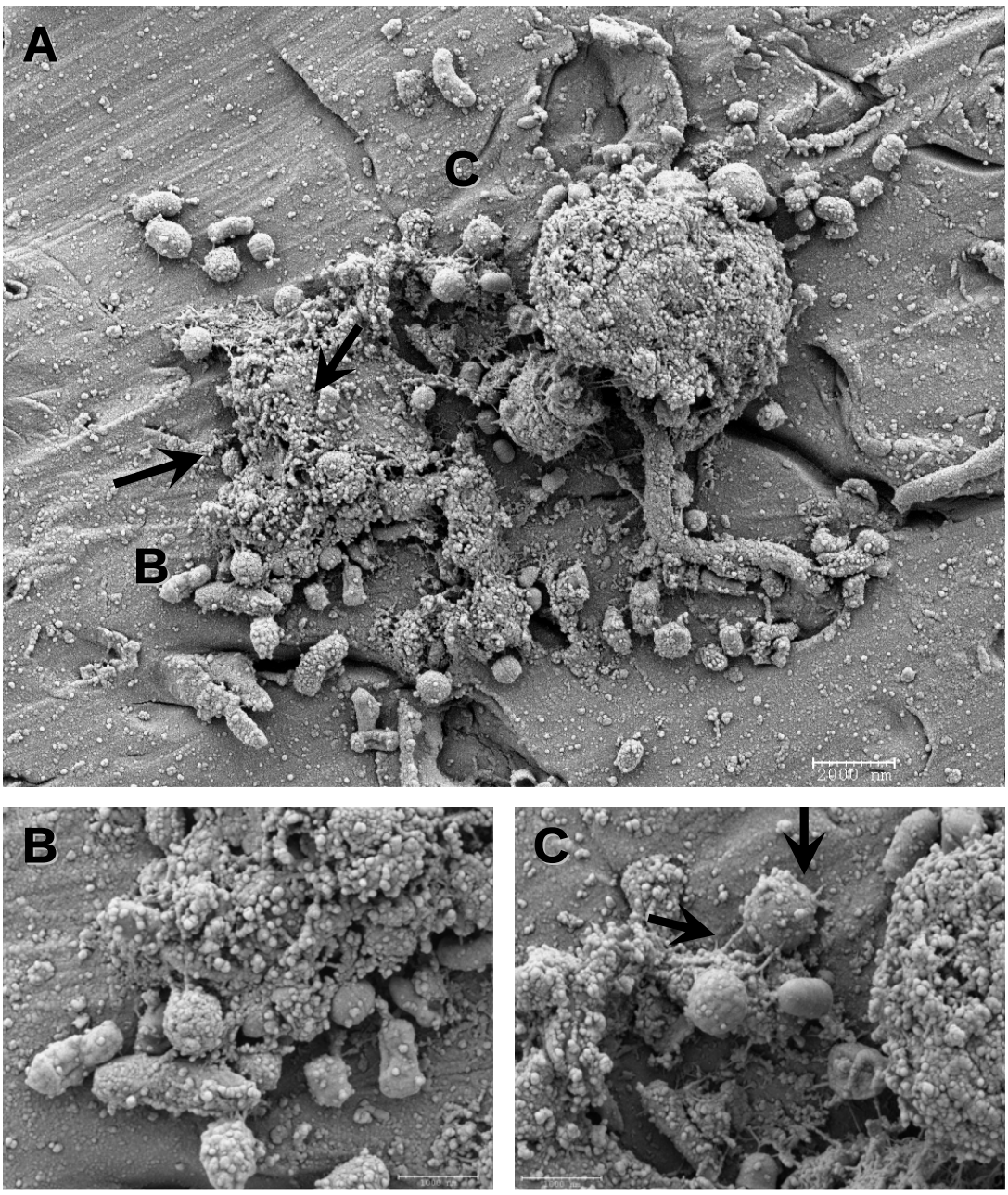
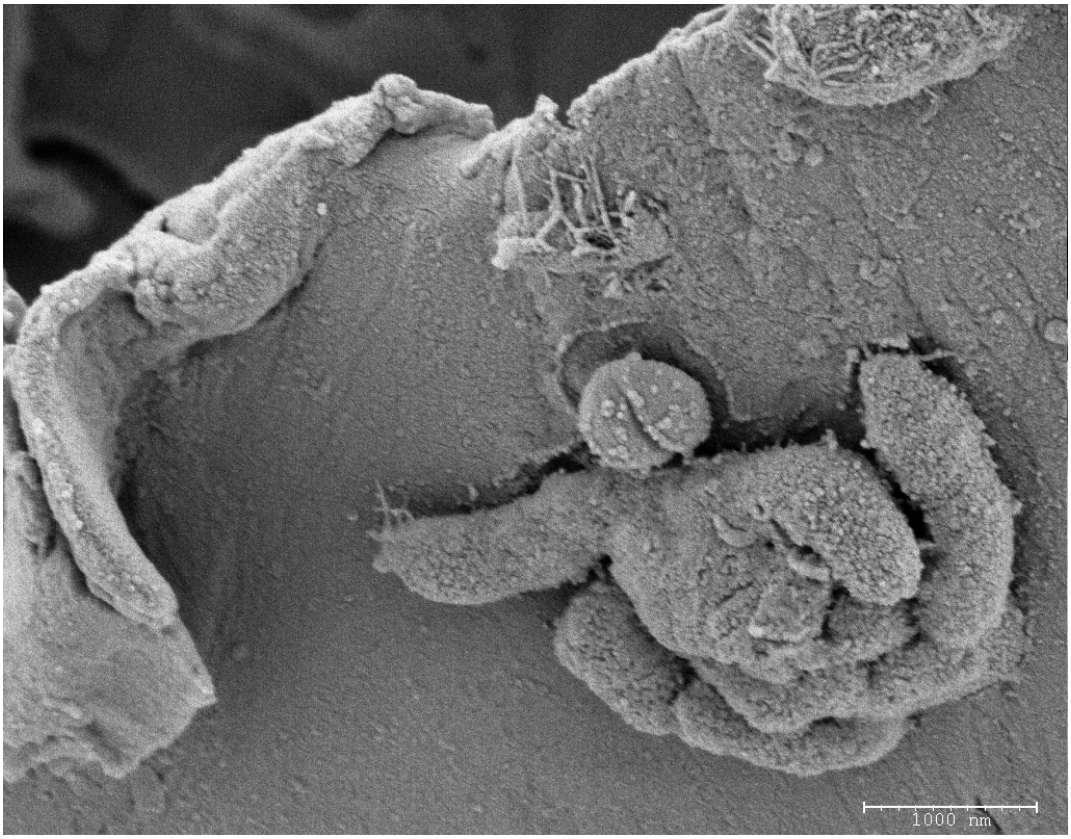

A
C
B
2000 nm
B
C

Figure 3.6. Scanning electron microscopy image of the bottom cover of milk meter 8-left showing a cluster of bacteria covered by exopolymeric matrix and attached to the surface of the milk meter.


1000 nm

DISCUSSION

Previously reported research on the attachment of bacteria to dairy equipment surfaces involved in vitro studies (Speers et al., 1984) or insertion of pieces of pipeline (Austin and Bergeron, 1995) or gaskets (Czechovski, 1990; Austin and Bergeron, 1995) into the milk line. To our knowledge, this is the first study that evaluated the presence of biofilms in milking equipment from samples removed directly from pieces of milking equipment that had been in use on an operating farm for almost 3 yr. In this study, despite the established CIP procedures, a bacterial biofilm as well as numerous bacteria were found attached to the surface of the milk meters as evidenced by the SEM micrographs. Bacteria were especially prominent in scratches on the inner surfaces of the plastic milk meters.

According to information provided by the producer, the milk meters were cleaned using an abrasive device (a wire brush) in 2006. Scratches on the surface of the milk meters could have facilitated the attachment and colonization of bacteria because roughness could limit the effectiveness of the CIP procedures. In a study of cleaning protocols on stainless steel surfaces, Wirtanen et al. (1995) demonstrated that smooth surfaces are easier to clean than rough surfaces. Additionally, the accumulation of milk residues on the surfaces of milking equipment may contribute to subsequent bacterial proliferation (Murphy and Boor, 2000).

The visualization of bacteria attached to the plastic surface by means of an apparent exopolymeric matrix (Carpentier and Cerf, 1993) indicated the presence of a biofilm in 2 milk meters. Rod-shaped bacteria were observed in SEM micrographs of the biofilm. The morphology and size were similar to *L. monocytogenes* on in vitro

biofilms (see Figure 1 in Chavant et al., 2004). Using the presented methods, however, it was not possible to definitively identify *L. monocytogenes* among the bacteria present in the observed biofilms.

Listeria monocytogenes was isolated from samples obtained from milk meters, a teat cup liner, and bulk tank outlet. The PFGE type T persisted in milking equipment over a 9-mo period, suggesting the presence of a persistent source of *L. monocytogenes*, consistent with our biofilm hypothesis. In addition, the large heterogeneity of PFGE types among *L. monocytogenes* isolates from fecal and environmental samples compared with the more limited heterogeneity in PFGE types in isolates from BTM, milk filter, milking equipment, and bulk tank outlet samples on this study farm (Latorre et al., 2009) also support the biofilm hypothesis.

In our study, although the presence of *L. monocytogenes* or bacterial biofilms could not be directly microscopically assessed on bulk tank surfaces, an *L. monocytogenes* isolate obtained from a bulk tank outlet sample showed the same PFGE type T that was found in milk meters and BTM.

The continuous sloughing of cells from a biofilm could explain the presence of 3 persistent *L. monocytogenes* PFGE types in samples of BTM and in-line milk filters collected over a period of 22 mo (Latorre et al., 2009).

During 1.7 mo of monitoring, the bulk tank washing temperatures were below the temperatures recommended by the manufacturer (73°C). Inappropriate temperatures during the cleaning cycle of the bulk tank may make the removal of milk residues difficult (National Mastitis Council, 2004). Accumulation of organic material debris in

the milking machines may create appropriate conditions for bacterial growth (Murphy and Boor, 2000) and could also help in the attachment of bacteria by creating a conditioning film (Zottola and Sasahara, 1994). High SPC and PIC counts in BTM on this farm could be explained by deficiencies in washing of the milking equipment (Murphy and Boor, 2000; Jayarao et al., 2004). This problem may have been further compounded by the presence of scratches on the surface of milk meters.

The presence of biofilms in dairy equipment may be a relevant finding for many dairy farms because CIP temperatures that are lower than the temperatures recommended by the equipment manufacturers are frequently observed (Elmoslemany et al., 2009). In addition, the wear of materials in the milking equipment that causes the appearance of cracks and crevices (Czechovski, 1990), or bacterial contamination during milking, could contribute to the presence of bacteria in milk (Murphy and Boor, 2000). And if these organisms present in milk find favorable conditions, they could eventually form a biofilm in the milking system. In the case of *L. monocytogenes*, the presence of water, nutrients, and cold temperatures found in bulk tanks provide favorable conditions for the organism not only to survive but to replicate. *Listeria monocytogenes* cells could then attach to the stainless steel (Norwood and Gilmour, 1999) and establish as a biofilm, causing subsequent continuous contamination of milk.

Raw milk from this particular farm is hauled to a milk processing plant for pasteurization and so does not present a risk to consumers. Nevertheless, raw milk contaminated with *L. monocytogenes* could pose a human health hazard, especially among consumers of raw milk. *Listeria monocytogenes* has been found several times during the past few years in milk from farms that sell certified raw milk in New York

State (http://www.agmkt.state.ny.us/AD/alertList.asp). Additionally, studies have shown that consumption of raw milk is not uncommon among dairy farm personnel (Jayarao et al., 2006), which could put them at risk for listeriosis if the raw milk at the farm is contaminated with *L. monocytogenes*.

Listeria present in raw milk could also pose a risk of contamination for a milk processing plant (Waak et al., 2002). The presence of *L. monocytogenes* in a processing plant could lead to postprocessing contamination, which also draws attention to the need to reduce the level of contamination of milk that will eventually be transported to a milk processing plant.

Prevention of biofilm establishment in milking equipment is a crucial step in fulfilling the requirement of safe, high-quality milk. Hygiene in the milking routine, correct implementation of milking equipment cleaning protocols (following manufacturer recommendations on duration, chemicals, and temperature), and replacing plastic and other materials in milking equipment that are susceptible to wear on a regular basis would help to prevent the establishment of biofilms and subsequent contamination of the bulk milk.

ACKNOWLEDGMENTS

Financial support for this work was provided by the USDA-ARS (Agreement No. 58-1265-3-155, 58-1265-3-156, 58-1265-3-158, 58-1265-4-020, and 58-1265-8-064) for the Regional Dairy Quality Management Alliance.

We thank the participating producer who kindly allowed this research to take place on his dairy farm. We also thank Ray Coolbaugh (Laboratory Operations, AHDC, Cornell University, Ithaca, NY) and Mandayam Parthasarathy (CIMC, Cornell University, Ithaca, NY) for their valuable insights and contributions to this study. We gratefully acknowledge all the help obtained from the Quality Milk Production Services (Ithaca, Cobleskill, Geneseo, and Canton laboratories, NY) and USDA-ARS (Beltsville, MD) teams during the course of this research.

Mention of a trade name, vendor, proprietary product, or specific equipment is not a guarantee or a warranty by the USDA and does not imply an approval to the exclusion of other products or vendors that also may be suitable.

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CHAPTER 4

MOLECULAR ECOLOGY OF *LISTERIA MONOCYTOGENES*: PREDOMINANT AND PERSISTENT STRAINS SHOWED INCREASED *IN-VITRO* ADHERENCE AND ON-FARM PERSISTENCE IN THE MILKING SYSTEM*

ABSTRACT

Dairy farms are a reservoir for *Listeria monocytogenes* and control of this pathogen at the farm level is critical for reducing human exposure. The objectives of this research were to study the diversity of *L. monocytogenes* strains on a single dairy farm, assess strain dynamics within the farm, identify potential sources of *L. monocytogenes* in bulk tank milk and milk filters, and assess adherence ability of representative isolates. A total of 248 *L. monocytogenes* isolates were analyzed by pulsed-field gel electrophoresis (**PFGE**). Combined AscI and ApaI restriction analysis yielded 40 PFGE types. The most predominant PFGE types were T (28.6 %), D (22.6 %), and F (14.9 %). A large heterogeneity of PFGE types among isolates from fecal (Simpson's Index of Diversity **SID**= 0.96) and environmental samples (SID= 0.96) was observed.

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A higher homogeneity of PFGE types was observed among isolates from milk filters (SID= 0.71) and bulk tank milk (SID= 0.65).

Six of 17 *L. monocytogenes* isolates (35.3%) were classified in an *in-vitro* assay as having "low adherence ability", nine (52.9%) as having "medium adherence ability", and two (11.8%) as having "high adherence ability". The *L. monocytogenes* types that were predominant and persistent showed significantly better adherence than types that were only sporadic, predominant, or persistent (p=0.0006). Our results suggest that the milking system was exposed to several *L. monocytogenes* types from different sources. Only 3 PFGE types, however, were successful in persisting within the milking system, suggesting that some strains are more suitable to that particular ecological environment than others.

INTRODUCTION

Foods contaminated with *Listeria monocytogenes* can be considered a threat to public health. Even though healthy adults may only develop a mild illness when *L. monocytogenes*-contaminated food is consumed (Riedo et al., 1994; Sim et al., 2002), listeriosis in more susceptible people, such as pregnant women, elderly, and immunocompromised individuals may have serious consequences (Fleming et al., 1985; Linnan et al., 1988; Centers for Disease Control and Prevention **CDC**, 2008). Several outbreaks of listeriosis have been linked to dairy products. These include pasteurized milk (Fleming et al., 1985; Dalton et al., 1997; CDC, 2008), butter (Lyytikäinen et al., 2000), on-farm manufactured fresh cheese (Carrique-Mas et al., 2003), and Mexican-style cheese (Linnan et al., 1988, MacDonald et al., 2005).

Unprocessed, raw milk from dairy farms is the starting point in the production-consumption chain; therefore prevention of bulk tank milk contamination with *L. monocytogenes* is crucial. Although pasteurization destroys pathogenic bacteria and makes milk safe for consumption, the presence of *L. monocytogenes* in bulk tank milk could represent a risk of contamination for milk processing plants. For example, in a study conducted by Waak et al. (2002), indistinguishable PFGE types were observed in *L. monocytogenes* isolates obtained from bulk tank milk and from the raw milk silos on a dairy plant. Furthermore, the presence of *L. monocytogenes* in dairy processing plants may represent a risk of post-pasteurization contamination of milk. It has been reported that persistent *L. monocytogenes* strains in food processing environments have been responsible for cross-contamination of finished products, such as ice cream and smoked fish (Miettinen et al., 1999; Vogel et al., 2001). However, one of the routes from which *L. monocytogenes* may initially enter food processing plants (with the subsequent colonization of the environment) is through raw materials (Vogel et al., 2001). Hence, control of *L. monocytogenes* at the farm level is crucial to prevent the entrance of the pathogen into the food chain through raw animal products, including milk.

Listeria monocytogenes is nearly ubiquitous in dairy farms (Nightingale et al., 2004) and its presence in milk and milk filters has been frequently reported (Van Kessel et al., 2004; Latorre et al., 2009; Mohammed et al., 2009). *Listeria monocytogenes* has been isolated from many locations within dairy farms including feces (Nightingale et al., 2004; Nightingale et al., 2005; Lyautey et al., 2007), animal drinking water (Nightingale et al., 2005; Latorre et al., 2009; Mohammed et al., 2009), feeds or feed components (Nightingale et al., 2005; Mohammed et al., 2009), and milking equipment (Latorre et al., 2009; Latorre et al., 2010). The potential link between

animal production systems and human listeriosis cases exists, as has been suggested by previous studies that have described the presence of *L. monocytogenes* ribotypes (Nightingale et al., 2004) or PFGE types (Borucki et al., 2004; Fugett et al., 2007) from farms that matched those observed in isolates from human listeriosis cases.

This study was a continuation of a series of studies conducted on a New York State dairy farm with a previously identified high prevalence of *L. monocytogenes* in in-line milk filters and bulk tank milk samples (Latorre et al., 2009; Latorre et al., 2010). In addition, the presence of a *L. monocytogenes*-containing biofilm on milking equipment surfaces has been suggested as a likely reservoir of *L. monocytogenes* and a potential source of bulk tank milk contamination (Latorre et al., 2009; Latorre et al., 2010) for this farm.

In these previous studies a useful but limited understanding of the molecular ecology of *L. monocytogenes* was gained. Particularly, little information was available on the bacterial sources of bulk tank milk contamination, sources of *L. monocytogenes* infection for cows, and potential reservoirs of the pathogen on the farm. In addition, although the presence of a *L. monocytogenes*-containing biofilm in milking equipment was previously suggested, no link was made to the in-vitro adherence ability of isolated strains. A potential difference in adherence ability between predominant, persistent, and sporadic strains isolated from the farm would further support the biofilm-in-milking-system hypothesis. The objectives of this research were therefore to study the diversity among *L. monocytogenes* isolates, assess strain dynamics within the farm over time, identify potential sources of *L. monocytogenes* strains in bulk tank milk and in-line milk filters, as well as assessing the adherence ability of representative *L. monocytogenes* strains *in-vitro*. To accomplish these objectives, we

expanded PFGE typing and analysis of *L. monocytogenes* isolates as compared with our previously published work (181 new isolates, plus 67 in our previous studies) (Latorre et al., 2009; Latorre et al., 2010). Moreover, in our previous studies, a major emphasis was given to *L. monocytogenes* strain dynamics within the milking system. In the present study *L. monocytogenes* isolates from all fecal and environmental-positive samples, as well as isolates from milking parlor, bulk tank milk, and milk filters collected during a 4-year period were analyzed by PFGE and multiplex PCR serotyping in order to investigate strain dynamics in space and time.

MATERIALS AND METHODS

Study Farm

The study was conducted on a New York State dairy farm. Detailed information about characteristics of this dairy farm has been previously reported by Latorre et al. (2009) and Pradhan et al. (2009). Briefly, the farm has approximately 330 lactating cows. The cows are milked three times per day and the milk is transported to a milk processing plant for pasteurization before distribution to consumers (Latorre et al., 2009; Latorre et al., 2010).

Sample Collection

Environmental and fecal samples were collected between February 2004 and April 2008. Environmental samples were collected every three months and fecal samples were collected on a six-month basis using the sample regime and protocol described by Latorre et al. (2009). In-line milk filter samples were collected weekly between April 2005 and the first week of June 2008. Three additional milk filter samples, 2 in

October 2004 and 1 in January 2005, were collected. Bulk tank milk samples were collected every week starting in February 2004 until March 2008. In-line milk filters and bulk tank milk samples were collected and transported to the laboratory as described in Latorre et al. (2009). Three samplings of the parlor environment and milking equipment were carried out in May 2007, and in January and February 2008 as previously described (Latorre et al., 2010).

Bacterial Analysis

Bacterial analysis for detection of *L. monocytogenes* in environmental, fecal, in-line milk filters, bulk tank milk, and parlor/milking equipment samples was conducted as previously described (Van Kessel et al., 2004; Latorre et al., 2009; Latorre et al., 2010).

Pulsed-field Gel Electrophoresis Typing

Pulsed-field gel electrophoresis was performed for one *L. monocytogenes* isolate from each positive sample as described by the CDC protocol (http://www.cdc.gov/pulsenet/protocols/pulsenet_listeria_protocol%20.pdf) with modifications (Latorre et al., 2009). Restriction digestion was carried out using AscI restriction endonuclease as previously described (Latorre et al., 2009). Digestion using ApaI was completed using ApaI restriction enzyme from New England Biolabs (New England BioLabs, Inc. Ipswich, MA) as previously described (Latorre et al., 2009) or ApaI from Roche Laboratories (Roche Diagnostics, Indianapolis, IN) as described in the standardized CDC protocol. Analysis of DNA band patterns was conducted by two independent observers using visual inspection according to the criteria described by Tenover et al (1995). Dendrograms were generated with the Bionumerics 3.5 software (Applied Maths,

Saint-Matins-Latem, Belgium) using a similarity score value of 100% (Latorre et al., 2009).

Pulsed-field gel electrophoresis types that were repeatedly found in any sample type (bulk tank milk, in-line milk filters, fecal, environmental, or milking equipment samples) were defined as "persistent" PFGE types (if found in at least three different samplings). Closely related PFGE types (i.e. 2 or 3 bands of difference between them, as described by Tenover et al., 1995) that were found in at least three different samplings were classified as "persistent groups". The PFGE types that were the most frequently identified (more than 5 isolates during the study period) were defined as "predominant" types. Pulsed-field gel electrophoresis types that were unique or only rarely found on the farm were classified as "sporadic" PFGE types.

Multiplex PCR Serotyping

One *L. monocytogenes* isolate from each PFGE type (n=40) was serotyped as described by Doumith et al. (2004). This PCR technique grouped *L. monocytogenes* serovars according to a serotype complex, allowing distinction and separation of serovars 1/2a, 1/2b, 1/2c, and 4b (2004).

Microtiter Plate Biofilm Assay

The adherence ability of 17 representative *L. monocytogenes* strains was determined using a microtiter plate assay (Djorjevic et al., 2002; Borucki et al., 2003; Merritt et al., 2005; Harvey et al., 2007). One *L. monocytogenes* isolate from each of the three most predominant PFGE types (T, D, and F) was selected for the assay. Remaining strains were selected based on their classification as predominant, persistent, non predominant-persistent, or sporadic, with the goal of including diverse, but

representative PFGE types (Table 4.1). The microtiter plate biofilm assay was conducted as described by Djorjevic et al. (2002) with modifications. Briefly, a loopful (1 µl inoculating loop) of isolated colonies was grown in 10 ml Trypticase Soy Broth (TSB. BD, Sparks, MD) at 32°C for approximately 18 h. Cultures were removed from incubation and 100 µl was transferred to 10 ml TSB (Harvey et al., 2007) and vortexed. After vortexing, 100 µl of each diluted culture was pipeted into each of 8 wells of a 96-well Polyvinyl Chloride (PVC) flat-bottom plate (BD Falcon, Franklin Lakes, NJ). Plates were covered with a PVC lid (BD Falcon) and incubated at 32°C for 48 h. The cultures were removed from the incubator and the plate wells were washed three times with 150 µl of sterile distilled water (Borucki et al., 2003; Harvey et al., 2007), air dried for 45 min, and stained with 150 µl of 1% crystal violet for 45 min. After staining, wells were washed three times using 150 µl of sterile distilled water (Borucki et al., 2003; Harvey et al., 2007). The microtiter plates were inverted and tapped on a paper towel to remove excess liquid, and allowed to dry. Dried plates were stored at room temperature (Merritt et al., 2005) until the quantitative assay was performed. High biofilm-forming *L. monocytogenes* FSL-J2-035 and low biofilm-forming *L. monocytogenes* FSL-N1-250 (Djorjevic et al., 2002) were used as control strains on each plate, as well as a TSB control (blank) (8 wells for each of the controls). All plates were prepared in duplicate and the overall experiment was repeated three times.

To quantify the remaining crystal violet as an indicator of adherence of isolates, 150 µl of 95% ethanol were added to each of the wells to solubilize the stained biofilm. Plates were covered with a PVC lid and incubated for 1 h at room temperature. After incubation, the crystal violet solutions were mixed with a pipet (~10 times/well). Immediately after mixing, the optical density of the solutions was read directly from

Table 4.1. Representative isolates, based on PFGE type, that were characterized for adherence ability.

		PFGE Type				
Isolate ID [a]	**Serotype**	**AscI**	**ApaI**	**CE**	**Source**	**Characteristics**
QMP L1-070 [b]	1/2a	5	5	T	Milk Meter	Pred/Perst [g]
QMP L1-021	1/2a	7	7	D	BTM	Pred/Perst
QMP L1-030	1/2a	3	3	F	MF	Pred/Perst
QMP L1-065	1/2a	5.1	5.1	U	Milk Meter	Pred/Perst
QMP L1-002	4b	4	4	J	Fecal	Pred/Perst
QMP L1-023 [c]	4b	2.1	2.2	O	Fecal	Non Pred/Perst [h]
QMP L1-001	4b	1	1	I	Flies	Non Pred/Perst
QMP L1-003	1/2b	6	6	A	Fecal	Non Pred/Perst
QMP L1-122	1/2b	6.1	14	Q	Fecal	Pred/Non Perst [i]
QMP L1-102	1/2a	11	11	W	Flies	Sporadic [j]
QMP L1-080	1/2a	9	9	B	Water	Sporadic
QMP L1-153	4b	22	20	AM	MF	Sporadic
QMP L1-073	1/2b	8	8	P	Fecal	Sporadic
QMP L1-103	1/2a	12	12	V	Water	Sporadic
QMP L1-109	1/2a	13	13	C	Water	Sporadic
QMP L1-251	N/T [e]	23	21	AN	MF	Sporadic (New) [k]
QMP L1-241	N/T	19	18	AJ	Fecal	Sporadic (New)
FSL N1-250 [d]	N/A [f]	N/A	N/A	N/A	Fish Processing Plant	Low Biofilm Former
FSL J2-035	1/2b	N/A	N/A	N/A	Animal (Goat)	High Biofilm Former

[a] Isolate identification number, serotype complex, PFGE type (visual inspection of AscI and ApaI restriction digest profiles according to the Tenover et al. (43) criteria, and combined AscI and ApaI restriction analysis [CE]), source (bulk tank milk [BTM], in-line milk filters [MF], or other sources) and characteristics of representative *L. monocytogenes* isolates used in microtiter plate biofilm assay.

[b] Quality Milk Production identification number (QMP ID).

[c] Isolate QMP L1-023 classified as "persistent" because it belongs to a "persistent group".

[d] Food Science Laboratory identification number (FSL ID). *Listeria monocytogenes* FSL N1-250 and FSL J2-035 were provided by Dr. M. Wiedmann, which were used as "low biofilm former" and "high biofilm former" (Djorjevic et al., 2002) controls, respectively.

[e] N/T= Non-serotypeable

[f] N/A= Not available

[g] Pred/Perst = Predominant and persistent *L. monocytogenes* strains

[h] Non Pred/Perst= Non-predominant and persistent *L. monocytogenes* strains

[i] Pred/Non Perst=Predominant and non-persistent *L. monocytogenes* strains

[j] Sporadic= Sporadic *L. monocytogenes* strains

[k] Sporadic (New)= New sporadic *L. monocytogenes* strains found in the last sampling on the study farm. Future recurrence is unknown.

the flat-bottom PVC plates at 595 nm using a BioTek Powerwave XS microplate spectrophotometer (BioTek, Winosski, VT) equipped with the software KC Junior Win (BioTek). The average OD_{595} of the ethanol extract was calculated for each of the *L. monocytogenes* strains and controls. For each plate, the average of the blank control was subtracted from the average of each *L. monocytogenes* strain. The test strain/positive control strain ratio (S/P ratio) was calculated by dividing the OD_{595} average of each of the *L. monocytogenes* strains by the average of the high biofilm-former control (FSL- J2-035) in every plate.

Listeria monocytogenes strains were classified as having "low adherence ability", "medium adherence ability", or "high adherence ability" if the average of S/P ratio of the three experiments were between 0 and 0.28 (average of low biofilm former control FSL- N1-250), 0.28 and 0.56 (twice the average of low biofilm former control), or >0.56, respectively.

Detailed information about the *L. monocytogenes* strains used for the microtiter plate biofilm assay is available at Pathogen Tracker (www.pathogentracker.net). General and source information about all *L. monocytogenes* isolates in this study are also available at Pathogen Tracker.

Data Analysis

To assess the relationship between persistence and adherence ability among *L. monocytogenes* strains used in the microtiter plate assay, the natural logarithm of the S/P ratio was used in a linear regression model. Predictor variables included strain characteristics that were previously classified as either persistent, predominant or sporadic. The interactions between strain classification, experimental replicate and

experiment number were also included as fixed effects. Statistical significance (α) was defined at 0.05. Diversity of *L. monocytogenes* PFGE types on the farm was calculated by using the Simpson's Index of Diversity (SID) as described by Hunter and Gaston (1988).

RESULTS

A total of 249 *L. monocytogenes* isolates obtained from fecal, environmental, in-line milk filters, bulk tank milk, and milking equipment samples were analyzed by PFGE. One *L. monocytogenes* isolate was typeable by AscI restriction endonuclease, but non-typeable by ApaI. This isolate was not included in the data analysis. After digestion with AscI restriction endonuclease, a total of 23 PFGE types and 14 subtypes (Tenover et al., 1995) were observed among 248 *L. monocytogenes* isolates. Digestion using ApaI yielded 22 PFGE types and 11 subtypes (Tenover et al., 1995). Combined restriction analysis showed 40 PFGE types (Figure 4.1).

The presence of predominant, persistent, and sporadic PFGE types was observed among *L. monocytogenes* isolates obtained from different farm sources. The most predominant PFGE types were PFGE type T (28.6 %), PFGE type D (22.6 %), and PFGE type F (14.9 %). Persistent PFGE types were A, I, J, S, T, U, F, and D. These PFGE types were repeatedly found in samples collected from different farm sources during the study period (Figure 4.1). Persistent groups were observed as well and are illustrated in Figure 4.1. The sources of *L. monocytogenes* isolates that had persistent PFGE types and the time period in which they were found are shown in Figure 4.2.

Figure 4.1. Dendrogram based on the combined analysis of AscI and ApaI digestion profiles of 40 representative *L. monocytogenes* isolates. The dendrogram shows all PFGE types observed on the study farm (visual inspection and combined enzyme analysis [CE]), the number of *L. monocytogenes* isolates (n) with a particular PFGE type and type of sample from which the isolates were obtained, time frame, and serotype complex (N/T=non typeable).

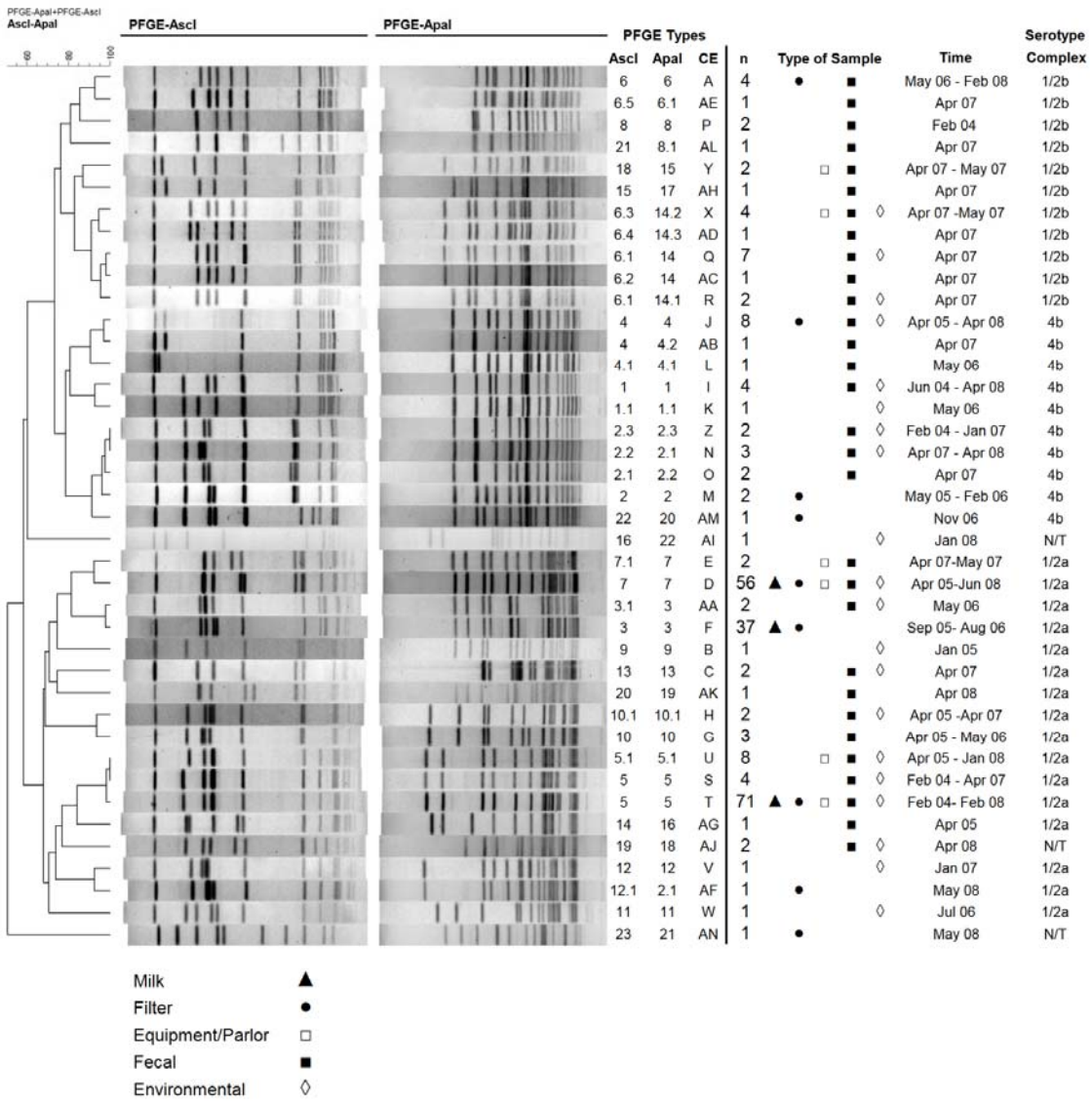
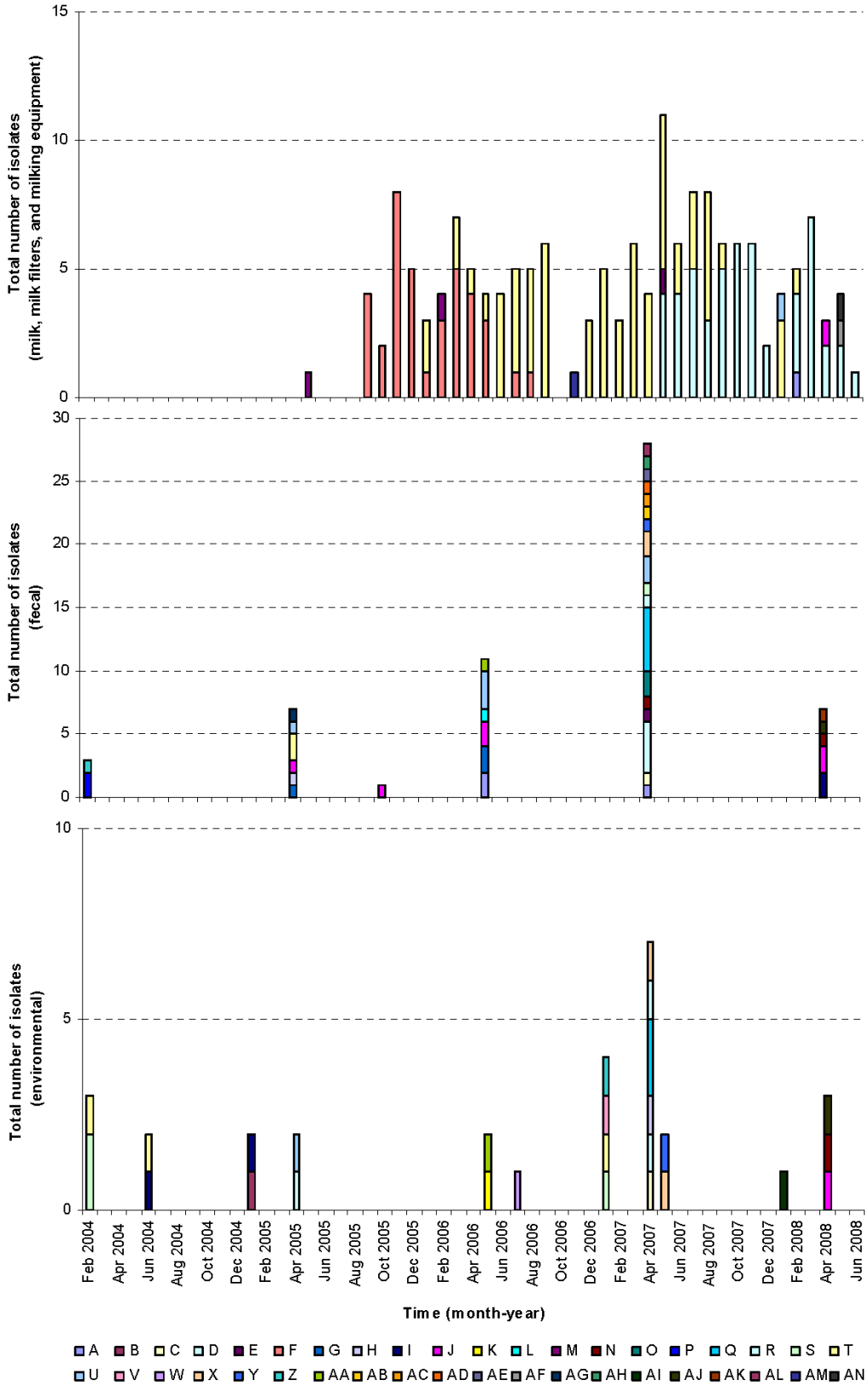

PFGE-ApaI+PFGE-AscI
AscI-ApaI
PFGE-AscI
PFGE-ApaI
PFGE Types
AscI ApaI CE n Type of Sample Time Serotype Complex
6 6 A 4 ● ■ May 06 - Feb 08 1/2b
6.5 6.1 AE 1 ■ Apr 07 1/2b
8 8 P 2 ■ Feb 04 1/2b
21 8.1 AL 1 ■ Apr 07 1/2b
18 15 Y 2 □ ■ Apr 07 - May 07 1/2b
15 17 AH 1 ■ Apr 07 1/2b
6.3 14.2 X 4 □ ■ ◊ Apr 07 -May 07 1/2b
6.4 14.3 AD 1 ■ Apr 07 1/2b
6.1 14 Q 7 ■ ◊ Apr 07 1/2b
6.2 14 AC 1 ■ Apr 07 1/2b
6.1 14.1 R 2 ■ ◊ Apr 07 1/2b
4 4 J 8 ● ■ ◊ Apr 05 - Apr 08 4b
4 4.2 AB 1 ■ Apr 07 4b
4.1 4.1 L 1 ■ May 06 4b
1 1 I 4 ■ ◊ Jun 04 - Apr 08 4b
1.1 1.1 K 1 ◊ May 06 4b
2.3 2.3 Z 2 ■ ◊ Feb 04 - Jan 07 4b
2.2 2.1 N 3 ■ ◊ Apr 07 - Apr 08 4b
2.1 2.2 O 2 ■ Apr 07 4b
2 2 M 2 ● May 05 - Feb 06 4b
22 20 AM 1 ● Nov 06 4b
16 22 AI 1 ◊ Jan 08 N/T
7.1 7 E 2 □ ■ Apr 07-May 07 1/2a
7 7 D 56 ▲ ● □ ■ ◊ Apr 05-Jun 08 1/2a
3.1 3 AA 2 ■ ◊ May 06 1/2a
3 3 F 37 ▲ ● Sep 05- Aug 06 1/2a
9 9 B 1 ◊ Jan 05 1/2a
13 13 C 2 ■ ◊ Apr 07 1/2a
20 19 AK 1 ■ Apr 08 1/2a
10.1 10.1 H 2 ■ ◊ Apr 05 -Apr 07 1/2a
10 10 G 3 ■ Apr 05 - May 06 1/2a
5.1 5.1 U 8 □ ■ ◊ Apr 05 - Jan 08 1/2a
5 5 S 4 ■ ◊ Feb 04 - Apr 07 1/2a
5 5 T 71 ▲ ● □ ■ ◊ Feb 04- Feb 08 1/2a
14 16 AG 1 ■ Apr 05 1/2a
19 18 AJ 2 ■ ◊ Apr 08 N/T
12 12 V 1 ◊ Jan 07 1/2a
12.1 2.1 AF 1 ● May 08 1/2a
11 11 W 1 ◊ Jul 06 1/2a
23 21 AN 1 ● May 08 N/T
Milk ▲
Filter ●
Equipment/Parlor □
Fecal ■
Environmental ◊

Figure 4.2. Number and PFGE types of *L. monocytogenes* isolates obtained from bulk tank milk, in-line milk filters, milking equipment, fecal, and environmental (including parlor) samples during the study period (February 2004-June 2008).


Total number of isolates (milk, milk filters, and milking equipment)
Total number of isolates (fecal)
Total number of isolates (environmental)
Time (month-year)
Feb 2004
Apr 2004
Jun 2004
Aug 2004
Oct 2004
Dec 2004
Feb 2005
Apr 2005
Jun 2005
Aug 2005
Oct 2005
Dec 2005
Feb 2006
Apr 2006
Jun 2006
Aug 2006
Oct 2006
Dec 2006
Feb 2007
Apr 2007
Jun 2007
Aug 2007
Oct 2007
Dec 2007
Feb 2008
Apr 2008
Jun 2008
A B C D E F G H I J K L M N O P Q R S T
U V W X Y Z AA AB AC AD AE AF AG AH AI AJ AK AL AM AN

Fecal samples

Many sporadic PFGE types were also observed among the *L. monocytogenes* isolates from several sample types as illustrated in Figure 4.1 and Figure 4.2.

A total of 2,934 fecal samples were collected between February 2004 and April 2008. Of these, 935 (31.8 %) were cultured for *L. monocytogenes*. Fifty seven (6 %) of the 935 cultured fecal samples were positive for *L. monocytogenes*. One isolate from each *L. monocytogenes*-positive fecal sample (n=57), was analyzed by PFGE. Combined AscI and ApaI restriction analysis yielded a total of 30 PFGE types for this category of sample (Figure 4.1). A high SID (SID=0.96) indicated that there was a large heterogeneity or diversity among *L. monocytogenes* isolates obtained from fecal samples (Figure 4.2).

Environmental samples

A total of 395 environmental samples were collected between February 2004 and April 2008. *Listeria monocytogenes* was isolated from 28 (7%) of these samples. Combined restriction enzymes AscI and ApaI analysis yielded a total of 20 PFGE types in 28 *L. monocytogenes* isolates (one from each of the *L. monocytogenes*-positive environmental samples) (Figure 4.1). One environmental isolate was typeable by AscI but non-typeable by ApaI (not counted among the 20 PFGE types from environmental samples, or the overall typing results). As with the fecal isolates, a large heterogeneity of PFGE types was observed among *L. monocytogenes* isolates obtained from environmental samples (SID=0.96) (Figure 4.2). No particular PFGE types were predominant in a specific sample site and a high diversity of PFGE types was found at each sampling time.

Milking equipment and parlor

A total of 11 *L. monocytogenes*-positive samples (9 from milking equipment and 2 from parlor) were obtained in three sample collections conducted between May 2007 and February 2008. Combined AscI and ApaI restriction enzyme analysis showed 6 PFGE types (Figure 4.1). A more limited heterogeneity than in fecal and environmental isolates was observed among isolates obtained from milking equipment and parlor (SID=0.73). Pulsed-field gel electrophoresis type T was the most predominant type (66.6%) observed in *L. monocytogenes*-positive milking equipment samples. The PFGE type T was observed in all 3 samplings of milk meters carried out within a 9 month period. Two isolates from each of two floor samples (parlor and storage room) had PFGE types X and Y, which were unrelated to the PFGE types isolated from the milking equipment.

In-line milk filters

One hundred and fifty in-line milk filter samples were collected weekly between April 2005 and the first week of June 2008. Three additional filters were collected, two in October 2004 and one in January 2005. A total of 102 (66.6%) of the samples were positive for *L. monocytogenes* and 101 isolates were analyzed by PFGE. Combined restriction enzymes AscI and ApaI analysis yielded 9 PFGE types (Figure 4.1). Less heterogeneity than in fecal and environmental isolates was observed among isolates obtained from in-line milk filter samples (SID=0.71) (Figure 4.2). The most predominant and persistent PFGE types were types T, D, and F which accounted for 39.6%, 26.7%, and 26.7%, respectively, of *L. monocytogenes* isolates within this category of sample.

Bulk tank milk

Two-hundred and four bulk tank milk samples were collected between February 2004 and March 2008. *Listeria monocytogenes* was isolated from 52 of these samples (25.4%). Fifty two *L. monocytogenes* isolates (one from each *L. monocytogenes*-positive sample) were analyzed by PFGE. Combined restriction digest analysis yielded 3 PFGE types (Figure 4.1). A more limited diversity than in milk filter, fecal, and environmental isolates was observed among *L. monocytogenes* isolates obtained from bulk tank milk samples (SID=0.65) (Figure 4.2). The predominant and persistent PFGE types F (19.2%), T (38.4%), and D (42.3%) were the only PFGE types observed within this category of sample (Figure 4.1).

Serotyping

A total of 40 *L. monocytogenes* isolates, one of each PFGE type were analyzed by multiplex PCR. Seventy-eight percent of the *L. monocytogenes* isolates collected from the farm corresponded to serotype complex 1/2a. Serotype complex 1/2b and 4b each accounted for 10% of the isolates. Three *L. monocytogenes* isolates (PFGE types AI, AJ, and AN) could not be grouped in a serotype complex with the PCR assay (Fig 1).

Microtiter Plate Biofilm Assay

Visual representation of the adherence ability of each of the *L. monocytogenes* isolates that were tested is shown in Figure 4.3, whereas average S/P ratios are shown in Figure 4.4. *Listeria monocytogenes* isolates QMP-L1-021 (PFGE type D) and QMP-L1-002 (PFGE type J) had the greatest S/P ratios among all isolates that were analyzed (average S/P ratio: 1.36 and 0.68, respectively). The lowest S/P ratios corresponded to the strains represented by isolates QMP-L1-080 (PFGE type B) and QMP-L1-003 (PFGE type A) (average S/P ratio: 0.19 and 0.20, respectively).

Figure 4.3. Visual representation of biofilm production of 17 representative *L. monocytogenes* isolates analyzed by microtiter plate assay. Pictures show stained biofilm (1% Crystal Violet) suspended in 150 µl of 95% ethanol. First row (top) shows Trypticase Soy Broth controls (TSB) and isolates ID. Second row (bottom) shows blank (B-C), high biofilm former (HBF-C), and low biofilm former (LBF-C) controls. Biofilm forming ability of each *L. monocytogenes* isolate (low-L, medium-M, or high-H) and classification (predominant and persistent-P/PS; non-predominant and persistent-NP/PS; predominant and non-persistent-P/NPS; Sporadic-S; or new sporadic-SN) are also shown. S/P ratio across all experiments for each of the isolates is shown in Figure 4.4.

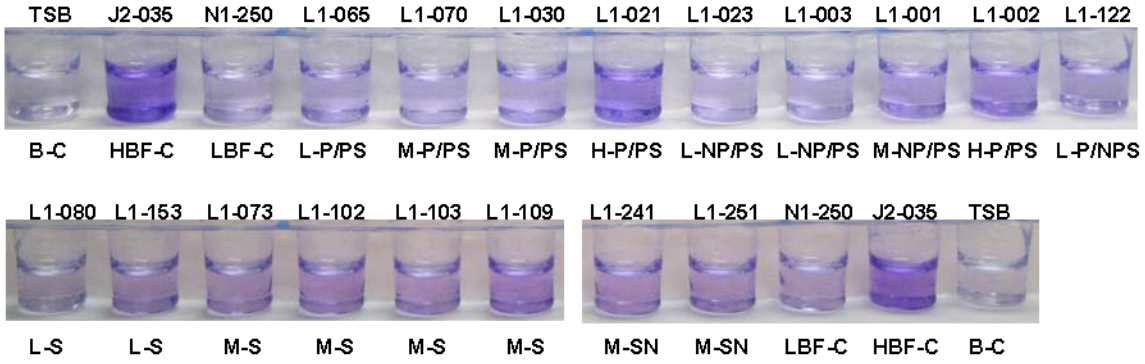
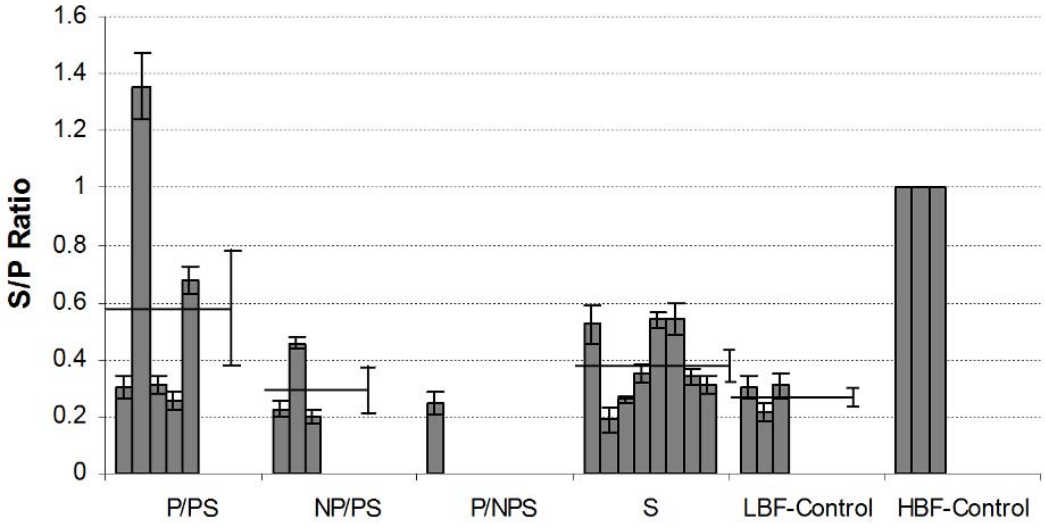

TSB J2-035 N1-250 L1-065 L1-070 L1-030 L1-021 L1-023 L1-003 L1-001 L1-002 L1-122
B-C HBF-C LBF-C L-P/PS M-P/PS M-P/PS H-P/PS L-NP/PS L-NP/PS M-NP/PS H-P/PS L-P/NPS
L1-080 L1-153 L1-073 L1-102 L1-103 L1-109 L1-241 L1-251 N1-250 J2-035 TSB
L-S L-S M-S M-S M-S M-S M-SN M-SN LBF-C HBF-C B-C

Figure 4.4. Average S/P ratios of *L. monocytogenes* for all three microtiter plate assay experiment repetitions. Each bar represents one *L. monocytogenes* isolate. From left to right: predominant and persistent isolates (P/PS) QMP-L1-070, L1-021, L1-030, L1-065, L1-002; Non-predominant and persistent isolates (NP/PS) QMP-L1-023, L1-001, L1-003; Predominant and non-persistent isolate (P/NPS) QMP-L1-122; Sporadic isolates (S) QMP-L1-102, L1-080, L1-153, L1-073, L1-103, L1-109, L1-251, and L1-241. *Listeria monocytogenes* FSL- N1-250 and FSL-J2-035 used as low biofilm former control (LBF-Control) and high biofilm former control (HBF-Control), respectively, show 3 bars each (average of each of the three experiment repetitions). Solid lines across bars show the average S/P ratio (approximate) for each group. Error bars show standard error of the mean.


S/P Ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P/PS
NP/PS
P/NPS
S
LBF-Control
HBF-Control

Six of 17 *L. monocytogenes* isolates (35.3%) had "low adherence ability", while nine (52.9%) had "medium adherence ability". Only two (11.8%) isolates had "high adherence ability" (Figure 4.4). Two sporadic isolates (QMP-L1-103 and QMP-L1-109) had a S/P ratio very close to the cutoff value to classify a *L. monocytogenes* isolate as having a "high adherence ability" (S/P ratio = 0.54 each; cutoff S/P ratio for high adherence isolates: 0.56).

Combined S/P ratios of *L. monocytogenes* isolates grouped by characteristic (Table 4.1) are shown in Figure 4.4. In a linear regression model, *L. monocytogenes* isolates that were predominant and persistent had better adherence ability as compared to isolates that were either predominant, persistent or sporadic (p=0.0006).

DISCUSSION

This longitudinal four-year study on the molecular ecology of *L. monocytogenes* was conducted to explain the long term prevalence of the pathogen in bulk tank milk and milk filters. Our data suggests that the milking system and, ultimately, the bulk tank, may have been exposed to multiple *L. monocytogenes* strains from different sources throughout the farm. However, only 3 PFGE types (T, D, and F) were successful in persisting within the milking system. This may suggest the formation of a biofilm by *L. monocytogenes* strains that were more suitable to a particular ecological environment.

Another potential explanation for the presence of 3 persistent PFGE types in bulk tank milk during the study period would be the presence of cows suffering from mastitis

caused by *Listeria monocytogenes.* In this case, we would be able to find persistent shedding of *L. monocytogenes* through infected udder quarters (Fedio et al., 1990) which may explain the repeated isolation of the pathogen from bulk tank milk samples. Mastitis caused by *L. monocytogenes*, however, is rare (Fedio et al., 1990; Jensen et al., 1996). On the study farm, milk from all cows suffering from mastitis is routinely cultured and *L. monocytogenes* was never isolated. Hence, the presence of cows shedding *L. monocytogenes* through infected quarters seems to be an unlikely explanation for persistence of specific PFGE types in the bulk tank milk. Furthermore, the previously reported presence of a biofilm in milking equipment on the study farm (Latorre et al., 2010) supports our hypothesis of colonization of the milk line by specific *L. monocytogenes* strains.

The three PFGE types observed in bulk tank milk (T, D, and F) were also observed in in-line milk filters. Furthermore, these PFGE types or closely related (Tenover et al., 1995) types were observed in feces and/or environmental isolates. With the exception of the unique PFGE types, AM and AN, all PFGE types isolated from in-line milk filters were also isolated from other sources such as feces, water, manure composite, bedding, etc. In the case of PFGE types M, F, and AF, closely related *L. monocytogenes* isolates were obtained from fecal and/or environmental samples. Hence, the *L. monocytogenes* types associated with milk and milk filter contamination were also associated with many locations throughout the farm.

The appearance of PFGE types F, T, and D in bulk tank milk and in-line milk filters showed a pattern of predominance of a particular PFGE type over time, followed by a period of overlapping, and finally the exclusion of the preceding PFGE type and replacement for a new PFGE type. *Listeria monocytogenes* strains isolated in the same

week from milk filter and bulk tank milk samples usually had the same PFGE type. On some occasions, however, when overlapping of *L. monocytogenes* strains occurred, PFGE types of isolates found in milk filters were different than those found in milk, but always corresponded to an overlapping type. Interestingly, *L. monocytogenes* PFGE type T (predominant PFGE type in milk filters and milk until May 2007 when the overlapping with PFGE type D started) was isolated for the last time from milk filters or milk in September 2007. Even though after September 2007 PFGE type D was the predominant type observed amongst in-line milk filter isolates and the only PFGE type observed in bulk tank milk, PFGE type T was isolated from milk meter samples collected in January and February 2008.

Strain competition (Moons et al., 2009) between *L. monocytogenes* within a biofilm in milking equipment could account for the detection of Type T in the meters long after it ceased being present in the milk and milk filters. The presence of PFGE type D as the predominant type from in-line milk filters would indicate that Type D is predominant in either existing biofilms, sources outside the milking equipment, or both. In addition, we only characterized by PFGE one isolate from each positive sample; multiple PFGE types in one sample, if present, would have not been detected using this approach (Döpfer et al., 2008).

Moreover, competition of *L. monocytogenes* strains during enrichment may result in an underrepresentation of the actual number of strains present on a given sample (Bruhn et al., 2005). For example, Bruhn et al. (2005) reported a greater proportion of lineage II *L. monocytogenes* strains than lineage I strains after enrichment in selective media (University of Vermont I and II media). Competition of certain *L. monocytogenes* strains during a single enrichment step was also reported by Gorski et

al. (2006), although no relationship between serotype and recovery after enrichment was found in their study.

The diversity of PFGE types in milk and milk filters over the course of the study (~4 years) was moderate (SID=0.65 and 0.71, respectively). When the SID was calculated for shorter time intervals, the diversity was lower because fewer PFGE types were observed within each of these shorter time periods than over the course of the study For example, SID's of milk isolates collected during 2005, 2006, 2007, or 2008 were 0, 0.54, 0.51, and 0, respectively. Furthermore, SID's for in-line milk filter isolates collected on the same years were 0.13, 0.55, 0.51, and 0.51, respectively.

In contrast with the more homogeneous PFGE types observed in milking equipment, bulk tank milk, and milk filters, a high heterogeneity of PFGE types was observed among fecal and environmental *L. monocytogenes* isolates (SID=0.96 for each). Heterogeneity of PFGE types in fecal *L. monocytogenes* isolates (Borucki et al., 2005; Esteban et al., 2009) as well as a large diversity of ribotypes (Ho et al., 2007) in individual cows' fecal samples have been previously described.

Source water used to fill water troughs (tap water) was always negative for *L. monocytogenes*. However, among the various samples collected from the farm environment, animal drinking water collected from the water troughs was most frequently *L. monocytogenes*-positive (64.3%). This percentage is similar to that found by Mohammed et al. (2009) in water trough samples from dairy farms in Central New York using a PCR detection method (66%). Other studies have also reported a high frequency of isolation of *L. monocytogenes* from water samples on dairy farms. For example, Nightingale et al. (2005) reported prevalences of *L. monocytogenes* in water samples from cattle farms that ranged between 18% and 25%.

After drinking contaminated water cows may shed *L. monocytogenes* back in the environment, creating a "recycling" of strains within the system. Although a high diversity of PFGE types was isolated from animal drinking water, the presence of a *L. monocytogenes*-containing biofilm in water troughs may also explain the greater prevalence of the pathogen in water.

No listeriosis in cows had ever been reported on the study farm even though animals were shedding the pathogen in their feces. This agrees with previous reports that cows can be healthy reservoirs of *L. monocytogenes* (Nightingale et al., 2004). Ho et al. (2007) suggested that *L. monocytogenes* can be shed in the feces by being passed through the gastric tract after the cow consumes contaminated silage. The same study showed that cows can shed *L. monocytogenes* 2-4 days after consuming contaminated silage, which suggests the presence of infection (Ho et al., 2007) or amplification (Nightingale et al., 2004) in animals. We observed the presence of *L. monocytogenes* in cows' feces but did not detect the pathogen in silage samples collected from the farm. *Listeria monocytogenes* was isolated from only one of 71 feed samples collected over a 4-year period. Hence, silage or other feedstuffs were unlikely to be the source of *L. monocytogenes* for cows in this study.

Besides fecal shedding of *L. monocytogenes* by cows, movement of animals or people may transport the pathogen to different locations throughout the farm. For example, PFGE type "X" was isolated from 2 individual fecal samples and from 1 water sample collected in April 2007, as well as from a swab of the milking parlor floor in May 2007. The presence of *L. monocytogenes* type "X" in the parlor may be explained by the presence of fecal material transported by cows on their feet or by defecation during milking. In addition, a PFGE type "Y" isolate was obtained from an individual fecal

sample in April 2007 and in May 2007 from a swab of the floor in the milk house storage room. As cows did not have access to the storage room, the presence of *L. monocytogenes* PFGE type "Y" in this location was likely due to the movement of farm personnel in the milk house and around the farm. The presence of *L. monocytogenes* on a farmer's rubber boots after a listeriosis outbreak in a sheep farm has been previously reported (Wagner et al., 2005). In addition, the presence of *L. monocytogenes* on employee's gloves and aprons, as well as on door handles, and switches in smoked fish processing plants have been described (Thimothe et al., 2004).

Wildlife and birds can be carriers of *L. monocytogenes* (Fenlon, 1985; Lyautey et al., 2007; Hellström et al., 2008). In our study farm, 11 bird/bird droppings samples were collected (Latorre et al., 2009) none of which tested positive for *L. monocytogenes*. Flies (16 samples) and other insects (5 samples) were also tested, and *L. monocytogenes* was isolated from 2 fly samples. The potential role of feral animals in the maintenance and/or spreading of the pathogen in the study farm cannot be ruled out as *L. monocytogenes* was isolated from one sample of feral animal feces collected from feed bunker in June 2004 (Latorre et al., 2009). One isolate from this sample was analyzed by PFGE and determined to be PFGE type T, which was the most predominant type on the farm having been found in feces, in-line milk filters, bulk tank milk, and milking equipment.

A greater prevalence of *L. monocytogenes* in the milking system than can be accounted for by the prevalence in environmental and fecal samples contrasts the results of another study conducted on New York State dairy farms (Mohammed et al., 2009). As we previously reported (Latorre et al., 2009; Latorre et al., 2010), a biofilm

in the milking equipment could act as a reservoir of *L. monocytogenes* and can account for the observed differences in prevalence.

Based on an *in-vitro* assay, only 2 *L. monocytogenes* strains were classified as having "high adherence ability", although all strains were able to adhere to the PVC surface used in the assay to various degrees (low, medium, or high adherence). One of the strains with high adherence ability was PFGE type D, and this was one of the three types that were persistent in in-line milk filters and bulk milk samples. This "high adherence ability" *L. monocytogenes* strain had an adherence ability that exceeded the ability of the strain used as the positive control (36% higher S/P ratio than control).

A statistically significant relationship between predominance and persistence, and adherence ability was found in our study. Although in our study we only performed a screening assay to determine biofilm forming ability, our results are similar to the findings reported by Borucki et al. (2003) who reported that persistent strains are better biofilm formers. Our findings, however, differ from the results obtained in the studies conducted by Djorjevic et al. (2002) and Harvey et al. (2007) as no relationship between persistence and biofim forming ability of strains was observed in their studies.

Adherence ability (and hence the potential to form biofilms) of persistent strains in our study may explain why they have been repeatedly found over time on the farm. In a study conducted in ready-to-eat meat processing plants (Kushwaha and Muriana, 2009), 76% of the *L. monocytogenes* isolates recovered from the plant with the highest percentage of *Listeria* spp.- and *L. monocytogenes*-positive samples had a moderate or high adherence ability. Molecular typing of isolates was not conducted, hence, the

repeated isolation of specific strains could not be assessed. The adherence ability of *L. monocytogenes* strains, however, may have played a role in the persistence of the pathogen in this food processing facility (Kushwaha and Muriana, 2009).

Pulsed-field gel electrophoresis types T and F, despite being persistent for a long time in bulk tank milk and milk filters, had only "medium adherence ability". Biofilm formation, however, may depend on multiple factors and there could be variations due to culture media used (Harvey et al., 2007) or the type of material to which *L. monocytogenes* strains are attached (Djorjevic et al., 2002). An important element to take into consideration is the fact that laboratory conditions for biofilm growth cannot mimic actual environmental conditions. The difference between the *in vitro* assay and *in vivo* conditions may impact the predictive value of *in vitro* biofilm formation ability (Jefferson, 2004).

Synergism or cooperation between different bacterial species within a biofilm has been previously described (Moons et al., 2009). Many bacterial species are found in the farm environment and have the potential to establish biofilms in the milking system. *Listeria monocytogenes* strains that do not have strong biofilm-forming capacity may still be part of the biofilm community. This may explain why some of the predominant and persistent PFGE types in our study had low or moderate adherence ability. Persistence of *L. monocytogenes* strains on a given niche may be also due to intrinsic characteristics of the strains, such as stress tolerance (van der Veen et al., 2008), better ability of certain strains to compete for nutrients (Gnanou Besse et al., 2010), or persistent re-introduction of a particular strain.

Listeria monocytogenes 1/2a, 1/2b, and 4b are the serotypes most frequently involved in human listeriosis (Farber and Peterkin, 1991) and all of them were isolated from the

study farm. The most predominant and persistent PFGE types (T, D, and F) corresponded to serotype complex 1/2a. According to a study conducted by Nightingale et al. (2006), *L. monocytogenes* strains that belong to lineage II (such as isolates characterized as serotype complex 1/2a) are most frequently found among isolates obtained from food. The same study (Nightingale et al., 2006) reported that isolates belonging to lineage I, such as 1/2b and 4b which combined accounted for 20% of the serotypes observed in our study, are frequently found in *L. monocytogenes* isolates from human listeriosis cases. In our study, PFGE type J (serotype complex 4b) was one of the predominant types and persisted on the farm for 3 years. Furthermore, one PFGE type J isolate (L1-002) showed high adherence ability. Although PFGE type J was mostly isolated from fecal samples, it was also isolated from an in-line milk filter sample in April 2008. As *L. monocytogenes* isolates belonging to Lineage I have been described to be more frequently involved in human listeriosis cases and have high virulence attributes (Nightingale et al., 2006), prevention of the appearance and establishment of *L. monocyogenenes* into the milking system on dairy farms is warranted.

Appropriate hygiene during milking helps to prevent *L. monocytogenes* entering into the milking system, while adequate milking equipment cleaning and regular replacement of sensitive milking equipment components would help in preventing the establishment of biofilms. Appropriate cleaning includes following the combination of water circulation time, water temperature and the use of cleaning and sanitizing agents in concentrations that are recommended by the manufacturer. Failure to follow these protocols may result in the establishment of biofilms within the milking system and subsequent contamination of the bulk milk.

Control of *L. monocytogenes* on dairy farms may be achieved by feeding cows with good quality silage because feedstuff and particularly poor quality silage can harbor *L. monocytogenes* (Fenlon, 1985). In addition, regular (and frequent) cleaning and disinfection of water troughs would reduce the number of *L. monocytogenes* in drinking water for animals as well as it would help preventing the establishment of potential biofilms in the water system. Control of *L. monocytogenes* on farms would benefit from pests and wild life control as these animals may transmit *L. monocytogenes* in their feces (Iida et al., 1991; Lyautey et al., 2007). Finally, implementation of biosecurity measures may help prevent the further spread of the pathogen on dairy farms.

ACKNOWLEDGEMENTS

Financial support for this work was provided by the USDA-Agricultural Research Service (Agreement No. 58-1265-3-155, 58-1265-3-156, 58-1265-3-158, 58-1265-4-020, and 58-1265-8-064) for the Regional Dairy Quality Management Alliance.

We thank the dairy producer who generously allowed the research to take place on his farm. We thank Dr. Martin Wiedmann (Food Science Department, Cornell University) for providing with control strains from his collection and Dr. Holger Sondermann (Veterinary Medical Center, Cornell University) for his insights and contributions to our microtiter plate biofilm assay. We gratefully acknowledge the valuable help received from the Quality Milk Production Services and USDA-Agricultural Research Service teams during the course of this research.

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CHAPTER 5

QUANTITATIVE RISK ASSESSMENT OF LISTERIOSIS DUE TO CONSUMPTION OF RAW MILK

ABSTRACT

The objectives of this study were to estimate the risk of illnesses for raw milk consumers due to *L. monocytogenes* contamination in raw milk sold by permitted raw milk dealers, and the risk of listeriosis for people on farms who consume raw milk. Three scenarios were evaluated for raw milk sold by dealers: raw milk purchased (i) directly from bulk tanks, (ii) from on-farm stores, and (iii) from retail stores. To assess the effect of mandatory testing of raw milk by regulatory agencies, the number of listeriosis cases per year were compared where (i) no raw milk testing was done, (ii) only a screening test to issue a permit was conducted, and (iii) routine testing was conducted and milk was recalled if it was found to be positive for *L. monocytogenes*. The median number of listeriosis cases associated with consumption of raw milk obtained from bulk tanks, farm stores, and retail stores for an intermediate-age population was estimated as 6.6×10^{-7}, 3.8×10^{-5}, and 5.1×10^{-5} cases/year, respectively.

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In populations with high susceptibility to listeriosis the estimated median number of cases per year was 2.7×10^{-7} (perinatal) and 1.4×10^{-6} (elderly) for milk purchased from bulk tanks, 1.5×10^{-5} (perinatal) and 7.8×10^{-5} (elderly) for milk purchased at farm stores, and 2.1×10^{-5} (perinatal) and 1.0×10^{-4} (elderly) for milk obtained from retail stores. For raw milk consumed on farms, the median number of listeriosis cases was estimated to be 1.4×10^{-7} cases/year. A reduction in the number of cases per year in all populations was observed when a raw milk testing program was in place, especially when routine testing and recalling of milk was conducted.

INTRODUCTION

Listeriosis is an uncommon but severe human disease caused by the foodborne bacterial pathogen, *Listeria monocytogenes*. Healthy adults are not at high risk for listeriosis and generally do not show serious symptoms of the disease. Listeriosis typically occurs in susceptible individuals such as pregnant women (and their unborn children), the elderly, and people with a weakened immune system (Linnan et al, 1988; Lyytikäinen et al, 2000; MacDonald et al, 2005). Milk and dairy products have been implicated in several foodborne listeriosis outbreaks in the United States (Fleming et al, 1985; Dalton et al, 1998; Linnan et al, 1988; MacDonald et al, 2005; CDC, 2008). Although none of the reported listeriosis outbreaks have been linked to consumption of raw milk, it is apparent that healthy adults may only exhibit flu-like or gastrointestinal symptoms (Riedo et al., 1994; Sim et al., 2002) that generally do not require medical attention. This may lead to an under-reporting or underestimation of the actual number of listeriosis cases.

Unfortunately, several reports of other foodborne illnesses outbreaks, such as *E. coli* O157:H7, *Campylobacter*, and *Salmonella* have been linked to consumption of raw milk (Keene et al., 1997; CDC, 2002; CDC, 2003; Denny et al, 2008; CDC, 2007) or dairy products manufactured with raw milk (CDC, 2000; CDC, 2007). Indeed, the majority of foodborne disease outbreaks attributed to raw milk consumption have occurred in states where raw milk sale is allowed (Headrick et al, 1998; Oliver et al., 2009). Despite the frequent reports of foodborne illnesses linked to consumption of raw milk, the sale of raw milk is currently legal in 29 states in the United States (Oliver et al., 2009).

Although raw milk sales are prohibited in several states, raw milk advocates may still obtain this product through cow-share or cow leasing programs (CDC, 2002; Denny et al, 2008; Oliver et al, 2009), as milk purchased "to feed animals" (Oliver et al, 2009; http://realmilk.com/happening.html), or by traveling to neighboring states where raw milk sales are legal. In addition, raw milk or other dairy products provided during farm tours (Anonymous, 1984; CDC, 1986) and raw milk used for cheese preparation which was illegally sold by a dairy (MacDonald et al, 2005) have also been linked to foodborne outbreaks. Furthermore, a large number of dairy producers have reported consuming raw milk (Jayarao et al, 2006; Jayarao and Henning, 2001; Hoe and Ruegg, 2006), which puts them at increased risk of acquiring listeriosis or other milk-borne illnesses.

A risk assessment **(RA)** conducted by the U.S. Food and Drug Administration **(FDA)** and the U.S. Department of Agriculture Food Safety and Inspection Service **(FSIS)**, which was published in 2003 (FDA/FSIS, 2003), estimated the relative risk of illness or death per serving and per annum due to consumption of 23 different ready-to-eat

(RTE) food categories (e.g., unpasteurized and pasteurized fluid milk, deli meats, and smoked seafood) contaminated with *L. monocytogenes.* Based on the relative risk ranking of these RTE food categories, this risk assessment classified unpasteurized fluid milk in the "high risk" group per serving basis and in the "moderate risk" per annum basis (which apparently reflects the low number of servings per annum in the total United States population for unpasteurized fluid milk).

Although a few risk assessment studies of listeriosis due to consumption of dairy products (i.e., soft cheese) (Bemrah, et al., 1998; Sanaa et al., 2004) have been conducted, reports for quantitative risk assessment of listeriosis due to consumption of raw milk are currently not available except for the FDA-FSIS risk assessment (FDA/FSIS, 2003). Although the FDA-FSIS risk assessment (FDA/FSIS, 2003) quantified relative risk of different categories of RTE foods (including unpasteurized fluid milk), it did not include assessment of risk of listeriosis attributed to consumption of raw milk purchased from different markets (i.e. directly from farm, farm stores, or retail stores) or estimation of the risk of listeriosis due to consumption of raw milk by dairy producers and farm personnel. The objectives of this study were (i) to estimate the risk of listeriosis for raw milk consumers due to the presence of *L. monocytogenes* in raw milk sold by permitted raw milk dealers and for people who consume raw milk on farms, (ii) to assess the effect of mandatory testing of raw milk by regulatory agencies, and (iii) to evaluate the risk of listeriosis associated with the consumption of raw milk from farms that have a known high prevalence of *L. monocytogenes* in bulk tank milk.

MATERIALS AND METHODS

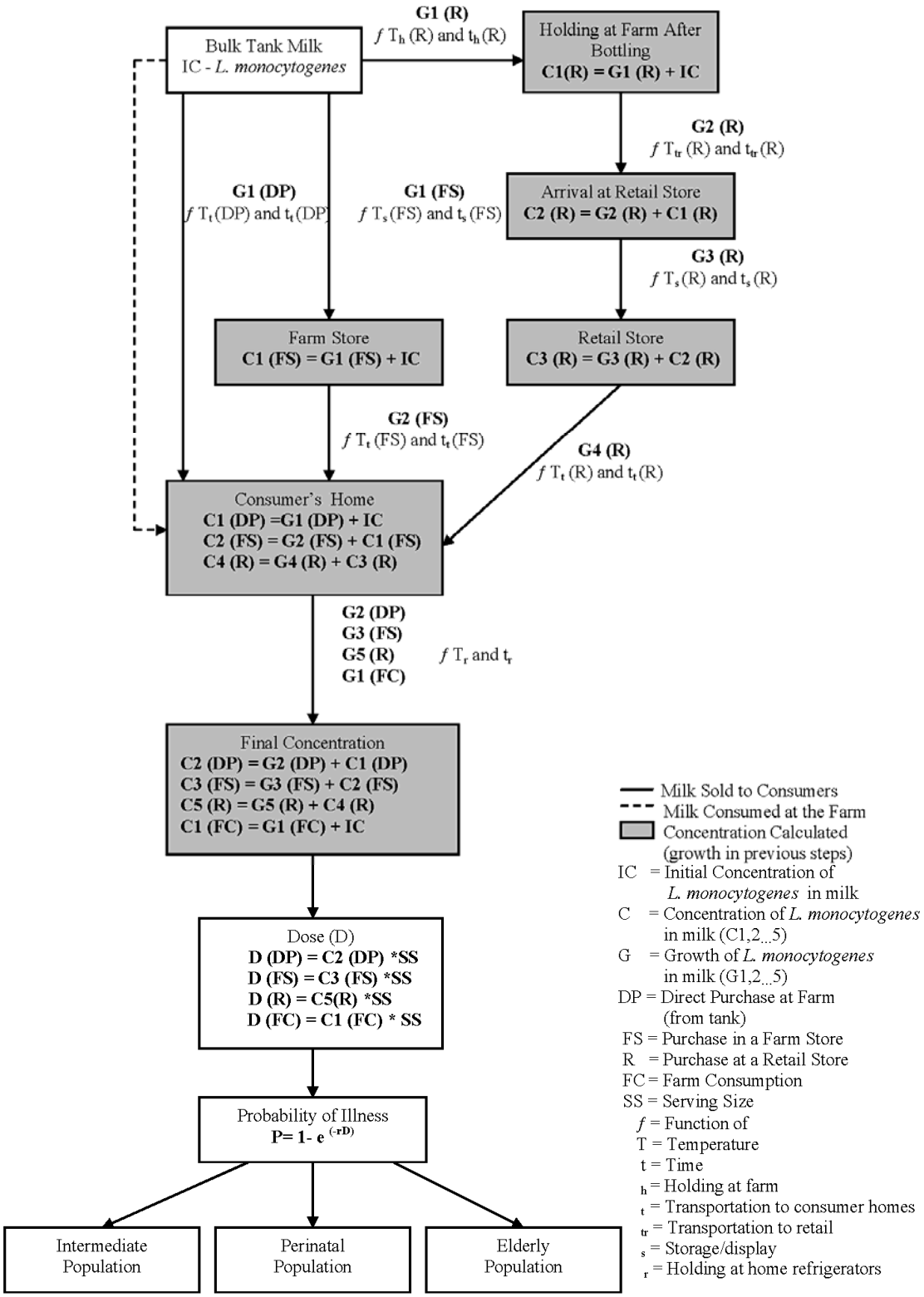
Overview of raw milk commercialization pathways and risk assessment model

Consumers can obtain raw milk from several different sources. For example, in some states raw milk sale by licensed dealers is allowed only on the farm premises. In New York State **(NYS)**, raw milk can be purchased at licensed farms, where consumers can either bring their own container and have it filled directly from the bulk tank in their presence or purchase bottled raw milk from on-farm stores. In other states, such as California, Maine, and Pennsylvania, raw milk can also be purchased from retail stores (http://farmtoconsumer.org/raw_milk_map.htm). To estimate the risk of listeriosis attributable to consumption of raw milk that may be obtained from different licensed raw milk commercialization pathways, we investigated and modeled the following three scenarios: (1) raw milk purchased directly from farm bulk tanks, (2) raw milk purchased from a farm store, and (3) raw milk purchased from a retail store (detailed in Figure 5.1). Additionally, we also modeled and estimated the risk of listeriosis due to consumption of raw milk on farms by dairy producers and farm personnel.

Scenarios of raw milk purchased directly from the farm bulk tank (DP), purchased from farm stores (FS), retail stores (R), and raw milk consumed on farms (FC)

In the first scenario (DP), it was assumed that customers brought their own containers to the farm and raw milk was purchased by filling their containers directly from the bulk tank. The growth and concentration of *L. monocytogenes* in milk at each of the time intervals between purchase and consumption were calculated as shown in Figure 5.1. For FS, an additional

Figure 5.1. Diagram of raw milk consumption pathways modeled in risk assessment model of listeriosis.


Bulk Tank Milk
IC - L. monocytogenes
G1 (R)
f Th (R) and th (R)
Holding at Farm After Bottling
C1(R) = G1 (R) + IC
G2 (R)
f Ttr (R) and ttr (R)
G1 (DP)
f Tt (DP) and tt (DP)
G1 (FS)
f Ts (FS) and ts (FS)
Arrival at Retail Store
C2 (R) = G2 (R) + C1 (R)
G3 (R)
f Ts (R) and ts (R)
Farm Store
C1 (FS) = G1 (FS) + IC
Retail Store
C3 (R) = G3 (R) + C2 (R)
G2 (FS)
f Tt (FS) and tt (FS)
G4 (R)
f Tt (R) and tt (R)
Consumer's Home
C1 (DP) =G1 (DP) + IC
C2 (FS) = G2 (FS) + C1 (FS)
C4 (R) = G4 (R) + C3 (R)
G2 (DP)
G3 (FS)
G5 (R)
G1 (FC)
f Tr and tr
Final Concentration
C2 (DP) = G2 (DP) + C1 (DP)
C3 (FS) = G3 (FS) + C2 (FS)
C5 (R) = G5 (R) + C4 (R)
C1 (FC) = G1 (FC) + IC
Dose (D)
D (DP) = C2 (DP) *SS
D (FS) = C3 (FS) *SS
D (R) = C5(R) *SS
D (FC) = C1 (FC) * SS
Probability of Illness
P= 1- e (-rD)
Intermediate Population
Perinatal Population
Elderly Population
Milk Sold to Consumers
Milk Consumed at the Farm
Concentration Calculated (growth in previous steps)
IC = Initial Concentration of L. monocytogenes in milk
C = Concentration of L. monocytogenes in milk (C1,2...5)
G = Growth of L. monocytogenes in milk (G1,2...5)
DP = Direct Purchase at Farm (from tank)
FS = Purchase in a Farm Store
R = Purchase at a Retail Store
FC = Farm Consumption
SS = Serving Size
f = Function of
T = Temperature
t = Time
h = Holding at farm
t = Transportation to consumer homes
tr = Transportation to retail
s = Storage/display
r = Holding at home refrigerators

growth step during storage and display at the farm stores [G1 (FS)] was modeled as compared to DP (Figure 5.1).

For quantification of the growth of *L. monocytogenes* in the R scenario, it was assumed that the raw milk farms were in the charge of handling the bottling, shipping, and distribution of their own products to retail stores. Growth of *L. monocytogenes* in this scenario was calculated during holding of bottled milk before shipping to retail stores [G1 (R)], during transportation of milk to retail stores [G2 (R)], and during storage at retail stores [G3 (R)]. Growth occurred during additional steps were calculated as described for DP and FS (Figure 5.1).

For raw milk consumed on farms by farm personnel (FC), it was assumed that milk was transported from the bulk tank to the house and it was also assumed that dairy producers or farm personnel lived on the farm premises. Hence, no transportation time or temperature was accounted for and it was assumed that no delay occurred in relocation of milk from the tank to the home refrigerator (Figure 5.1). Concentrations of *L. monocytogenes* in the milk for all scenarios were calculated after each growth step as shown in Figure 5.1.

Initial concentration and growth of L. monocytogenes in raw milk

Initial concentration of *L. monocytogenes* in bulk tank milk **(IC)** was characterized as a cumulative distribution (Table 5.1) using reported data of *L. monocytogenes* contamination in raw cow milk (FDA/FSIS, 2003; Meyer-Broseta et al., 2003). No further growth of *L. monocytogenes* was calculated because the initial concentration was obtained from data where bulk tank milk was sampled at dairy plants before pasteurization (FDA/FSIS, 2003) or during pick up (Meyer-Broseta et al., 2003).

Table 5.1. Details of parameters and their values or formulas for distributions used in the quantitative risk assessment model of listeriosis associated with consumption of *L. monocytogenes*-contaminated raw milk

Parameter	Units	Values or Formulas for Distributions	Reference
Prevalence of *L. monocytogenes*			
Overall prevalence on U.S. Dairy Farms	%	6.5	Van Kessel et al., 2004.
Prevalence in Raw Milk from Licensed Farms	%	2.1	NYS Department of Agriculture and Markets
Prevalence in Commercialized Raw Milk from Licensed Farms	%	1.3	NYS Department of Agriculture and Markets
Contamination prevalence, raw milk farm (high)	%	35.3	NYS Department of Agriculture and Markets
Contamination prevalence, traditional farm (high)	%	25.4	Latorre et al. (unpublished data)
Initial Concentration of *L. monocytogenes* in Bulk Tank Milk (IC)	log (cfu/ml)	RiskCumul(-1.4,2.18,{-1.4,1,2},{0.926,0.972,0.999})	FDA/FSIS, 2003; Meyer-Broseta, 2003.
Maximum Population Density (MPD)			
MPD <5°C	log (cfu/g)	RiskUniform(6.5,7.5)	FDA/FSIS, 2003.
MPD 5-7°C	log (cfu/g)	RiskUniform(7,8)	FDA/FSIS, 2003.
MPD >7°C	log (cfu/g)	RiskUniform(7.5,8.5)	FDA/FSIS, 2003.
Temperature Distributions			
Farm Milk Tank (T_{mt})	°C	RiskUniform(7,10)	PMO, 2009
Farm Store Refrigerator [T_s(FS)]	°C	RiskTriang(-6.1787,4.4444,14.473,RiskTruncate(0,))	Ecosure, 2007
Holding Temperature at Farm Before Shipping to Retail [T_h (R)]	°C	RiskUniform(2.2,4.4)	Personal Communication [a]
Transportation to Retail [T_{tr} (R)]	°C	RiskTriang(3.6,6.5,10.9)	Koutsomanis et al., 2010
Storage/Display at Retail Store [T_s (R)]	°C	RiskTriang(-6.1787,4.4444,14.473,RiskTruncate(0,))	Ecosure 2007
Home Arrival Temperature (T_{ha})	°C	RiskTriang(-3.3804,8.8889,21.156,RiskTruncate(0,))	Ecosure, 2007
Transportation Farm to Home [T_t (DP)]	°C	(T_{mt} + T_{ha})/2	
Transportation Farm Store to Home [T_t (FS)]	°C	([T_s(FS)] + T_{ha})/2	
Transportation Retail Store to Home [T_t (R)]	°C	([T_s (R)]+ T_{ha})/2	
Home Refrigerator (T_r)	°C	RiskTriang(-5.0221,2.7778,17.238,RiskTruncate(0,))	Ecosure, 2007
Time Distributions			
Storage/Display at Farm Store [t_s (FS)]	day	RiskUniform(1,7)	Personal Communication [b]
Holding at Farm Before Shipping to Retail [t_h (R)]	h	RiskUniform(4,12)	Personal Communication [a]
Transportation to Retail [t_{tr} (R)]	h	RiskTriang(1.7181,2.1333,5.9224)	Raw Milk Farm, website [c]
Storage/Display at Retail Store [t_s (R)]	day	RiskUniform(1,7)	Raw Milk Farm, website [d]
Transportation Farm/ Farm Store/Retail to Home [t_t (DP), t_t (FS), t_t (R)]	h	RiskTriang(0.28801,0.65,3.1552)	Ecosure, 2007
Home Refrigerator (t_r)	day	RiskPert(0.5,2.5,8.5)	FDA/FSIS, 2003; Heidinger et al., 2009

[a] Personal communication with vice-president of manufacturing of a retail chain that collects, pasteurizes, and sells milk from dairy producers.

[b] Personal communication with dairy plant manager of a farm that sells raw milk from a farm store. The minimum storage time of raw milk in this farm store before sale corresponded to the 24 h holding time while waiting for results of milk laboratory tests and the maximum corresponded to the "sell by" date of raw milk.

[c] Distance and time of transportation were estimated using the address of a raw milk farm and the address of each of the retail stores that sell their raw milk provided in the farm's website (http://claravaledairy.com/store_list.html. Accessed on May 6, 2010).

[d] Based on the approximate "purchase by" date reported by a raw milk farm that distributes raw milk to retail stores (http://claravaledairy.com/faq.html. Accessed on June 24, 2010).

Hence, any growth that may have occurred while milk was stored in the tank was assumed to be accounted for.

For all four of the above mentioned scenarios, growth of *L. monocytogenes* in raw milk was calculated based on the equation described by Koutsomanis et al. (2010) for *L. monocytogenes* growth in pasteurized milk, with modifications:

After milk is collected from milk tank (C1, Figure 5.1):

$\log (N_t) = \log (N_0) + [\mu_{max}/\ln(10)](t)$

If $\log (N_t) > MPD$, then $\log (N_t) = MPD$ (Equation 1)

For all following steps (C2-5, Figure 5.1):

If $\log (C_{prev}) > MPD$, then $\log (N_t) = \log (C_{prev})$

Else, $\log (N_t) = [\mu_{max}/\ln(10)](t) + \log (C_{prev})$

and, if $\log (N_t) > MPD$, then $\log (N_t) = MPD$ (Equation 2)

Where N_t is the concentration of *L. monocytogenes* (cfu/ml) at time t, (N_0) is the initial concentration of *L. monocytogenes* in bulk tank milk (cfu/ml), and C_{prev} is the concentration of *L. monocytogenes* (cfu/ml) reached in the previous step of the model. In the equation, μ_{max} is the maximum specific growth rate (h^{-1}) and $\sqrt{\mu_{max}} = b(T-T_{min})$, where b=0.024 (Xanthiakos et al., 2006; Koutsomanis et al., 2010) and T_{min} = -2.32 (Xanthiakos et al., 2006) were the values assigned to the constant b and to the minimum growth temperature (T_{min}) of *L. monocytogenes*, respectively.

T corresponds to the temperature (˚C) and t is the amount of time (h) in each of the model steps (holding, storage, transportation, etc.). Maximum population densities (MPD) were calculated at different temperatures (< 5˚C, between 5 and 7˚C, and >

7˚C) as described in the FDA/FSIS risk assessment model for unpasteurized milk (FDA/FSIS, 2003) (Table 5.1).

Because *L. monocytogenes* is a psychrotolerant bacterial pathogen, it can be assumed that if it is present in the bulk tank milk, *L. monocytogenes* would have already adapted to the holding temperature (~4C) and would not be expected to go through a lag phase prior to exponential growth. Hence, lag phase was not considered in the calculation of *L. monocytogenes* growth (Yang et al., 2006; Franz et al., 2010) and growth of *L. monocytogenes* was considered to be exponential in each of the subsequent steps of the model. This approach represented a conservative choice from a public health standpoint because underestimation of growth may have occurred if lag phase was included in the growth calculation without accounting for holding time of milk on farm tanks.

Time and Temperature Distributions

Time and temperature data used to describe the distributions of these parameters in our model were obtained from the literature, from on-line sources, and through personal communication with a raw milk producer, a raw milk plant manager, and from a retail chain that sells their own dairy (pasteurized) products (to protect confidentiality, names of individuals and retail chain were not disclosed). Compiled data were fitted with BestFit software (Palisade Corporation, Ithaca, NY) to characterize time and temperature distributions. Triangular distribution frequently provided the best fit to our data (Chi-square test), therefore this distribution was used to define time and temperature distributions. Also, at some steps where not enough data were available for fitting procedure to characterize these distributions, uniform distributions were

used. Distributions for time and temperature, parameters and their values are detailed in Table 5.1.

Insufficient data were available on storage temperature of milk at farm stores. Hence, the temperature of raw milk prior to sales in farm stores was assumed to be equal to the temperatures reported in the "U.S. cold temperature evaluation" conducted by Ecosure (2007) for semi-solid dairy products (yogurt) sold at the retail level (http://foodrisk.org/exclusives/EcoSure/). For scenario R, information regarding holding time and temperature of milk at the farm before shipping to retail stores was provided by the vice-president of manufacturing of a retail chain that sells pasteurized milk (Table 5.1). The temperature of the milk during transportation from farms to retail stores was modeled using data for milk transportation from a distribution center to retail stores reported by Koutsomanis et al. (2010) (Table 5.1).

Time of transportation from farm to retail stores was calculated using a licensed raw milk farm from California that distributes milk to retail stores as an example. By means of a web-based map system (http://maps.google.com), the distance and time of transportation was estimated using the location of the farm and the location of each of the retail stores that sell their raw milk, the latter which were provided on the farm's website (http://claravaledairy.com/store_list.html). To model this distribution (Table 5.1), direct transportation of raw milk from farm to the destination store was assumed (i.e. stops during transportation, and delivery of milk to more than one store were not considered because of unavailability of data) and the fastest route was used when more than one route was available.

The minimum time of display in retail stores was set at 24 h to account for the time it took to unload the trucks, back room storage, and display before purchase. The maximum time of raw milk display in retail stores was defined based on the approximate "purchase by" date reported by a raw milk farm in California (Table 5.1).

Calculation of L. monocytogenes dose per serving

For all scenarios, the *L. monocytogenes* dose per serving was calculated by multiplying the final concentration of *L. monocytogenes* in raw milk (cfu/ml) after storage in home refrigerators by the serving size. The raw milk serving size was modeled as a cumulative distribution using the data of unpasteurized milk consumption reported by the FDA/FSIS RA model (same as serving size of pasteurized milk) (FDA/FSIS, 2003). The FDA/FSIS model reported consumption of milk in g instead of mL, therefore serving size in our model was converted to mL using a milk density value of 1.025g/ml (Heidinger et al., 2009).

To estimate the probability of illnesses due to consumption of raw milk from licensed raw milk dealers, an exponential dose-response model was used (WHO/FAO, 2004; Ross et al., 2009), and the probability of illness per serving was calculated by combining the dose estimate and contamination prevalence (Ross, 2009):

$$P = 1 - e^{(-rD)}$$

$$P_{serv} = P \times prev$$

(Equation 3)

Where:

P is the probability of illnesses (severe listeriosis), D is the dose per serving (cfu/ml), and r is the parameter describing the probability that one *L. monocytogenes* cell causes illness: $r = 8.5 \times 10^{-16}$ for intermediate-age population, 5.0×10^{-14} for

perinatal population, and 8.4×10^{-15} for elderly population (WHO/FAO, 2004). P_{serv} is the probability of illnesses per serving and *prev* corresponds to the prevalence of *L. monocytogenes* in raw milk.

For raw milk sold by licensed dealers, P_{serv} was calculated for each of the three scenarios using the overall prevalence of *L. monocytogenes* in raw milk (2.1%), based on the results of laboratory testing of milk samples collected between 2003 and 2009 from all licensed raw milk farms in NYS. This data were provided by the NYS Department of Agriculture and Markets and corresponded to the results of monthly tests that are conducted on every licensed raw milk farm in NYS (http://www.agmkt.state.ny.us/AI/sheep_goats/Raw_Milk_Sales_Start_Up_and_Guidance_0508.pdf).

P_{serv} for dairy producers and farm personnel on US dairy farms was calculated assuming an overall prevalence of *L. monocytogenes* in bulk tank milk of 6.5% (Van Kessel et al., 2004). Additionally, the number of listeriosis cases per year due to consumption of licensed raw milk were calculated by multiplying P_{serv} in each scenario by the number of servings per year for every target population (intermediate-age, perinatal, and elderly populations) (Ross et al., 2009). The number of annual raw milk servings used in our model were 3.60×10^8, 2.5×10^6, and 7.5×10^7 for the intermediate, perinatal, and elderly populations, respectively (FDA/FSIS, 2003).

The number of listeriosis cases per year due to raw milk consumption on farms was calculated using the number of servings per year among dairy producers and farm workers. For this calculation, it was estimated that this population had 0.91 raw milk servings/person/day, based on the annual number of pasteurized milk servings in the US population reported in the FDA/FSIS RA model (8.7×10^{10} raw milk

servings/person/year) (FDA/FSIS, 2003). In addition, the number of pasteurized milk servings per year instead of raw milk servings was used to calculate the number of listeriosis cases per year for farm consumers to better reflect the regular raw milk consumption behavior by this specific population.

The number of raw milk consumers on US dairy farms was estimated to be 8.2×10^4 based on the estimate by Jayarao and Hening (2001) and Hoe and Ruegg (2006) that 60% of dairy producers drink raw milk and that 24.2% of producers allowed their employees to consume raw milk on the farm (Jayarao et al. 2006). The total population of US dairy producers in our model was assumed to be 69,890 (one producer per farm) based on the number of dairy farms reported in the Census of Agriculture (2007). The number of farm personnel was assumed to be 165,688 (Total number of milk cows reported in the Census of Agriculture, 2007 = 9,894,291, divided by the average number of cows per worker in NYS reported in Dairy Farm Business Summary, 2008 = 42, minus the number of producers). No data about the number of elderly or perinatal people on farms were available, and dairy producers and farm personnel were assumed to belong to the intermediate-age population.

Prevalence of L. monocytogenes in raw milk and effect of testing by regulatory agencies on the number of listeriosis cases per year

The NYS raw milk regulations were used as an example to assess the impact of testing and monitoring for the presence of *L. monocytogenes* on licensed raw milk farms. A dairy producer must submit a milk sample to be tested for the presence of pathogens, including *L. monocytogenes*, to the NYS Department of Agriculture and Markets before being permitted to sell raw milk (http://www.agmkt.state.ny.us/AI/sheep_goats/Raw_Milk_Sales_Start_Up_and_Guidance_0508.pdf). If a raw milk permit is granted,

the permit holders must submit a monthly sample to the Food Laboratory Division to be tested for the presence of *L. monocytogenes* and other zoonotic pathogens. If a milk sample from a particular farm is positive for *L. monocytogenes*, then the milk from that farm is recalled. Once a positive test is obtained, milk sales are prohibited until a subsequent milk sample is negative, or until four consecutive samples are negative if the pathogen was found in two consecutive samples. After milk sales are resumed, weekly samples are collected from the farm for a 4-week period. A key assumption in our model was that if a bulk tank milk sample is *L. monocytogenes*-positive, the levels of contamination will be at least 0.04 cfu/mL of milk, which is the detection limit for culturing a 25 mL sample of milk.

To assess the effect of raw milk testing on the number of listeriosis cases associated with raw milk consumption, the probability of a listeriosis case per raw milk serving and the annual cases of listeriosis were compared when i) no raw milk testing was done ii) only an initial screening test for the purpose of issuing a permit was conducted, and iii) routine testing was conducted and milk was recalled if found positive for *L. monocytogenes*. For this comparison, P_{serv} (equation 3) was calculated using the prevalence of *L. monocytogenes* in raw milk in each of the testing conditions.

The effect of not testing the raw milk prior to sales was calculated using the overall prevalence of *L. monocytogenes* in bulk tank milk of US farms (6.5%) based on the results of the National Animal Health Monitoring System survey conducted on US dairy farms in 2002 (Van Kessel et al., 2004). To account for the effect of a screening test, P_{serv} was calculated using a 2.1% prevalence of *L. monocytogenes* in raw milk (overall prevalence in licensed raw milk in NYS). Finally, P_{serv} was calculated using a

1.3% prevalence of *L. monocytogenes* in raw milk, which corresponded to the prevalence from milk that was actually commercialized (i.e., samples collected from raw milk farms during recall periods were not accounted for) based on the NYS guidelines described earlier for raw milk samples that test positive for *L. monocytogenes*.

The annual number of listeriosis cases due to raw milk consumption under different raw milk testing conditions were calculated using the number of raw milk servings per year, as described earlier.

Risk assessment model of listeriosis due to consumption of raw milk from farms with high prevalence of L. monocytogenes in bulk tank milk

Two "what if" scenarios of the risk of listeriosis associated with consumption of raw milk from farms with a known high prevalence of *L. monocytogenes* in bulk tank milk were analyzed.

The first scenario described the risk of listeriosis due to raw milk consumption from a licensed raw milk farm, including the impact of *L. monocytogenes* prevalence on each of the commercialization pathways (direct purchase, farm stores, retail purchase). For this scenario, a prevalence of 35.3% was assumed, which corresponded to the highest prevalence of *L. monocytogenes* found among the licensed farms in NYS (data provided by the NYS Department of Agriculture and Markets). Milk samples were collected monthly from this high-prevalence raw milk farm between November 2006 and October 2008.

The second scenario described the risk of listeriosis among dairy producers, farm personnel and their families if the milk consumed is from a dairy farm (whose milk is not intended to be sold as raw milk) in which *L. monocytogenes* is frequently found in milk samples (Latorre et al., 2009; Pradhan et al., 2009; Latorre et al., 2010). For this scenario, a 25.4% prevalence of *L. monocytogenes* in milk was assumed based on the laboratory results of bulk tank milk samples collected between February 2004 and March 2008 from a NYS dairy farm (Latorre et al., unpublished data).

Model simulations and analyses

The risk assessment models for all scenarios (as detailed in Table 5.2) were simulated with the Monte Carlo simulation technique using risk analysis software @Risk 5.5 (Palisade Corp.). Each simulation was performed for 100,000 iterations and the Latin Hypercube sampling method was utilized to sample different values for input parameters from their corresponding distributions. Sensitivity analyses were performed to identify important parameters affecting the risk of listeriosis by running @Risk sensitivity analysis. For sensitivity analysis, the Spearman's correlation coefficients were used to determine the effect of model parameters on the probability of listeriosis per raw milk serving and the number of illnesses on the US population per year.

Table 5.2. Distributions and formulas used to calculate *L. monocytogenes* growth and concentrations in raw milk purchased/obtained from different commercialization pathways/sources.

***L. monocytogenes* Growth and Concentration**		
Raw milk purchased directly from farm tank	**Units**	**Formula**
MPD During Transportation Farm to Home [MPD_1 (DP)]	log (cfu/g)	IF(T_t (DP)<5,RiskUniform(6.5,7.5),(IF(T_t (DP)>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Transportation Farm to Home [G1 (DP)]	log (cfu/ml)	((((0.024*(T_t (DP)-(-2.32)))^2)/LN(10))*(t_t (DP))
Concentration After Transportation Farm to Home [C1 (DP)]	log (cfu/ml)	IF ((IC+ [G1 (DP)])> [MPD1 (DP)],[MPD1 (DP)],(IC+ [G1 (DP)]))
MPD During Storage in Home Refrigerator [MPD2 (DP)]	log (cfu/g)	IF(Tr<5,RiskUniform(6.5,7.5),(IF(Tr>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Storage at Home Refrigerator [G2 (DP)]	log (cfu/ml)	((0.024*(T_r-(-2.32)))^2)/LN(10))*(t_r*24)
Concentration After Storage at Home Refrigerator [C2 (DP)]	log (cfu/ml)	IF([C1 (DP)]> [MPD2 (DP)],[C1 (DP)],IF(([C1 (DP)]+[G2 (DP)])> [MPD2 (DP)], [MPD2 (DP)],([C1 (DP)]+[G2 (DP)])))
Raw milk purchased from farm stores		
MPD During Storage/Display at Farm Store [MPD_1 (FS)]	log (cfu/g)	IF([T_s (FS)]<5,RiskUniform(6.5,7.5),(IF([T_s (FS)]>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth during Storage/Display at Farm Store [G1 (FS)]	log (cfu/ml)	(((0.024*([Ts(FS)]-(-2.32)))^2)/LN(10))*([ts (FS)]*24)
Concentration during Storage/Display at Farm Store [C1 (FS)]	log (cfu/ml)	IF((IC+[G1 (FS)])> [MPD1 (FS)] , [MPD1 (FS)] ,(IC+[G1 (FS)]))
MPD During Transportation Farm Store to Home [MPD_2 (FS)]	log (cfu/g)	IF(T_t (FS)<5,RiskUniform(6.5,7.5),(IF(T_t (FS)>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Transportation Farm Store to Home [G2 (FS)]	log (cfu/ml)	((((0.024*(T_t (FS)-(-2.32)))^2)/LN(10))*(t_t (FS))
Concentration After Transportation Farm Store to Home [C2 (FS)]	log (cfu/ml)	IF([C1 (FS)]> [MPD2 (FS)],[C1 (FS)],IF(([C1 (FS)]+[G2 (FS)])> [MPD2 (FS)], [MPD2 (FS)],([C1 (FS)]+[G2 (FS)])))
MPD During Storage in Home Refrigerator [MPD3 (FS)]	log (cfu/g)	=IF(Tr<5,RiskUniform(6.5,7.5),(IF(Tr>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Storage at Home Refrigerator [G3 (FS)]	log (cfu/ml)	((((0.024*(T_r-(-2.32)))^2)/LN(10))*(t_r*24)
Concentration After Storage at Home Refrigerator [C3 (FS)]	log (cfu/ml)	IF([C2 (FS)]>[MPD3 (FS)],[C2 (FS)],IF(([C2 (FS)]+ [G3 (FS)])>[MPD3 (FS)],[MPD3 (FS)],([C2 (FS)]+ [G3 (FS)])))

Table 5.2. (Continued)

***L. monocytogenes* Growth and Concentration**		
Raw milk purchased at retail stores	**Units**	**Formula**
MPD During Holding at Farm Before Shipping to Retail [MPD_1 (R)]	log (cfu/g)	IF([Th (R)]<5,RiskUniform(6.5,7.5),(IF([Th (R)]>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Holding at Farm Before Shipping to Retail [G_1(R)]	log (cfu/ml)	(((0.024*([Th (R)]-(-2.32)))^2)/LN(10))* (th (R))
Concentration After Holding at Farm Before Shipping to Retail [C1 (R)]	log (cfu/ml)	IF((IC+[G1(R)])>[MPD1 (R)],[MPD1 (R)],(IC+[G1(R)]))
MPD During Transportation Farm to Retail [MPD_2 (R)]	log (cfu/g)	IF([T_{tr} (R)]<5,RiskUniform(6.5,7.5),(IF[T_{tr} (R)]>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Transportation Farm to Retail [G2 (R)]	log (cfu/ml)	(((0.024*([Ttr (R)]-(-2.32)))^2)/LN(10))*(ttr (R))
Concentration After Transportation Farm to Retail [C2 (R)]	log (cfu/ml)	IF([C1 (R)]> [MPD2 (R)], [C1 (R)],IF(([C1 (R)]+[G2 (R)])> [MPD2 (R)], [MPD2 (R)], ([C1 (R)]+[G2 (R)])))
MPD During Storage/Display at Retail [MPD_3 (R)]	log (cfu/g)	IF([Ts (R)]<5,RiskUniform(6.5,7.5),(IF([Ts (R)]>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth during Storage/Display at Retail [G3 (R)]	log (cfu/ml)	(((0.024*([Ts (R)]-(-2.32)))^2)/LN(10))*([ts (R)]*24)
Concentration after Storage/Display at Retail [C3 (R)]	log (cfu/ml)	IF([C2 (R)]>[MPD3 (R)] ,[C2 (R)],IF(([C2 (R)]+[G3 (R)])>[MPD3 (R)] ,[MPD3 (R)] , ([C2 (R)]+[G3 (R)])))
MPD During Transportation Retail to Home [MPD_4 (R)]	log (cfu/g)	IF([Tt (R)]<5,RiskUniform(6.5,7.5),(IF[Tt (R)]>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Transportation Retail to Home [G4 (R)]	log (cfu/ml)	(((0.024*([Tt (R)]-(-2.32)))^2)/LN(10))*(t_t (R))
Concentration After Transportation Retail to Home [C4 (R)]	log (cfu/ml)	IF([C3 (R)]> [MPD4 (R)],[C3 (R)],IF(([C3 (R)]+[G4 (R)])> [MPD4 (R)], [MPD4 (R)], ([C3 (R)]+[G4 (R)])))
MPD During Storage in Home Refrigerator [MPD5 (R)]	log (cfu/g)	=IF(Tr<5,RiskUniform(6.5,7.5),(IF(Tr>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Storage at Home Refrigerator [G5 (R)]	log (cfu/ml)	(((0.024*(T_r-(-2.32)))^2)/LN(10))*(t_r*24)
Concentration After Storage at Home Refrigerator [C5 (R)]	log (cfu/ml)	IF([C4 (R)]> [MPD5 (R)], [C4 (R)],IF(([C4 (R)]+[G5 (R)])> [MPD5 (R)], [MPD5 (R)], ([C4 (R)]+[G5 (R)])))
Raw milk consumed on farms		
MPD During Storage in Home Refrigerator [MPD1 (FC)]	log (cfu/g)	=IF(Tr<5,RiskUniform(6.5,7.5),(IF(Tr>7,RiskUniform(7.5,8.5),RiskUniform(7,8))))
Growth During Storage at Home Refrigerator [G1 (FC)]	log (cfu/ml)	(((0.024*(T_r-(-2.32)))^2)/LN(10))*(t_r*24)
Concentration After Storage at Home Refrigerator [C1 (FC)]	log (cfu/ml)	IF((IC+ [G1 (FC)])>[MPD1 (FC)],[MPD1 (FC)],(B2+ [G1 (FC)]))

RESULTS

Estimated number of listeriosis cases due to consumption of raw milk sold by permitted raw milk dealers and due to consumption of raw milk on farms by farm personnel

The probability of illnesses per raw milk serving from each commercialization pathway as well as the number of cases per year is shown in Table 5.3. The median number of listeriosis cases per year associated with consumption of raw milk purchased directly from licensed farms, farm stores, and retail stores for intermediate susceptible populations was 6.6×10^{-7}, 3.8×10^{-5}, and 5.1×10^{-5}, respectively. In more susceptible populations, the median number of cases per year was 2.7×10^{-7} (perinatal), 1.4×10^{-6} (elderly) for DP, 1.5×10^{-5} (perinatal), 7.8×10^{-5} (elderly) for FS, and 2.1×10^{-5} (perinatal), 1.0×10^{-4} (elderly) for R.

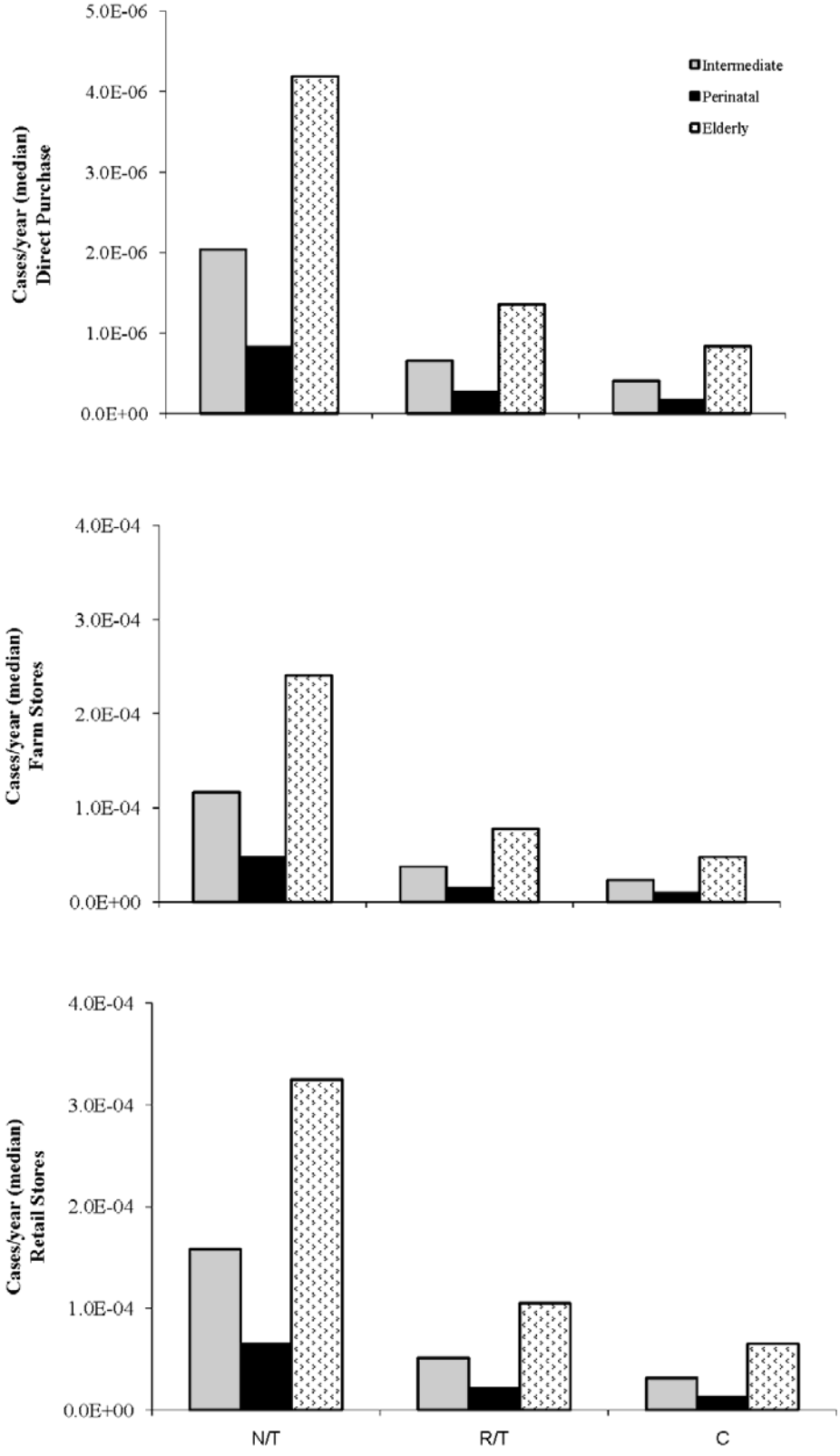
Both the probability of illnesses per serving and the number of listeriosis cases per year were greater for raw milk purchased from retail stores than for milk purchased from farm stores. The lowest probabilities of listeriosis per serving and the number of annual cases were observed when raw milk was purchased directly from farm tanks (Table 5.3). For all scenarios modeled, the probability of illness per serving was greater for perinatal populations than for elderly or intermediate populations, while the number of listeriosis cases per year was greater in elderly populations (Table 5.3).

A reduction in the number of cases per year for all populations was observed when a raw milk testing program was in place, especially when routine testing and recall of milk was conducted (Figure 5.2). For all three scenarios (DP, FS, R), a 5-fold decrease in the median number of listeriosis cases per year was observed when raw milk was

Table 5.3. Probability of illness per serving and number of listeriosis cases per year in the US associated with consumption of raw milk purchased directly from farm bulk tanks (DP), from farm stores (FS), and from retail stores (R). Data presented here corresponds to the median, 5th percentile, and 95th percentile obtained after 100,000 iterations using @Risk 5.5.

Population/Raw Milk Commercialization	**Probability of Illness/ Serving** Median (5th, 95th percentiles)	**Number of Cases/ Year** Median (5th, 95th percentiles)
Intermediate Population-DP	1.8×10^{-15} (6.3×10^{-17}, 4.8×10^{-11})	6.6×10^{-7} (2.3×10^{-8}, 1.7×10^{-2})
Perinatal population-DP	1.1×10^{-13} (3.8×10^{-15}, 2.8×10^{-9})	2.7×10^{-7} (9.4×10^{-9}, 7.0×10^{-3})
Elderly-DP	1.8×10^{-14} (6.3×10^{-16}, 4.7×10^{-10})	1.4×10^{-6} (4.7×10^{-8}, 3.5×10^{-2})
Intermediate Population-FS	1.0×10^{-13} (4.0×10^{-16}, 3.2×10^{-8})	3.8×10^{-5} (1.4×10^{-7}, 12)
Perinatal population-FS	6.2×10^{-12} (2.3×10^{-14}, 1.9×10^{-6})	1.5×10^{-5} (5.8×10^{-8}, 4.8)
Elderly-FS	1.0×10^{-12} (3.9×10^{-15}, 3.2×10^{-7})	7.8×10^{-5} (2.9×10^{-7}, 24)
Intermediate Population-R	1.4×10^{-13} (5.5×10^{-16}, 4.0×10^{-8})	5.1×10^{-5} (2.0×10^{-7}, 14)
Perinatal population-R	8.3×10^{-12} (3.2×10^{-14}, 2.3×10^{-6})	2.1×10^{-5} (8.0×10^{-8}, 5.8)
Elderly-R	1.4×10^{-12} (5.4×10^{-15}, 3.9×10^{-7})	1.0×10^{-4} (4.0×10^{-7}, 29)

Figure 5.2. Effect of raw milk testing (N/T= no testing, R/T= testing as a screening to issue a raw milk permit, C= routine testing and recall of raw milk) on the number of listeriosis cases (median value) per year associated with raw milk consumption obtained by direct purchase from the farms, farm stores, and retail stores. N/T, R/T, and C were calculated based on the prevalence of *L. monocytogenes* in bulk tank milk on US farms (6.5%), the overall prevalence of *L. monocytogenes* in raw milk from NYS (2.1%), and prevalence of the pathogen in raw milk from NYS that was commercialized between 2003 and 2009 (1.3%) (milk under recall not included).


5.0E-06
4.0E-06
3.0E-06
2.0E-06
1.0E-06
0.0E+00
Cases/year (median)
Direct Purchase
Intermediate
Perinatal
Elderly
4.0E-04
3.0E-04
2.0E-04
1.0E-04
0.0E+00
Cases/year (median)
Farm Stores
4.0E-04
3.0E-04
2.0E-04
1.0E-04
0.0E+00
Cases/year (median)
Retail Stores
N/T
R/T
C

subjected to initial screening and routine testing (i.e. monthly milk testing) and was recalled if *L. monocytogenes* was present, as compared to no testing at all (Figure 5.2). When requiring only an initial milk screening test prior to issuing a raw milk sales permit, a 3.1-fold reduction in the median number of listeriosis cases per year was observed, compared with raw milk in which no initial testing was performed. Adding routine testing, monitoring, and recall (if positive) of milk after a raw milk permit was issued in addition of the initial screening of milk resulted in a 1.6-fold reduction of the annual listeriosis cases (Figure 5.2).

The probability of listeriosis per raw milk serving and the annual number of listeriosis cases associated with consumption of raw milk on dairy farms are shown in Table 5.4. The median number of listeriosis cases per year associated with raw milk consumed by dairy producers and farm personnel on farms corresponded to 1.4×10^{-7}.

Estimated number of listeriosis cases due to consumption of raw milk from farms with high prevalence of L. monocytogenes in bulk tank milk

The probability of listeriosis per raw milk serving associated with consumption of licensed raw milk from a high prevalence farm for each of the commercialization pathways is shown in Table 5.5. For all scenarios and in all target populations, the probability of listeriosis per raw milk serving if milk came from this high prevalence farm was approximately 17 times greater than the probability of listeriosis associated with milk from all raw milk farms (Table 5.3).

Probabilities of listeriosis per serving and the number of listeriosis cases among dairy producers and farm personnel who consume raw milk from a farm with high prevalence of *L. monocytogenes* in raw milk are shown in Table 5.4. A similar trend as

Table 5.4. Probability of illnesses per serving and number of listeriosis cases per year associated with consumption of raw milk on farms by dairy producers and farm personnel based on the overall prevalence of *L. monocytogenes* in bulk tank milk from US dairy farms, and raw milk from a farm with a high prevalence of *L. monocytogenes*.

	Overall Dairy Farms[a]		**High Prevalence Dairy Farm[b]**	
Population	**Probability of Illness/ Serving**	**Number of Cases/ Year**	**Probability of Illness/ Serving**	**Number of Cases/ Year**
	Median (5^{th}, 95^{th} percentiles)	Median (5^{th}, 95^{th} percentiles)	Median (5^{th}, 95^{th} percentiles)	Median (5^{th}, 95^{th} percentiles)
Intermediate	5.0×10^{-15} (1.8×10^{-16}, 1.4×10^{-10})	1.4×10^{-7} (4.9×10^{-9}, 3.9×10^{-3})	2.0×10^{-14} (7.0×10^{-16}, 5.6×10^{-10})	5.4×10^{-7} (1.9×10^{-8}, 1.5×10^{-2})
Perinatal	3.0×10^{-13} (1.1×10^{-14}, 8.4×10^{-9})	N/A[c]	1.2×10^{-12} (4.1×10^{-14}, 3.3×10^{-8})	N/A
Elderly	5.0×10^{-14} (1.8×10^{-15}, 1.4×10^{-9})	N/A	1.9×10^{-13} (6.9×10^{-15}, 5.5×10^{-9})	N/A

[a] Prevalence of *L. monocytogenes* in bulk tank milk samples = 6.5% (Van Kessel et al., 2004)

[b] Prevalence of *L. monocytogenes* in bulk tank milk samples = 25.4% (Latorre et al., unpublished data)

[c] N/A= Not available

Table 5.5. Probability of listeriosis per serving associated with consumption of raw milk purchased directly from farm tanks (DP), from farm stores (FS), and from retail stores (R) using as an example raw milk from a licensed farm with a high prevalence of *L. monocytogenes* in bulk tank milk (35.3%)

Population/Raw Milk Commercialization	**Probability of Illness/ Serving** Median (5^{th}, 95^{th} percentiles)
Intermediate Population-DP	3.1×10^{-14} (1.1×10^{-15}, 8.0×10^{-10})
Perinatal population-DP	1.8×10^{-12} (6.3×10^{-14}, 4.7×10^{-8})
Elderly-DP	3.0×10^{-13} (1.1×10^{-14}, 7.9×10^{-9})
Intermediate Population-FS	1.8×10^{-12} (6.7×10^{-15}, 5.4×10^{-7})
Perinatal population-FS	1.0×10^{-10} (3.9×10^{-13}, 3.2×10^{-5})
Elderly-FS	1.7×10^{-11} (6.6×10^{-14}, 5.4×10^{-6})
Intermediate Population-R	2.4×10^{-12} (9.2×10^{-15}, 6.7×10^{-7})
Perinatal population-R	1.4×10^{-10} (5.4×10^{-13}, 3.9×10^{-5})
Elderly-R	2.4×10^{-11} (9.0×10^{-14}, 6.6×10^{-6})

that observed in licensed raw milk was observed in raw milk consumed on a high prevalence farm. The probability of listeriosis and the number of cases were approximately 4 times greater than those observed in all dairy farms. This increase is a direct function of the increase in the prevalence of *L. monocytogenes* in milk from 6.5% to 25.4% (Table 5.4).

Effect of model parameters affecting the risk of listeriosis cases

Sensitivity analyses indicated that the parameter with the greatest impact on the probability of listeriosis and the annual number of listeriosis cases for all scenarios (licensed raw milk and raw milk that was consumed on farms by dairy producers and farm personnel) was the temperature of the home refrigerator (Figure 5.3). The order of importance of the parameters following home refrigerator temperature was time of storage in the home refrigerator and serving size for raw milk purchased directly from milk tanks and milk consumed on farms (Figures 5.3 and 5.4). For raw milk that was commercialized in stores (farm stores and retail), the temperature and time of storage and display at the store were the second and third most important parameters affecting the risk of listeriosis, respectively (Figure 5.3).

DISCUSSION

In this risk assessment model of listeriosis associated with consumption of raw milk, the probability of illnesses per raw milk serving was low, although this risk was increased as more potential growth steps were included in the model. For example, the probability of illness and the median number of cases was greater for raw milk that was purchased from retail stores than for milk purchased directly from farm tanks. Given the overall prevalence of *L. monocytogenes* in raw milk and the number of

Figure 5.3. Tornado graphs showing the impact of the different parameters on the probability of illnesses and the number of listeriosis cases per year associated with consumption of licensed raw milk purchased from different sources.

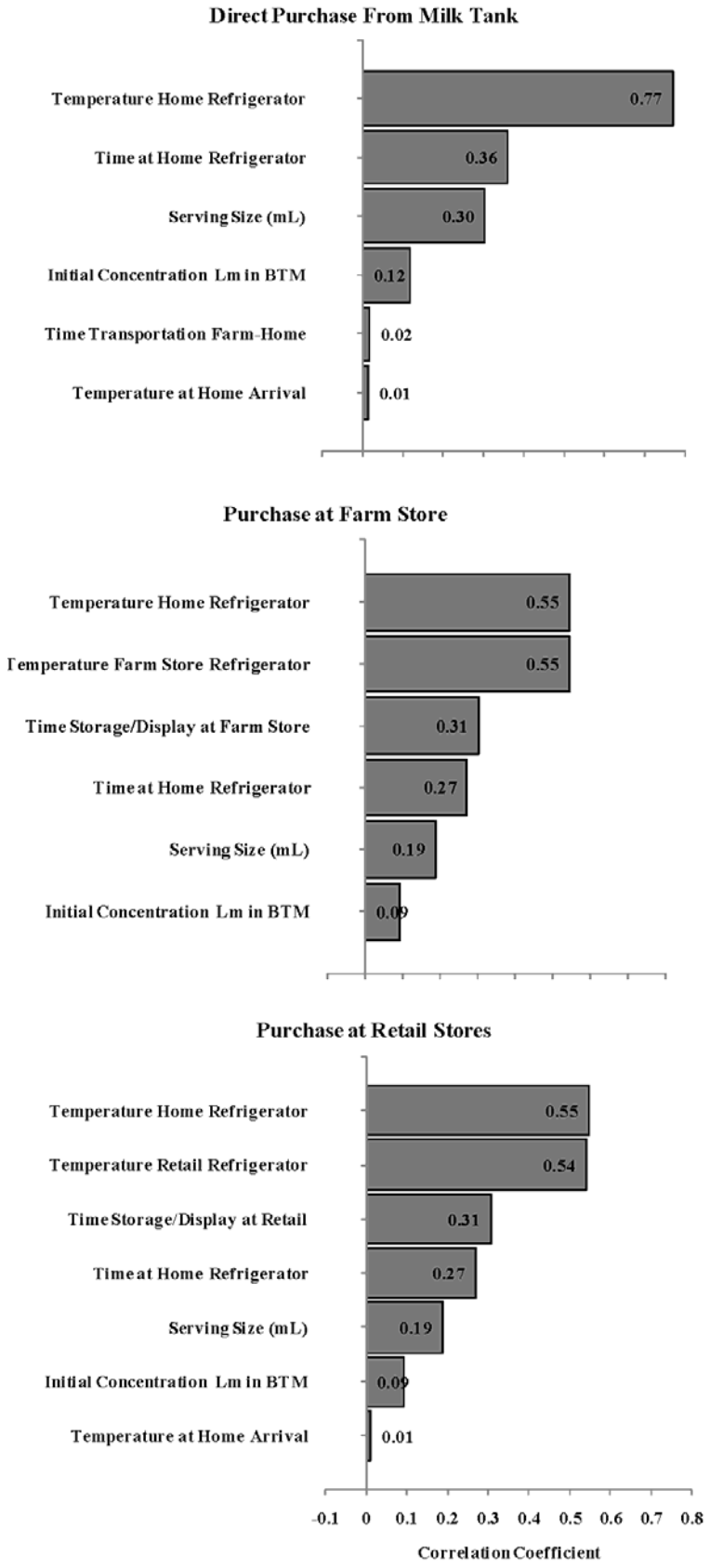
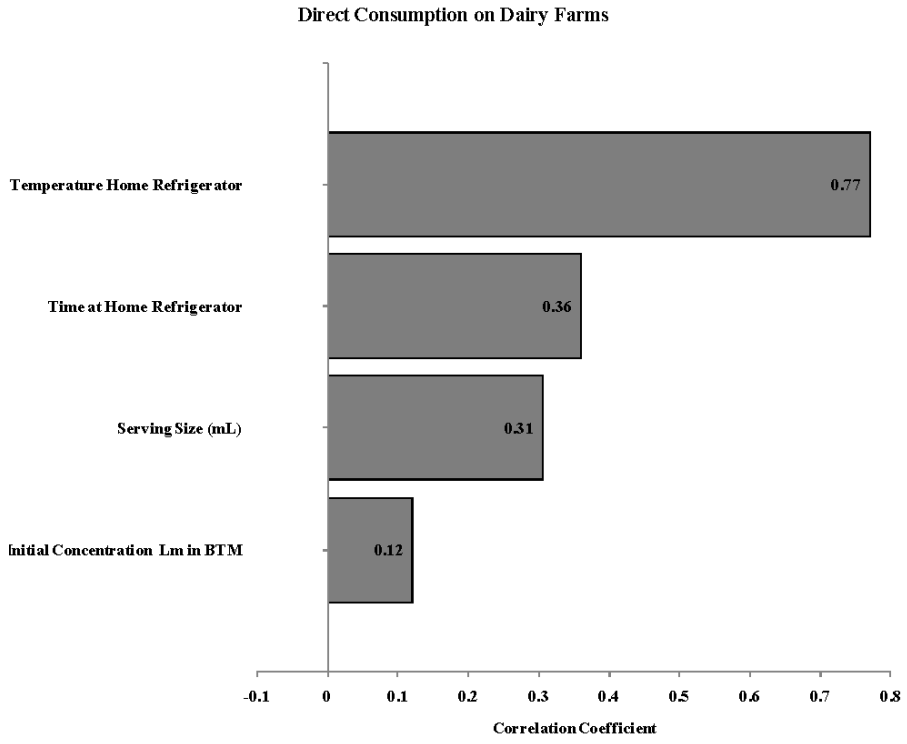

Direct Purchase From Milk Tank
Temperature Home Refrigerator
0.77
Time at Home Refrigerator
0.36
Serving Size (mL)
0.30
Initial Concentration Lm in BTM
0.12
Time Transportation Farm-Home
0.02
Temperature at Home Arrival
0.01
Purchase at Farm Store
Temperature Home Refrigerator
0.55
Temperature Farm Store Refrigerator
0.55
Time Storage/Display at Farm Store
0.31
Time at Home Refrigerator
0.27
Serving Size (mL)
0.19
Initial Concentration Lm in BTM
0.09
Purchase at Retail Stores
Temperature Home Refrigerator
0.55
Temperature Retail Refrigerator
0.54
Time Storage/Display at Retail
0.31
Time at Home Refrigerator
0.27
Serving Size (mL)
0.19
Initial Concentration Lm in BTM
0.09
Temperature at Home Arrival
0.01
-0.1 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Correlation Coefficient

Figure 5.4. Tornado graph generated by @Risk 5.5 during the sensitivity analysis of the risk assessment model of listeriosis. Graph shows the effect of the most important parameters on the probability of illness and the annual number of listeriosis cases associated with consumption of raw milk on dairy farms.


Direct Consumption on Dairy Farms
Temperature Home Refrigerator
Time at Home Refrigerator
Serving Size (mL)
Initial Concentration Lm in BTM
0.77
0.36
0.31
0.12
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Correlation Coefficient

servings per year used in this model; the expected risk of listeriosis (95th percentile) for the intermediate-age population is approximately 1 case in the population every 58 years if raw milk is purchased directly from milk tanks. The risk of listeriosis for the perinatal and elderly populations was estimated as 1 case every 142 and every 28 years, respectively, if raw milk is obtained from this source. On the contrary, if raw milk is purchased from a retail store, the estimated risk of listeriosis for the intermediate, perinatal, and elderly populations corresponds to 14, 5.8, and 29 cases per year, respectively.

This increased risk may be explained by the ability of *L. monocytogenes* to grow at refrigeration temperatures (Swaminathan, 2001). In our model, sensitivity analysis showed that the home refrigerator temperature was the most important parameter affecting the number of cases associated with raw milk consumption. For example, increasing the temperature of the home refrigerator from 4˚C to 8˚C resulted in a ~7-fold increase in the number of cases (data not shown). Another important consideration is that, in our model, raw milk purchased directly from farms was more likely to be consumed sooner than milk purchased from a retail store because of additional storage time during display in the latter. As more time/temperature combination steps are included in the model, the chances of growth of *L. monocytogenes* in the raw milk increases as well, with a subsequent increase in probability of illness per serving.

Although outbreaks of milk-borne listeriosis are infrequent, when they occur a large number of cases are usually involved. The annual number of cases in our model reflects what has actually happened in past listeriosis outbreaks that were linked to milk consumption (Fleming et al., 1985; Dalton et al., 1997; CDC, 2008). Due to the

skewness of the probability distributions (right-skewed) most of the time the probability of having listeriosis cases due to raw milk consumption is very low but the risk of sporadic outbreaks with large number of cases exists, as reflected in the upper quartile of distributions.

Overall the annual number of listeriosis cases due to raw milk consumption is predicted to be low by this model. It is difficult to validate this finding as there is a lack of data about the actual number of raw milk consumers. In our study, the number of annual servings for raw milk corresponded to 0.5% of the total milk consumption in the US (FDA/FSIS, 2003). This estimated number of servings was based on studies conducted over a decade ago, which may not reflect the current trends in raw milk consumption. For example, in NYS the number of licensed raw milk farms increased from 3 farms in 2003 to 25 in 2009 (NYS Department of Agriculture and Markets, unpublished data), probably as a consequence of a growing number of raw milk consumers. Hence, because of this underestimation in the consumption of raw milk, the predicted number of listeriosis cases per year may be underrepresented in our model.

The number of listeriosis cases associated with raw milk consumption on farms may be also underestimated as a consequence of insufficient data about raw milk intake in farm worker populations. In our model, only dairy producers and farms personnel are considered as raw milk consumers because no data are available regarding raw milk consumption by their families. In a study conducted by Hoe and Ruegg (2006), 40% of producers declared that people associated with surveyed farms consumed raw milk or raw milk products on a regular basis. Information regarding the number of people

related to dairy farms, or number of family members of producers/farm personnel, however, is not available.

Since there is no field data to anchor the number of cases in our model, it was not possible to precisely estimate the absolute risk of severe listeriosis. It is therefore also not possible to conclude whether our model predictions or the FDA model predictions are closer to the observed listeriosis cases in the US. As expected, a close relationship between prevalence of *L. monocytogenes* in raw milk and the values of disease incidence was observed. As the prevalence in raw milk increased, the risk of severe listeriosis due to raw milk consumption increased as well. In our model, we assumed a *L. monocytogenes* detection limit of 0.04 cfu/mL when culturing 25 mL of milk. A negative test implies either the absence or the presence of *L. monocytogenes* under the detection level of the test. This likely results in an underestimation of the actual risk of listeriosis.

Raw milk testing performed by regulatory agencies dramatically decreased both the probability of illness per serving and the number of annual cases of listeriosis. In addition, routine testing identifies farms with a high prevalence of *L. monocytogenes* in the milk. For example, the probability of listeriosis per serving if raw milk came from the farm with the highest prevalence in NYS (35.3%) was 17 times greater than the probability observed in all raw milk farms (all milk samples collected from all farms, between 2003 and 2009). Routine testing allowed the recall of milk from this farm which reduced the risk for the consumers. As farms that tested positive had a higher risk of testing positive again, routine testing with prohibition of raw milk sales upon identification of a positive sample effectively lowered the prevalence of *L. monocytogenes* contamination in the raw milk supply. As a consequence of the testing

policy, one high-prevalence farm actually stopped selling raw milk to customers in 2008.

Screening tests before issuing raw milk permits also proved to be a useful tool for reduction of the risk of listeriosis associated with raw milk consumption, although not as effective as a continuous raw milk surveillance program (i.e. monthly testing of raw milk). Other studies conducted by our group (Latorre et al., 2009; Latorre et al., 2010) have also shown the importance of herd monitoring programs. For example, repeated bulk tank milk testing monitored the presence or absence of pathogens over time, and identified potential sources and reservoirs of *L. monocytogenes* on dairy farms and in bulk tank milk.

On the other hand, milk that it is not intended to be sold as licensed raw milk, is transported to dairy processors for pasteurization. Hence, this milk is not routinely tested for *L. monocytogenes* and other major foodborne pathogens such as *E. coli* 0157:H7, *Campylobacter*, or *Salmonella*. The results of the effect of testing in our model also suggest that an increased risk of listeriosis (and other milk-borne zoonotic diseases) may be expected for people who consume raw milk on farms or for raw milk consumers who obtain their milk through cow leasing programs since raw milk is not usually tested in this situation.

Our risk assessment model characterized the risk of listeriosis associated with licensed raw milk consumption for three different markets. Our model also allowed the comparison of the impact of each of the different commercialization pathways on the risk of listeriosis for consumers. In addition, to our knowledge, this is the first study designed to assess the risk of listeriosis among people who consume raw milk on

farms, behavior that has been reported to be common (Jayarao and Henning, 2001; Hoe and Ruegg, 2006; Jayarao et al., 2006).

Prevalence of *L. monocytogenes* in licensed raw milk in our model was based on laboratory results of raw milk samples from all licensed farms in NYS over a 7-year period, providing a good insight into the actual prevalence of the pathogen in raw milk. Furthermore, all components of the path that raw milk normally follows between when it is collected from the cows and when it reaches the consumer's table were included, as an attempt to accurately reproduce real life scenarios. An effort was made to include as much data and information specific for the raw milk industry as possible.

An important short coming in our model, however, was the lack of data available regarding the proportion of raw milk that is sold directly to consumers, sold in on-farm stores, and in retail stores in the US. For example, in states in which retail sales of raw milk are allowed, raw milk may also be acquired directly from farms or other sources, as reported in the study conducted by Headrick et al. (1997) in California. Although a greater percentage of raw milk is probably sold in retail stores in such states, no data about the contribution of each of the markets to the total percentage of raw milk sales are currently available. Hence, in our model the probability and cases of listeriosis due to raw milk consumption can only be analyzed as if raw milk was sold in one of the markets (i.e. farms, farm stores, or retail) or another.

Another limitation of our model is that there is no information available on the number of highly susceptible people that work on dairy farms, or susceptible family members of farm personnel/producers who may drink raw milk on farms. Due to the lack of

data, it was assumed that all producers/farm personnel in our model belong to the intermediate-age population, which may not accurately reflect the composition of farm populations.

Both the probability of listeriosis and number of annual cases in our study differ from those reported in the FDA/FSIS RA (2003) for the same food category. The median number of listeriosis cases per raw milk serving in the FDA/FSIS model were 2.9×10^{-9}, 9.9×10^{-7}, and 2.2×10^{-8} for intermediate, perinatal, and elderly populations, respectively. In our study, these figures in all scenarios were lower than those obtained in the FDA/FSIS RA (2003). Differences in the risk model and in the data used in each model, for example temperature distributions, time distributions, as well as the prevalence of *L. monocytogenes* in raw milk (FDA/FSIS, 2003), may explain this disparity.

Pasteurization of milk effectively eliminates *L. monocytogenes* and other pathogenic organisms that may cause disease without causing significant change to the nutritional properties of the milk (Potter et al., 1984; LeJeune and Rajala-Schultz, 2009). In a study conducted in 2000 (Frye and Donnelly, 2005), only 0.018% of pasteurized milk samples obtained from retail stores in the US were positive for *L. monocytogenes*, which is considerably lower than the prevalence of the pathogen observed in raw milk in our study (2.1% overall prevalence) or other bulk tank milk studies conducted in different states of the US (ranging from 2.8% to 4.6%) (Jayarao and Henning, 2001; Jayarao et al., 2006) and nationwide (6.5%) (Van Kessel et al., 2004). Both the significantly low prevalence of *L. monocytogenes* as well as the low concentrations of the pathogen in pasteurized milk (if found) (Frye and Donnelly, 2005) explain the low risk of listeriosis associated with consumption of pasteurized milk.

Raw milk has been associated with many outbreaks involving pathogens such as *Campylobacter*, *Salmonella*, *E. coli* O157: H7 (Denny et al., 2008; Keene et al., 1997; CDC, 2002; CDC, 2003). In addition, the presence of pathogenic bacteria in raw milk, including *L. monocytogenes,* has been frequently reported (http://www.agmkt.state.ny.us/AD/alertList.asp). Hence, consumption of raw milk from any source is not recommended. However, there are currently 29 states that allow raw milk sales and many people believe raw milk consumption to be a healthier alternative to pasteurized milk consumption. Despite the low probability of acquiring listeriosis from a single raw milk serving, the serious consequences of the disease such as miscarriages, stillbirths, meningitis, or even death (Fleming et al., 1985, Linnan et al., 1988) and the high hospitalization rates attributed to listeriosis (CDC 2010) should not be disregarded by raw milk consumers.

Our study quantified the risk of listeriosis among raw milk consumers. Quantification of the risks associated with consumption of raw milk is necessary from a public health perspective as it helps to increase the awareness of potential dangers for the health of consumers, which may contribute to reduce the risk of milk-borne illnesses. For example, in a study conducted by Jayarao et al (2006), raw milk was more frequently consumed among dairy producers who did not have knowledge about the presence of pathogenic bacteria in milk.

Even though the consumption of raw milk is not recommended (NEHA, 2008), there is still a growing number of people who choose and prefer to drink raw milk. Raw milk sales regulations and surveillance by regulatory agencies contribute to the improvement of raw milk production standards which help protect consumers' health by reducing the risks associated with raw milk consumption. In addition to the

contribution of monitoring programs, our study showed that measures taken by consumers and sellers to maintain raw milk at proper refrigeration temperatures and to consume milk more immediately after purchase would also help in reducing the risk of listeriosis associated to consumption of this product.

ACKNOWLEDGEMENTS

Financial support for this work was provided by the USDA-Agricultural Research Service (Agreement No. 58-1265-3-155, 58-1265-3-156, 58-1265-3-158, 58-1265-4-020, and 58-1265-8-064) for the Regional Dairy Quality Management Alliance.

We thank the raw milk producer, raw milk plant manager, and the vice-president of a milk retailer, who kindly provided valuable information for our study.

We gratefully acknowledge the help received during this research by the laboratory personnel and staff at Quality Milk Production Services-Cornell University and the Food Laboratory at the New York State Department of Agriculture and Markets.

Mention of a trade name, vendor, proprietary product, or specific equipment is not a guarantee or a warranty by the U.S. Department of Agriculture and does not imply an approval to the exclusion of other products or vendors that also may be suitable.

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CHAPTER 6

DISCUSSION

The major contributions of the studies in this thesis to the current knowledge of L. monocytogenes on dairy farms

Longitudinal studies are helpful to better understand the epidemiology of *L. monocytogenes* (Latorre et al., 2009) and other pathogens (Van Kessel et al., 2007; Pradhan et al., 2009) on dairy farms. On the study farm, the present longitudinal study allowed us to analyze changes in prevalence of *L. monocytogenes* in feces, environment, in-line milk filters, or bulk tank milk samples over time. We were also able to investigate the appearance of a potential new bacterial reservoir in milking equipment (Latorre et al., 2009). For example, a cross-sectional study conducted on the study farm before November 2005 would have detected the presence of *L. monocytogenes* only in environmental and fecal samples. On the contrary, if a cross-sectional study would have been carried out after the appearance of *L. monocytogenes* in bulk tank milk (November 2005), the observation that the pathogen was never isolated from in-line milk filters and bulk tank milk in the earlier stages of the study would have been missed.

In combination with molecular subtyping using pulsed-field gel electrophoresis (**PFGE)** our longitudinal epidemiologic study allowed us to characterize *L. monocytogenes* isolates collected over time, detecting thus persistence of certain PFGE types which suggested the presence of a biofilm in milking equipment.

Persistence of *L. monocytogenes* (Miettinen et al., 1999; Vogel et al., 2001; Thimothe et al., 2004) as well as the presence of bacterial biofilms in food processing environments (Carpentier and O'Cerf, 1993; Holah and Gibson, 2000) has been a problem frequently reported in the food industry. To our knowledge, our study is the first reporting the presence of *L. monocytogenes*-containing biofilms on a dairy farm.

Our studies showed that the presence of *L. monocytogenes* in milk may be caused by *L. monocytogenes* that colonize and persist in milking equipment, and not only by mastitic cows shedding *Listeria* in milk (Fedio et al., 1990; Jensen et al., 1996; Winter et al., 2004) or by the introduction of feces (or environmental materials) into the milk line due to inappropriate milking hygiene. Surface materials and environmental conditions within a milking system (i.e low temperature in milk tank, humidity, presence of nutrients) can provide a permissive habitat for colonization and subsequent attachment of bacteria. Hence, milking harvesting equipment should be acknowledged as a potential reservoir of *L. monocytogenes* on farms (Latorre et al., 2010) along with other well documented sources such as healthy cattle (Nightingale et al., 2004) or poorly fermented silage (Fenlon, 1985).

With the use of PFGE and our *L. monocytogenes* collection, our study provided an opportunity to gain insight about the ecology of *L. monocytogenes* strains in the farm environment. For example, we were able to analyze persistence and changes in the ecology of *L. monocytogenes* strains on the farm over time (Latorre et al., unpublished). In addition, we were able to observe phenomena such as an apparent strain dominance or competition in biofilms (reviewed by Moons et al., 2009) and the ability of certain strains to adapt to specific environments, such as the milking system, and the consequences of such adaptation.

Finally, our study was the first in investigating the risk of listeriosis associated with consumption of raw milk from licensed farms and raw milk consumed on dairy farms by farm personnel. Although a previous risk assessment model (FDA/FSIS, 2003) reported the risk of listeriosis due to consumption of unpasteurized milk, this previously published study did not assess the impact of each commercialization pathway (nor of milk consumed on farms). Although the consumption of raw milk is not recommended (NEHA, 2008), there is still a growing number of raw milk advocates and a large number of people who consume raw milk on farms (Jayarao and Henning, 2001; Jayarao et al., 2006; Hoe and Ruegg, 2006). As consumption of raw milk may continue, suggested measures that may be taken by regulatory agencies as well as guidelines for raw milk consumers aimed to reduce the risk of listeriosis are an important contribution of our study to public health.

Benefits and drawbacks of performing L. monocytogenes research on a single farm

In our study on the epidemiology of *L. monocytogenes*, the focus on a single dairy farm allowed an in-depth analysis of very precise and comprehensive data collected over time from the same sources. Analysis of the prevalence of *L. monocytogenes* in different locations throughout the farm, identification of a potential reservoir, as well as dynamics of strains on the farm was possible because of a consistent and frequent monitoring of the study farm.

Sufficient data to better understand the dynamics of *L. monocytogenes* strains on dairy farms cannot be obtained reliably by performing sporadic samplings on many farms. Therefore, a more intensive monitoring was necessary, an inherent disadvantage is that it would be difficult to implement this on many farms.

In addition, repeated sampling on a single farm allowed us to obtain significant knowledge of the farm and animals management, farm environment, milking routine, etc., which is useful to implement "customized" measures aimed to the control of the pathogen on a particular premise. For example, in our study, we identified milking equipment as a potential source of *L. monocytogenes* in bulk tank milk. Hence, guidelines for control of *L. monocytogenes* on the study farm were not only focused on the farm environment, but especially on milking equipment. Moreover, by means of a consistent monitoring on this single farm, we were able to see the impact of interventions or changes aimed to control *L. monocytogenes* in bulk tank milk, as discussed later.

Intensive research on a single farm may or may not provide us with insights that may be common to many dairy farms. Dynamics of microorganisms vary from farm to farm (Pradhan et al., 2009) and conclusions and recommendations for control of *L. monocytogenes* that are drawn from our single-farm study may therefore not be generalizable to other farms. Furthermore, unique characteristics of each farm, such as environment, number of animals, herd composition, management etc. may be relevant for the epidemiology of *L. monocytogenes*. Therefore, our single- farm study may not have been able to analyze some of the potential effect that these factors may have on the ecology and dynamics of *L. monocytogenes* on other dairy farms. Hence, collection of comprehensive data obtained from only one farm may be considered a starting point for further research on other farms.

Potential sources of L. monocytogenes in bulk tank milk

Bulk tank milk samples collected from the farm in a weekly regime were negative for *L. monocytogenes* from February 2004 until November 2005. *Listeria monocytogenes*

was isolated from bulk tank milk samples on a regular basis starting November 2005, and it was also isolated on a regular basis from in-line milk filters samples since earlier that year (September 2005) (Figure 6.1). Interestingly, *L. monocytogenes* was only isolated sporadically from feces and environmental samples. The low prevalence of the pathogen in this category of samples contrasted with the high frequency of isolation from samples collected from bulk tank milk or in-line milk filter samples (Latorre et al., 2009). Furthermore, we observed less diversity of PFGE types among *L. monocytogenes* isolates from bulk tank milk (Simpson's Index of Diversity, **SID**= 0.65) (Hunter and Gaston, 1988) as compared to fecal and environmental isolates (SID= 0.96 for each) and the persistence of 3 PFGE types over the study period (Latorre et al., unpublished).

The presence of cows suffering from mastitis caused by *L. monocytogenes* may be a potential source of the pathogen in bulk tank milk, as they may shed the pathogen through infected udder quarters (Fedio et al., 1990; Jensen et al., 1996; Winter et al., 2004). Mastitis caused by *Listeria*, however, is very infrequent (Fedio et al., 1990). For example, *L. monocytogenes* was isolated from milk of only 0.04% of 1,132,958 cows in a study conducted over a 23-year period in Denmark (Jensen et al., 1996). On the study farm, milk from all cows that present clinical mastitis is routinely analyzed, and *L. monocytogenes* has never been isolated. Hence, in our study, it was unlikely that the persistent presence of *L. monocytogenes* in the milking system was due to cows suffering from mastitis caused by *L. monocytogenes*.

Fecal contamination may be a potential source of *L. monocytogenes* in bulk tank milk as well (Wagner et al., 2005). If cows are shedding *L. monocytogenes*, and inappropriate hygiene during milking occurs, fecal material containing the pathogen

Figure 6.1. Bulk tank somatic cell count (SCC), standard plate count (SPC), and preliminary incubation count (PIC) on bulk tank milk from the study farm over a 6.3-year period. Red arrows show the date of appearance of *L. monocytogenes* in in-line milk filter and bulk tank milk samples. Blue arrow show the date that weekly manual cleaning of milk tank started. Pink arrows show the dates of change of wash system and installation of new hot water unit in the milk tank room, respectively.

Source: Pennsylvania Quality Milk Program, Department of Veterinary Science. Pennsylvania State University.

SCC
SPC
PIC
Counts/mL
1.4e+6
1.2e+6
1.0e+6
4.0e+5
3.0e+5
2.0e+5
1.0e+5
0.0
L. monocytogenes in milk filter
L. monocytogenes in bulk tank milk
Change wash system
New hot water unit
Manual cleaning
of bulk tank milk
2/04
5/04
8/04
11/04
2/05
5/05
8/05
11/05
2/06
5/06
8/06
11/06
2/07
5/07
8/07
11/07
2/08
5/08
8/08
11/08
2/09
5/09
8/09
11/09
2/10
5/10
Date (month/year)

may enter the milking system and cause contamination of milk. In the study farm, PFGE typing of *L. monocytogenes* isolates from fecal samples revealed a large heterogeneity of PFGE types (Latorre et al., Chapter 4). Other studies (Ho et al., 2007a; Borucki et al., 2005) have also described a high diversity among *L. monocytogenes* isolates obtained from cow's feces. As much less diversity of PFGE types was observed among *L. monocytogenes* isolates from bulk tank milk, fecal contamination of milk seems to be an unlikely source of persistent *L. monocytogenes* in bulk tank milk in our study. Our study suggests, however, that fecal strains may have initially entered into the milking system with a subsequent colonization and persistence within a biofilm.

Biofilms can be defined as a group of microorganisms attached to a surface by means of an exopolymeric matrix (Carpentier and O'Cerf, 1993; Hall-Stoodley et al., 2004). Biofilm growth confers upon bacteria increased resistance to diverse sanitizing compounds (Pan et al., 2006) and studies have even shown that bacteria attached to surfaces are more resistant to heat, sanitizers, and the combination of both (Frank and Koffi, 1990). This resilience of bacteria in biofilms to adverse environments may enhance their ability to persist for long periods of time on a given niche.

Persistence of *L. monocytogenes* in food processing plants (Miettinen et al., 1999; Vogel et al., 2001; Thimothe et al., 2004) and in a drain of the milking parlor on a sheep farm (Ho et al., 2007b) has been previously reported. Although these studies did not confirm the presence of *L. monocytogenes* biofilms within the food processing premises, in some of the premises specific niches harboring persistent *L. monocytogenes* strains were identified (Miettinen et al., 1999; Vogel et al., 2001; Thimothe et al., 2004; Ho et al., 2007b).

In our study, by using molecular methods combined with scanning electron microscopy (**SEM**) we were able to confirm our hypothesis of a biofilm in the milking equipment as a potential source of *L. monocytogenes* on the study farm (Latorre et al., 2009; Latorre et al., 2010). Although we were not able to confirm the presence of *L. monocytogenes* within the biofilm visualized in pieces of milking equipment (Latorre et al., 2010), the isolation of persistent PFGE types from the milking system and bulk tank milk support our biofilm hypothesis. Moreover, the isolation of persistent *L. monocytogenes* types from samples obtained from milking equipment after routine washing cycles plus the presence of PFGE types in milk meters and milk tank that matched those visualized in milk filters and bulk tank milk samples, further suggest the presence of a *L. monocytogenes*-containing biofilm.

Among the potential causes of the presence of a biofilm in milking equipment, we hypothesized the intrinsic ability of certain *L. monocytogenes* strains to form biofilms. We investigated the biofilm forming ability of selected *L. monocytogenes* strains from the study farm (Latorre et al., unpublished). Only one of the three persistent strains observed in bulk tank milk had increased ability to form biofilms in an *in-vitro* assay (Latorre et al., unpublished). However, participation of the other two strains as a part of a bacterial community within a biofilm (Jefferson, 2004) along with other bacteria species more capable of forming biofilm likely explains why they were repeatedly found during the study period.

Another explanation for the presence of a biofilm in milking equipment may be the presence of a favorable environment for bacterial colonization (Jefferson, 2004) in milk meters. Milk meters were cleaned out with a wire brush in May 2006, which caused multiple scratches that were readily observed once that pieces removed for

SEM analysis were dry (Figure 6.2). It is likely that the milk meters were mechanically cleaned because of visible milk residues, which may have been an indication that some problems with the milking equipment washing were occurring. Scratches on the surface of milking equipment may make their cleaning more difficult as rough surfaces are harder to clean as demonstrated by Wirtanen et al. (1995) in stainless steel surfaces.

The presence of scratches combined with inadequate washing procedures may cause the accumulation of milk residues, which may be a source of nutrients for bacteria. In addition, SEM pictures obtained in our study suggest that bacteria attached to these scratches may have used them to "protect" themselves from the environment (Figure 6.3).

High standard plate count (**SPC**) and preliminary incubation count (**PIC**) were observed during the study period (Figure 6.1), which have been reported as an indicator of inadequate washing of milking equipment (Murphy and Boor, 2000).

During an approximately 1.7 month period (September – October 2007), temperature of both milk pipeline and milk tank washes were monitored. Representative temperature data for the pipeline and tank washes are shown in Figures 6.4 and 6.5.

During the monitoring period, the milk pipeline washing temperatures were relatively consistent and seemed to be within the recommendations of the manufacturer of the milking equipment. The water temperatures used for the milk tank wash, however, were considerably lower (range from 9°C to 47°C) than those suggested by the

Figure 6.2. Scratches in the bottom cover of a milk meter removed from the milking parlor for scanning electron microscopy. Panel A shows scratches in a half milk meter cover. Panel B shows magnification of the area.


A


B

Figure 6.3. Scanning electron microscopy images of scratches in the bottom cover of milk meters. Panels A and B correspond to the bottom cover of a milk meter removed from milking unit 3, located at the left side of parlor pit. Panels C and D show scratches in the bottom cover of a milk meter removed from milking unit 8, also located at the left side of parlor pit. Arrows show the presence of bacteria attached to the surface of milk meters. Scale: 1000nm.

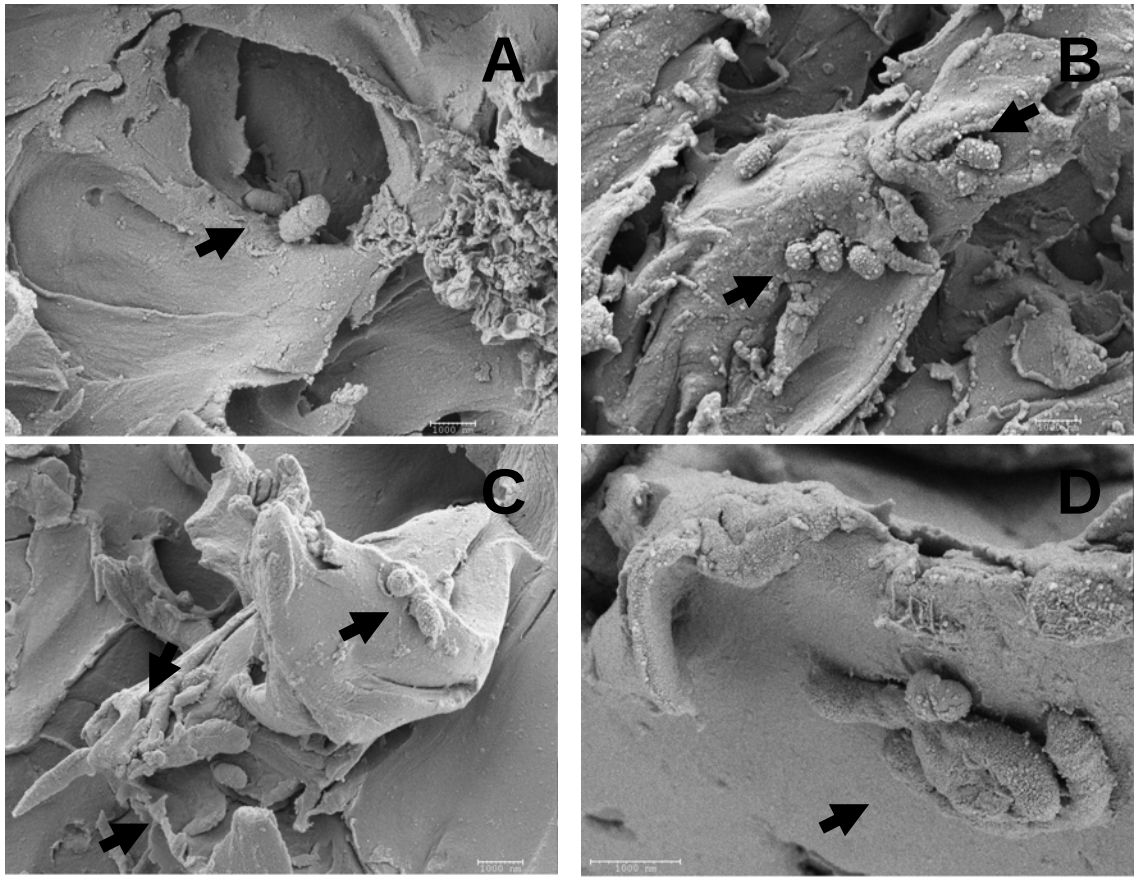

A
B
C
D

Figure 6.4. A one-day (September 4, 2007) representation of the average time and temperature of routine washes of the milk pipeline as registered by a MilkGuard ® probe located at the outlet of the milk pipeline. Arrows show pre-rinse cycle, wash cycle, and acid rinse time durations.

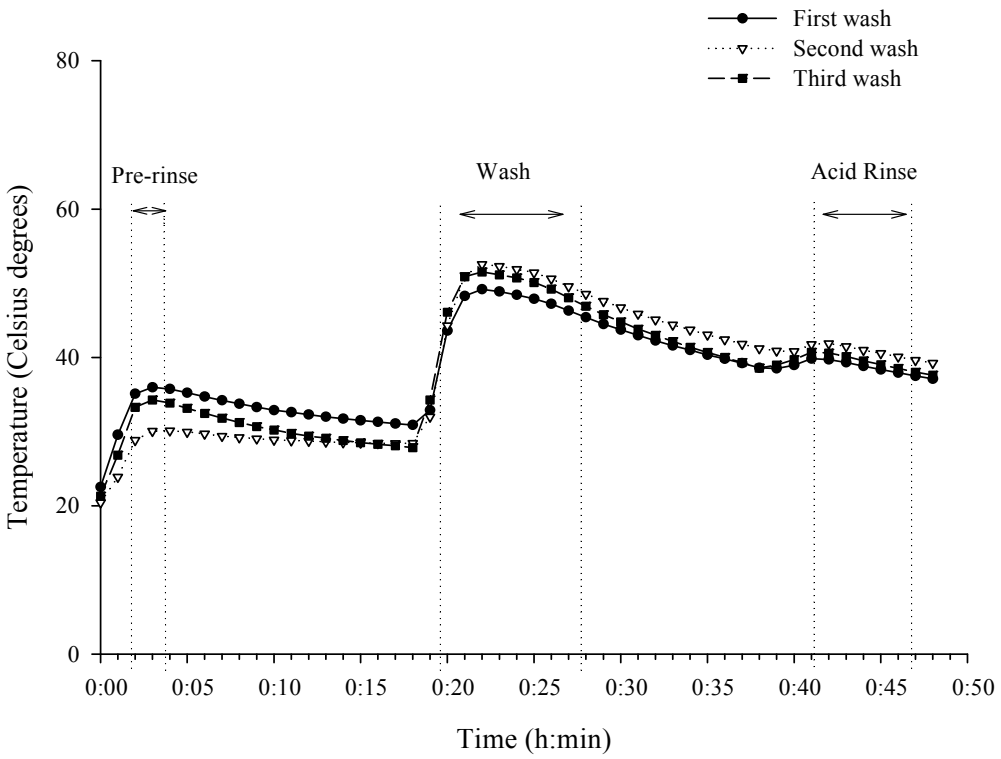
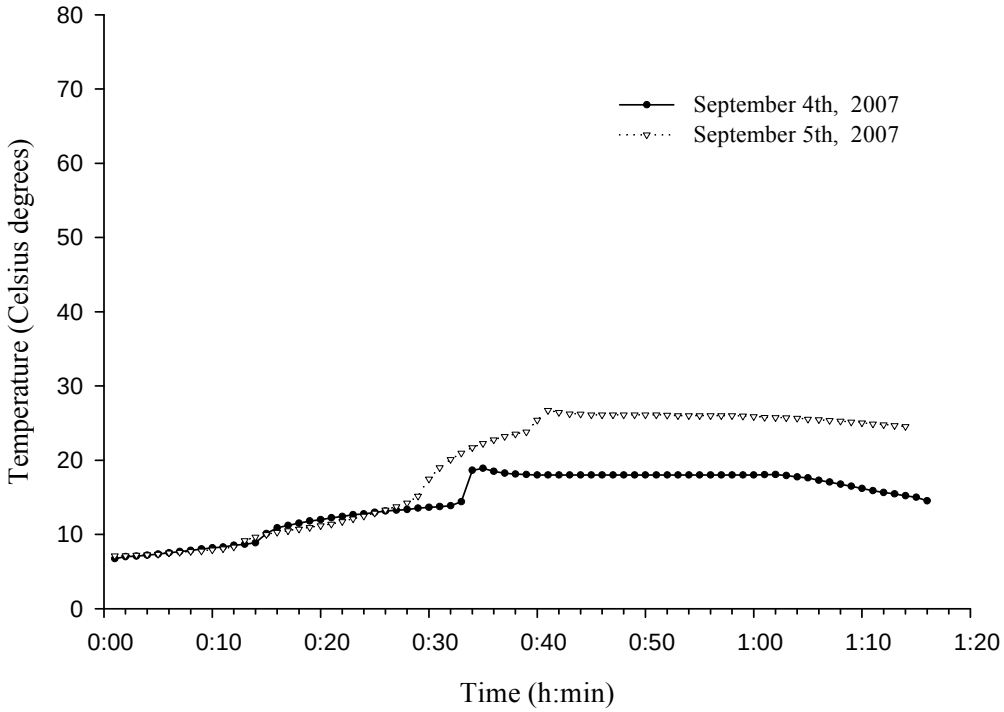

First wash
Second wash
Third wash
Pre-rinse
Wash
Acid Rinse
Temperature (Celsius degrees)
80
60
40
20
0
0:00
0:05
0:10
0:15
0:20
0:25
0:30
0:35
0:40
0:45
0:50
Time (h:min)

Figure 6.5. Average time and temperatures of two routine bulk tank milk washes performed during two different days.


80
70
60
50
40
30
20
10
0
Temperature (Celsius degrees)
0:00
0:10
0:20
0:30
0:40
0:50
1:00
1:10
1:20
Time (h:min)
September 4th, 2007
September 5th, 2007

manufacturer (recommended temperatures for pre-rinse cycle: between 38 and 49°C; wash cycle: reaching at least 73°C; acid wash cycle: between 38 and 49°C).

During a visit to the farm in January 2008 with the purpose of collecting sponge swab samples from milking equipment for our study, the presence of severe issues with milking equipment washes was observed, as shown in figure 6.6. In this occasion, a large percentage of milking equipment samples was positive for *Listeria spp*. and *L. monocytogenes* (Latorre et al., 2010).

Furthermore, although high SPC and PIC were observed throughout the study period, PIC counts as high as >100,000,000 cfu/mL were more frequently observed starting in 2008 (Figure 6.1), despite efforts that were made to improve milking equipment washing procedures, which included the purchase of new water heaters.

An inspection conducted to the milk tank on April 27 2009 revealed that extensive areas inside the tank had the presence of a film. This film was not only found inside the milk tank, but also attached to the surface of the agitator's blades (Figure 6.7). Temperature sensors inside the bulk tank milk were tested and they showed to be working properly. However, a clog in one of the sprinklers (Figure 6.8) caused an insufficient amount of water to be released inside the tank during washing, which may explain the insufficient cleaning and low temperatures during bulk tank wash cycle.

The clog was removed from the sprinkler, a manual cleaning of the inside of the milk tank with hot water and a detergent solution was carried out, and manual cleaning of the tank once per week was recommended.

Figure 6.6. Milk residues in the top cover (A) and the bowl component (B) of two different milk meters. Pictures were taken after the routine washing cycle was completed.


A


B

Figure 6.7. Images of the agitator blades located inside the milk tank. Panels A and B show the presence of a thick film attached to the surface of blades.

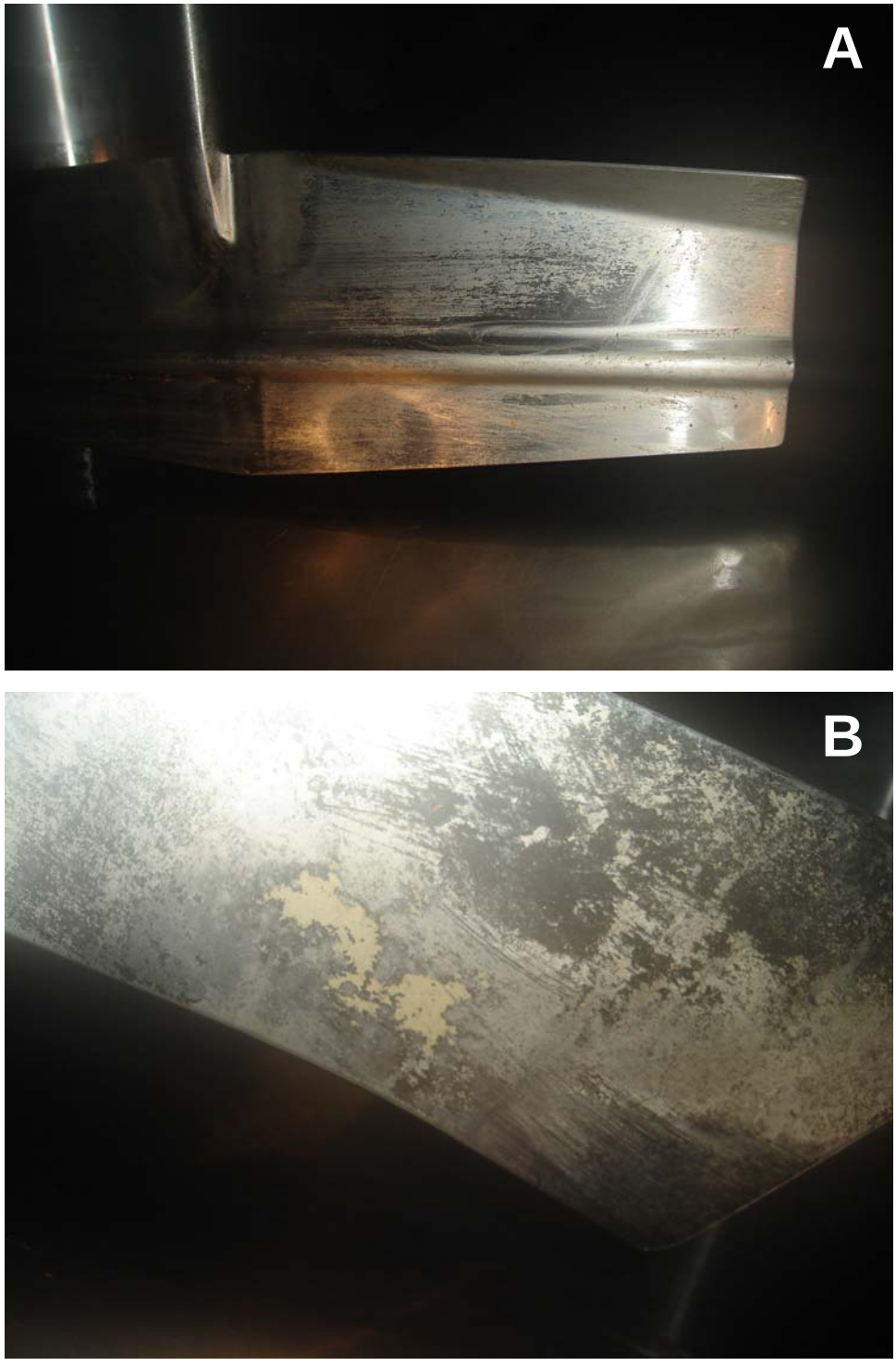

A
B

Figure 6.8. Images of foreign material (a broken gasket) obstructing a milk tank water sprinkler. Panel A shows the obstructed sprinkler and the position of the clog. Panel B shows the clog once it was removed from the sprinkler.


A


B

After the introduction of the weekly cleaning of the milk tank as a routine procedure on the farm, both SPC and PIC declined, being this reduction more marked in PIC counts which have been consistently low, showing only occasional spikes (Figure 6.1). Moreover, analysis of *L. monocytogenes* isolation data from in-line milk filters and bulk tank milk samples collected in 2009 revealed that the isolation of the pathogen from such samples ceased in June and at the beginning of May, respectively (Figure 6.9).

Although *L. monocytogenes* was not isolated from bulk tank milk samples between April and November 2008, it was still being repeatedly isolated from in-line milk filter samples during the same time-period (Figure 6.9), suggesting multiple biofilm locations within the milk line.

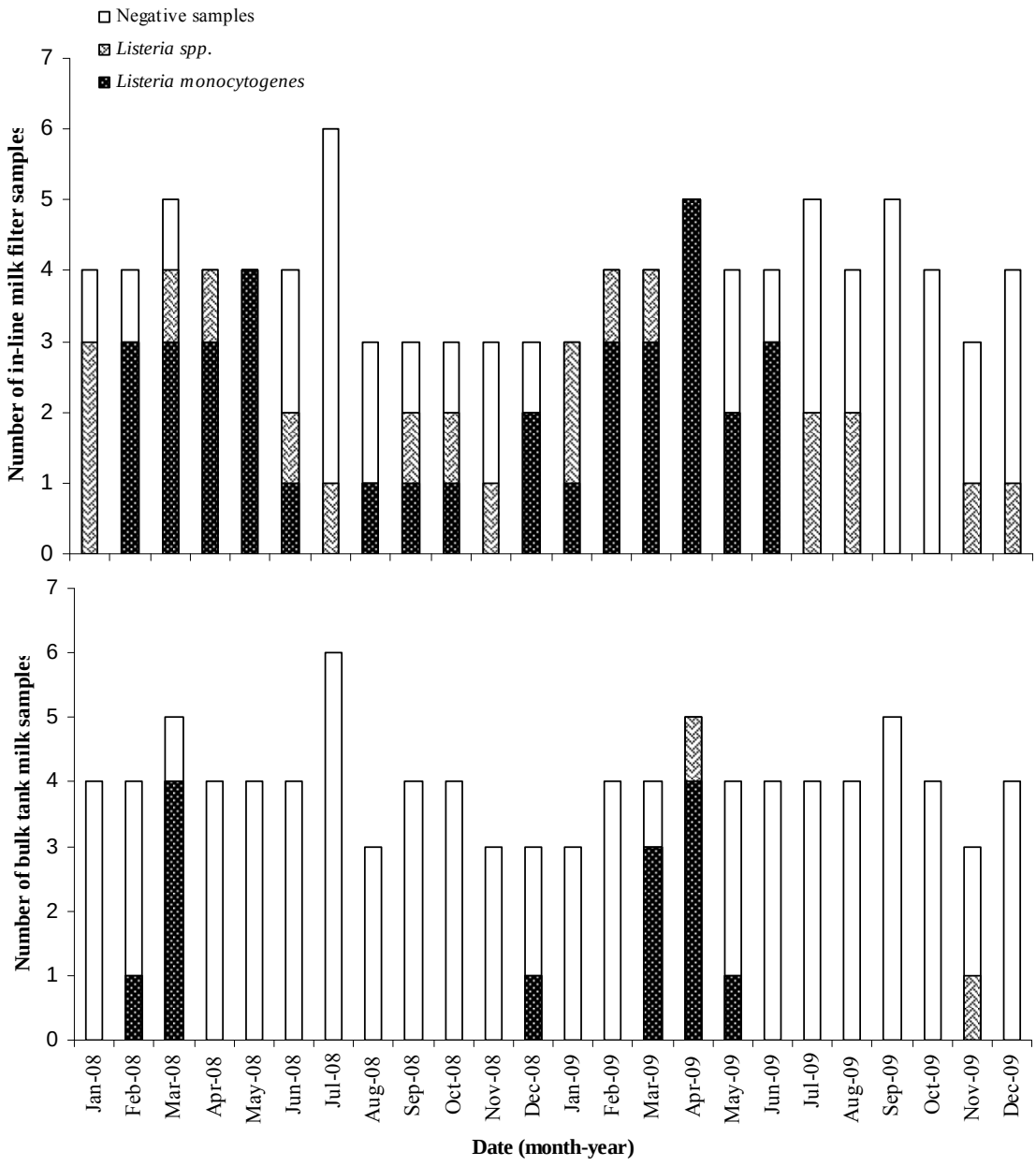
Listeria monoytogenes was isolated again in a milk sample collected in December 2008, followed by a high frequency of isolation between March and April 2009 (Figure 6.9). The manual cleaning of milk tank and removal of the film attached to surfaces resulted in reduction of *L. monocytogenes*, although sporadic isolation of *Listeria spp.* in milk and milk filters was still observed (Figure 6.9).

Changes in the milking equipment washing procedures (including purchase of new equipment to ensure proper washing temperatures plus implementation of a weekly program of manual cleaning of the milk tank), resulted in a reduction of the frequency of isolation of *L. monocytogenes* from in-line milk filters and bulk tank milk samples.

Potential sources of L. monocytogenes on farms

The presence of *L. monocytogenes* on the dairy farm environment has been frequently

Figure 6.9. Number of *Listeria* spp. and *L. monocytogenes*-positive samples obtained from in-line milk filters and bulk tank milk samples collected weekly between January 2008 and December 2009.


Negative samples
Listeria spp.
Listeria monocytogenes
Number of in-line milk filter samples
Number of bulk tank milk samples
0
1
2
3
4
5
6
7
Jan-08
Feb-08
Mar-08
Apr-08
May-08
Jun-08
Jul-08
Aug-08
Sep-08
Oct-08
Nov-08
Dec-08
Jan-09
Feb-09
Mar-09
Apr-09
May-09
Jun-09
Jul-09
Aug-09
Sep-09
Oct-09
Nov-09
Dec-09
Date (month-year)

reported (Nightingale et al., 2004; Mohammed et al., 2009; Borucki et al., 2005) as well as the shedding of the pathogen in feces of healthy animals (Husu, 1990; Nightingale et al., 2004; Ho et al., 2007a). Furthermore, it has been suggested that dairy cows may play a role in the amplification and dissemination of *L. monocytogenes* on farms (Nightingale et al., 2004), which may explain the presence of indistinguishable or closely related (Tenover et al., 1995) PFGE types that were often found in individual fecal samples and the farm environment in our study (Latorre et al., unpublished). Fecal shedding of *L. monocytogenes* as well as listeriosis in ruminants has been associated with the consumption of contaminated silage (Ho et al., 2007a; Wagner et al., 2005).

In our study, however, we observed the presence of *L. monocytogenes* in cows' feces but absence of the pathogen in silage samples collected from the farm.

Of 71 feed samples collected over a 4-year period, *L. monocytogenes* was isolated from only one sample. Hence, in our study silage or feedstuff was unlikely to be the source of *L. monocytogenes* for cows. Drinking water for animals, however, was frequently found to be *L. monocytogenes*-positive in agreement with previous studies (Mohammed et al., 2009; Nightingale et al., 2004). Our research suggested that the presence of *L. monocytogenes* in drinking water was likely a result of defecation of animals into water troughs. If animals are shedding *L. monocytogenes* in their feces, they may contribute to the persistence of the pathogen on farms trough a continuous cycle of defecation in water followed by ingestion of water contaminated with *L. monocytogenes*. Hence, our study suggested that drinking water should be considered along with poorly fermented silage among potential reservoirs of *L. monocytogenes* on farms.

Interestingly, in our study one sample of feral animal's feces that was collected in June 2004, not only was positive for *L. monocytogenes*, but also PFGE typing of one isolate obtained from this sample showed the same persistent PFGE type (T) that was found in feces, farm environment, and later in the milking system. Furthermore, *L. monocytogenes* was isolated from 2 out of 16 flies' samples as well. The presence of *L. monocytogenes* in wildlife has been described previously (Lyautey et al., 2007) as well as the presence of other bacteria (i.e. *Salmonella*, *E. coli*, *Enterococcus*) carried by flies (Pradhan et al., 2009). In our study, it was not possible to determine if feral animals or insects played a role in transmission or persistence of *L. monocytogenes* on the farm or if the presence of the pathogen on feral animal's feces and flies was due to their close contact with cow's feces or other contaminated materials. In any case, the presence of *L. monocytogenes* on samples collected from these sources is of concern, and wildlife, pests, and insects should be also considered as a potential source of the pathogen on farms.

Although in the study farm *L. monocytogenes* was frequently isolated from individual fecal samples (overall prevalence of 6%), no listeriosis cases in cows have ever been reported on the farm. Nightingale et al (2004) reported that cattle may be a healthy reservoir of *L. monocytogenes* on farms.

Apparently, fecal shedding of *L. monocytogenes* in lactating cows would not be a cause of decrease in milk production or potential economic losses as it has been described for other pathogens (Smith et al., 2009; Smith et al., 2010). However, efforts should be made to control *L. monocytogenes* on farms not only from an animal health perspective, but ultimately to prevent the appearance of the pathogen in bulk tank milk.

Link between L. monocytogenes in dairy production systems and human health

Although ready-to-eat foods are considered of highest risk and are the category of food more frequently involved in listeriosis outbreaks (FDA/FSIS, 2003), dairy products have been linked to foodborne *L. monocytogenes* infections as well (Fleming et al., 1985; Linnan et al., 1988; Dalton et al., 1997; Lyytikäinen et al., 2000; Carrique-Mas et al., 2003; MacDonald et al., 2005, CDC 2008).

The potential link between animal production systems and human listeriosis cases exists, as it has been suggested by previous studies that have described the presence of *L. monocytogenes* ribotypes (Nightingale et al., 2004) or PFGE types (Borucki et al., 2004, Fugett et al., 2007) from farms that matched those observed in isolates from human listeriosis cases.

The common presence of *L. monocytogenes* on dairy farms (Nightingale et al., 2004) may explain why the pathogen is frequently found in bulk tank milk with prevalences ranging from 2.8% to 25.4% (Jayarao and Henning, 2001; Jayarao et al., 2006; Van Kessel et al., 2004; Mohammed et al, 2009; Latorre et al., unpublished data).

The presence of *L. monocytogenes* in raw bulk tank milk could represent a risk of contamination for milk processing plants. For example, in a study conducted by Waak et al. (2002), indistinguishable PFGE types were observed in *L. monocytogenes* isolates obtained from bulk tank milk and from the raw milk silos on a dairy plant.

Furthermore, the presence of *L. monocytogenes* on dairy processing plants may represent a risk of post-pasteurization contamination of milk. It has been reported that *L. monocytogenes* already established in food processing environments have been responsible for cross-contamination of finished products, such as ice cream and

smoked fish (Miettinen et al., 1999; Vogel et al., 2001). However, one of the routes from which *L. monocytogenes* may initially enter processing plants (with the subsequent colonization of the environment) is through raw materials (Vogel et al., 2001). Hence, control of *L. monocytogenes* at the farm level is crucial to prevent the entrance of the pathogen into the food chain through raw animal products, including milk.

Risk of listeriosis associated with raw milk consumption

The potential risk of listeriosis in humans associated to raw milk consumption constitutes a public health issue. Currently, 29 states allow the sale of raw milk (Oliver et al., 2009) despite numerous milk-borne diseases outbreaks linked to raw milk consumption that have been reported during the past decade (CDC, 2002; CDC, 2003; CDC, 2007; Denny et al, 2008).

Although pasteurization of milk does not significantly affect the nutritional properties of milk (Potter et al., 1984) and effectively eliminates *L. monocytogenes*, an increasing number of consumers prefer drinking raw milk. Furthermore, an important percentage of dairy producers (between 42.3% and 60%) have reported consuming raw milk on farms (Jayarao and Henning, 2001; Jayarao et al., 2006; Hoe and Ruegg, 2006).

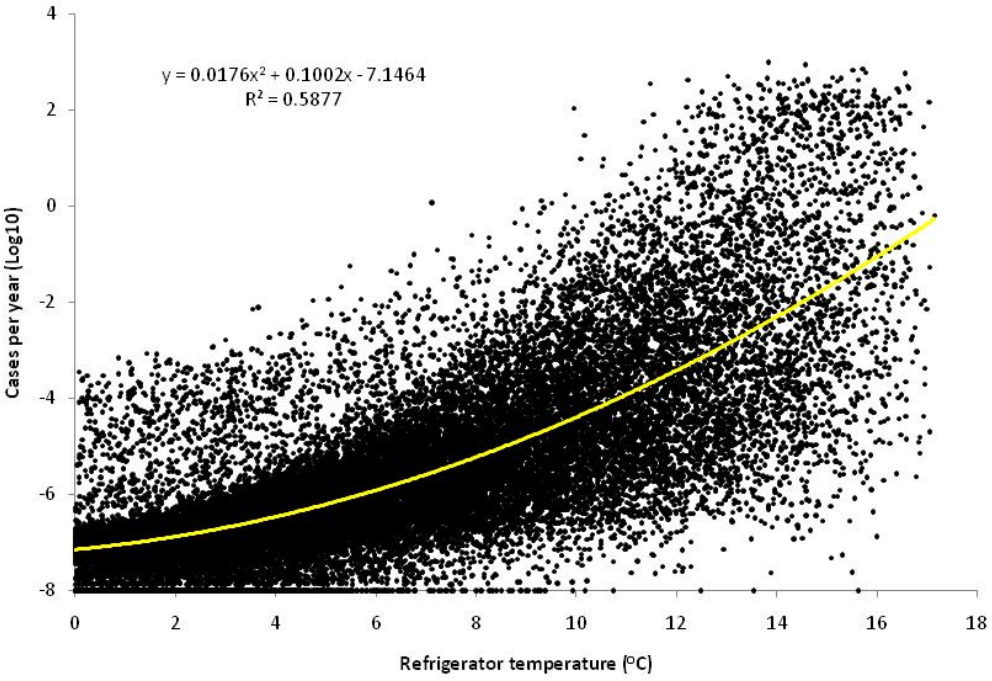
The frequent isolation of *L. monocytogenes* from bulk tank milk samples (Jayarao et al., 2001; Van Kessel et al., 2004; Jayarao and Henning, 2006; Latorre et al, 2009), the large number of people who drink raw milk on farms, combined with the growing number of licensed raw milk farms (New York State **NYS** Department of Agriculture and Markets, unpublished data) that have increased the availability of the product to consumers, draw attention to the potential risk of listeriosis for these populations.

Even though the practice of drinking raw milk will probably not be eradicated despite recommendations to avoid the consumption of the product (NEHA, 2008), our study showed that the risk of listeriosis among consumers can be reduced. As the relative risk of listeriosis was greatly influenced by the prevalence of *L. monocytogenes* in raw milk (Latorre et al., unpublished), implementation of raw milk monitoring programs may significantly reduce the risk for raw milk consumers.

Moreover, home refrigeration temperatures were of significant impact in the number of listeriosis cases. For example, for the general population that is at immediate risk that purchased milk directly on farms, an increase in the home refrigerator temperature from 4˚C to 6˚C resulted in a 2.3-fold increase in the number of cases per year (Figure 6.10). An increase of the temperature in the home refrigerator from 4˚C to 8˚C, results in approximately 7.1-fold increase in the number of listeriosis cases (Figure 6.10). Prevalence of *L. monocytogenes* in raw milk had a linear relationship with the number of cases rather than the exponential relationship observed in refrigeration temperatures. For example, an increase in the prevalence of *L. monocytogenes* in raw milk from 2.1% (overall raw milk) to 35.3% (highest prevalence observed among raw milk farms) resulted in a ~17-fold increase in the number of annual listeriosis cases (Latorre et al., Chapter 5).

Our results show that not only regulatory agencies, but also raw milk consumers and sellers can contribute to the reduction of the risk of listeriosis. This can be achieved by reducing *L. monocytogenes* prevalence in raw milk, ensuring proper refrigeration temperatures during display at stores, during transportation, and at home. A reduction of the time that milk is kept at refrigeration temperatures would also benefit the reduction of risk, as suggested by our model in which the risk of listeriosis increased

Figure 6.10. Number of annual listeriosis cases in intermediate population associated with consumption of raw milk purchased on farms and stored at different refrigerator temperatures at home. Graph show 32,000 data points generated by @Risk 5.5 in the quantitative risk assessment model of listeriosis due to raw milk consumption (Latorre et al., unpublished).


y = 0.0176x² + 0.1002x - 7.1464
R² = 0.5877
Cases per year (Log10)
Refrigerator temperature (°C)
4
2
0
-2
-4
-6
-8
0
2
4
6
8
10
12
14
16
18

as more time/temperature combination steps were included.

Potential for future research

One important challenge for future research is the development of techniques that allow for the detection *in-situ* of *L. monocytogenes* within a biofilm. For example, a short-coming in our study was that we were not able to directly identify the presence of *L. monocytogenes* in the biofilm visualized in milking equipment.

The use of confocal-laser scanning microscopy has been successfully used for visualization of the structure of bacterial biofilms in human tissue (Kania et al., 2007). Furthermore, the combination of confocal-laser scanning microscopy and the use of fluorescence *in situ* hybridization (**FISH**) probes targeting specific bacteria in *in vivo* biofilms in humans have been recently reported (Al-Ahmad et al., 2010). These techniques could be adapted in the future for use on field studies of *L. monocytogenes*-containing biofilms as well.

Peptid Nucleic Acid (**PNA**) probes have been successfully used for detection of *Listeria* (Brehm-Stecher et al., 2005) and they may be potentially used for identification of *Listeria* spp. in biofilms. The particular structure of these probes (reviewed by Stender et al., 2002) may facilitate hybridization with target sequences in *L. monocytogenes*, avoiding thus the need of permeabilization of the cell wall as sometimes is needed in Gram-positive bacterial cells (Moter and Göbel, 2000). The increased cost of these probes, however, can be a limitation. An important consideration for future research is the design of specific probes that allow for detection of *L. monocytogenes* in a "real life" environment, such as milking equipment on farms. For example, a hybridization study conducted by Wang et al. (1991) using a

16s rRNA probe (RL-2 probe) successfully identified *L. monocytogenes* strains and showed a high specificity when tested with other bacterial species, such as *Escherichia coli* or *Bacillus subtilis* strains (among others) (Wang et al., 1991). Adaptation of this probe for detection of *L. monocytogenes* in pieces of milking equipment by FISH, however, would not be recommended as a BLAST search showed significant alignment with sequences of other bacteria, such as some *Lactococcus*, *Campylobacter,* or *Pseudomonas* sp. that may be found in the milk-line environment.

In-situ detection of *L. monocytogenes* in biofilms may benefit not only from specificity and ability of the probe to target the organism, but also from previous analysis of the characteristics of surface materials as they may difficult visualization of bacteria. For example, a high autofluorescence of plastic materials was observed during analysis of plastic pieces from milking equipment under the fluorescence microscope in our study, which interfered with *in-vitro* biofilm visualization (unpublished data). Furthermore, the shape of some of the pieces obtained from rubber liners made observation of parts under microscope very difficult (unpublished data).

Another potential for future research is the analysis of the structure of bacterial populations in a *L. monocytogenes*-containing biofilm community. The identification and characterization of microorganisms present in a *L. monocytogenes*-containing biofilm as well as their relative frequency within the community would help to better understand the structure of such biofilms.

The use of molecular methods to investigate the ability of *L. monocytogenes* to form biofilms is another promising field for future research. The use of molecular methods in the study of biofilms may allow the detection of specific genes responsible for

biofilm production in *L. monocytogenes*. Although many genes have been described as involved in biofilm production in bacteria (Jefferson, 2004; Beloin and Ghigo, 2005), specific proteins [namely diguanyl cyclases (**DGCs**) and cyclic dimeric GMP (**c-di-GMP**) phosphodiesterases (**PDEs**)] may also play a role in biofilm formation by mean of regulation of bis-(3'-5') c-di-GMP concentrations (Kulasakara et al., 2006).

In *L. monocytogenes* strain EGD-e, only 3 hypothetical proteins in the EAL domain (putative PDEs) and 5 hypothetical proteins in the GGDEF domain (putative DGCs) have been identified (Personal communication, Dr. Holger Sondermann. Avail able at http://www.ncbi.nlm.nih.gov/Complete_Genomes/SigCensus/EALfirmi2008.html#169963 and http://www.ncbi.nlm.nih.gov/Complete_Genomes/SigCensus/GGDEFfirmi2008.html#169963).

By sequencing the genome of field *L. monocytogenes* strains, we would be able to investigate the presence of genes involved in the codification of these proteins with GGDEF and EAL domain (Kulasakara et al., 2006). Phenotypic analysis of the attachment of *L. monocytogenes* strains in a microtiter plate assay (Latorre et al., unpublished) after overexpression or suppression of genes encoding DGC's and PDE's would provide valuable information about the role of these proteins in biofilm forming ability of *L. monocytogenes* strains. Ultimately, the identification of genes responsible for biofilm formation in *L. monocytogenes* strains would help in the control of *L. monocytogenes* biofilms on farms and in the food industry.

Prevention of biofilm formation in milking equipment or eradication of existing biofilms it is also an area that may benefit from further research. Studies on the effect of chemical compounds or the combination of chemicals and mechanic methods on

biofilms already established in milking equipment are suggested. As *L. monocytogenes* in biofilms have been reported to be resistant to sanitizers (Frank and Koffi, 1990; Pan et al., 2006), finding a product or combination of products that are equally effective and safe for use in milk harvesting equipment represents a challenge.

Further investigation about other potential reservoirs of *L. monocytogenes* such as water troughs, wildlife, birds, or insects would be helpful to gain a better understanding of their contribution to the presence of the pathogen on farms. For example, additional PFGE typing of *L. monocytogenes* isolates obtained from water samples would allow to investigate the overall diversity of PFGE types in water or detection of persistent types that may be an indication of biofilms in water troughs. Moreover, additional PFGE typing of fecal and environmental isolates would be needed to identify all *L. monocytogenes* types that may be present in a given sample (Döpfer et al., 2008).

One of the many advantages of the molecular typing technique used in our study (PFGE) is that it is a highly standardized method that not only allows for comparison of *L. monocytogenes* restriction digest profiles over time within a laboratory, but also across laboratories (CDC, 2010). Hence, comparison of DNA banding patterns between *L. monocytogenes* isolates from our study, isolates obtained from dairy processors, and isolates from human listeriosis cases would also be of interest in future research.

As shown in our quantitative risk assessment of listeriosis associated with consumption of raw milk, the overall prevalence of *L. monocytogenes* in raw milk from licensed raw milk farms in NYS was relatively low. However, repeated isolation

of *L. monocytogenes* was observed in some of the farms, causing prevalences of the pathogen in milk as high as 35.3% (Latorre et al., unpublished) increasing thus the risk of listeriosis for consumers. Molecular studies about potential sources of *L. monocytogenes* in bulk tank milk in those farms, including assessment of the potential presence of biofilms in the milk line are recommended.

Finally, risk assessment of listeriosis due to raw milk consumption would benefit from data about volume of raw milk sales, creation of customers records to quantify the actual consumption of the product by the population (and for quick notification in the event of raw milk recall), and from information about the contribution of each market (i.e. farms, farm stores, and retail) to raw milk sales. Hence, future studies aimed to compile missing information about raw milk marketing in the United States would be very valuable for the estimation of the actual risk of listeriosis associated with raw milk consumption.

Conclusions

Our study allowed the identification of a *L. monocytogenes*-containing biofilm in milking equipment, suggesting the presence of a potential reservoir of *L. monocytogenes* on dairy farms that had not been documented previously. Furthermore, our results indicate that the presence of *L. monocytogenes*-containing biofilms in the milk line may have been responsible for a high prevalence of the pathogen in in-line milk filter and bulk tank milk samples throughout the 4-year study period (and beyond).

A large heterogeneity of *L. monocytogenes* types was observed among isolates obtained from cows and the environment, whereas more homogeneity of PFGE types

was observed in samples from milking equipment, milk filters, and bulk tank milk. Persistent PFGE types observed in the bulk tank milk were also observed throughout the farm, suggesting initial contamination of the milk line from the farm environment. Subsequently, milking equipment may have been colonized by *L. monocytogenes* strains that were better-adapted to persist in the milk line and bulk tank environment.

Our study provided science based guidelines to help dairy producers in the control of *L. monocytogenes* on farms. Appropriate milking practices would prevent the entrance of *L. monocytogenes* (and other pathogens) into the milking system, reducing thus the risk of biofilm establishment in milking equipment. Regular replacement of milking equipment parts that are more susceptible to wear such as rubber liners, gaskets, milk meters' parts, etc., and correct milking equipment washing would also help in the prevention of colonization and subsequent biofilm formation by *L. monocytogenes* and other bacteria.

Control of *L. monocytogenes* in the farm environment may be achieved by feeding cows with properly fermented silage, regular cleaning and disinfection of water troughs, control of wild animals and pests on the farm premises, and implementation of biosecurity measures to prevent spreading of the pathogen on the farm.

Reduction the presence of *L. monocytogenes* on dairy farms and, hence, in bulk tank milk would have a public health impact not only on farms that supply milk to dairy processors, but particularly for farms that sell raw milk to consumers.

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APPENDIX

Table 1A. *Listeria monocytogenes* isolates analyzed in our study. Table shows Quality Milk Production Services ID (QMP ID), USDA-ARS Culture Collection number (CC#), isolate patch number (Isolate#), source (MF=milk filter, BTM=bulk tank milk, MM=milk meter, TC=teat cup liner, and other sources), internal project ID (EMSL ID), and date of sample collection. Right columns show PFGE types (AscI and ApaI) assigned by visual inspection by two independent observers, and combined enzyme analysis (C.E.) by using Bionumerics software (BN). Serotype complex (Doumith et al., 2004) is displayed on the table as well.

Information for these isolates is also available at www.pathogentracker.net

Table 1A.

QMP ID	CC#	Isolate #	Source	EMSL ID	Date	PFGE Type		C.E	Serotype
						AscI	ApaI	BN	Complex
L1-001	325	07/08/04-1p #19	Flies (calf area)	A-S1-1	6/29/2004	1	1	I	4b
L1-079	2299	1/14/05-1p#2	Water (pen 3+4)	A-4-3	1/11/2005	1	1	I	4b
L1-243	2610	4/21/08-4p #36	Fecal (1478)	A-17-165	4/14/2008	1	1	I	4b
L1-245	2612	4/21/08-4p #43	Fecal (1600)	A-17-204	4/14/2008	1	1	I	4b
L1-091	2312	5/11/06-1P 17	Water (dry cow)	A-9-5	5/1/2006	1.1	1.1	K	4b
L1-009	333	5/20/05-8P 44	MF	A-MS-61	5/10/2005	2	2	M	4b
L1-192	2389	3/6/06-1P #7	MF	A-MS-102	2/28/2006	2	2	M	4b
L1-114	2336	04/23/07-5P #11	Fecal(1478)	A-13-38	4/16/2007	2.1	2.2	O	4b
L1-023	349	4/24/07-5P #40	Fecal (1829)	A-13-260	4/16/2007	2.1	2.2	O	4b
L1-134	2357	04/24/07-6P #1	Fecal (1860)	A-13-278	4/16/2007	2.2	2.1	N	4b
L1-237	2604	4/21/08-1p #12	Water (dry cow)	A-17-5	4/14/2008	2.2	2.1	N	4b
L1-242	2609	4/21/08-4p #19	Fecal (1760)	A-17-114	4/14/2008	2.2	2.1	N	4b
L1-072	2291	02/26/04-1p # 8	Fecal (1155)	A-1-133	2/17/2004	2.3	2.3	Z	4b
L1-104	2326	01/16/07-1P #25	Water (pen 3+4)	A-12-3	1/8/2007	2.3	2.3	Z	4b
L1-030	600	9/27/05-1P#1	MF	A-MS-78	9/8/2005	3	3	F	1/2a
L1-031	602	9/26/05-1P#1	MF	A-MS-79	9/14/2005	3	3	F	1/2a
L1-032	604	9/26/05-1P#31	MF	A-MS-80	9/20/2005	3	3	F	1/2a
L1-179	2372	10/3/05-1P #2	MF	A-MS-81	9/29/2005	3	3	F	1/2a
L1-207	2373	10/11/05-2P #2	MF	A-MS-82	10/4/2005	3	3	F	1/2a
L1-010	334	10/31/05-1P 1	MF	A-MS-85	10/26/2005	3	3	F	1/2a
L1-180	2375	11/7/05-1P #1	MF	A-MS-86	11/1/2005	3	3	F	1/2a
L1-181	2376	11/14/05-1P #1	MF	A-MS-87	11/9/2005	3	3	F	1/2a
L1-004	328	11/16/05-1P 2	BTM	A-M-87	11/9/2005	3	3	F	1/2a
L1-182	2377	11/21/05-1P #22	MF	A-MS-88	11/16/2005	3	3	F	1/2a
L1-209	2460	11/21/05-1P #7	BTM	A-M-88	11/16/2005	3	3	F	1/2a
L1-183	2378	11/30/05-1P #3	MF	A-MS-89	11/22/2005	3	3	F	1/2a
L1-184	2379	12/6/05-1P #39	MF	A-MS-90	11/30/2005	3	3	F	1/2a
L1-210	2461	12/6/05-1P #31	BTM	A-M-90	11/30/2005	3	3	F	1/2a
L1-011	335	12/12/05-2P 13	MF	A-MS-91	12/7/2005	3	3	F	1/2a
L1-211	2462	12/12/05-1P #1	BTM	A-M-91	12/7/2005	3	3	F	1/2a
L1-277	2381	12/19/05-1p #5	MF	A-MS-92	12/14/2005	3	3	F	1/2a
L1-012	336	1/3/06-1P 17	MF	A-MS-93	12/21/2005	3	3	F	1/2a
L1-187	2383	1/3/06-1P #21	MF	A-MS-94	12/29/2005	3	3	F	1/2a
L1-188	2384	01/17/06-1P #1	MF	A-MS-95	1/11/2006	3	3	F	1/2a
L1-191	2387	02/24/06-1P #1	MF	A-MS-100	2/15/2006	3	3	F	1/2a
L1-013	337	2/27/06-1P 12	MF	A-MS-101	2/21/2006	3	3	F	1/2a
L1-212	2463	2/27/06-1P #1	BTM	A-M-101	2/21/2006	3	3	F	1/2a
L1-193	2390	3/13/06-1P #1	MF	A-MS-103	3/8/2006	3	3	F	1/2a
L1-213	2464	3/15/06-1P #13	BTM	A-M-103	3/8/2006	3	3	F	1/2a
L1-194	2392	3/27/06-1P #1	MF	A-MS-105	3/21/2006	3	3	F	1/2a
L1-214	2465	3/27/06-1P #11	BTM	A-M-105	3/21/2006	3	3	F	1/2a
L1-215	2466	4/3/06-1P #13	BTM	A-M-106	3/29/2006	3	3	F	1/2a
L1-195	2394	4/10/06-1P #1	MF	A-MS-107	4/4/2006	3	3	F	1/2a
L1-216	2467	4/10/06-1P #7	BTM	A-M-107	4/4/2006	3	3	F	1/2a
L1-275	2395	4/24/06-1p #1	MF	A-MS-109	4/19/2006	3	3	F	1/2a
L1-217	2468	4/24/06-1P #16	BTM	A-M-109	4/19/2006	3	3	F	1/2a
L1-197	2397	5/15/06-1P #9	MF	A-MS-112	5/9/2006	3	3	F	1/2a
L1-198	2398	5/22/06-1P #6	MF	A-MS-113	5/16/2006	3	3	F	1/2a
L1-199	2399	5/30/06-1P #7	MF	A-MS-114	5/24/2006	3	3	F	1/2a
L1-204	2406	7/31/06-1P #1	MF	A-MS-123	7/26/2006	3	3	F	1/2a
L1-205	2407	8/7/06-1P #1	MF	A-MS-124	8/1/2006	3	3	F	1/2a
L1-095	2317	5/10/06-9P 4	Fecal (1624)	A-9-141	5/1/2006	3.1	3	AA	1/2a
L1-090	2311	5/11/06-1P 12	Water (trough 3+4)	A-9-3	5/1/2006	3.1	3	AA	1/2a
L1-088	2308	04/19/05-2p # 20	Fecal (1484)	A-5-245	4/12/2005	4	4	J	4b
L1-002	326	10/11/05-1P 41	Fecal (1250)	A-7-170	10/3/2005	4	4	J	4b
L1-094	2316	5/8/06-2P 45	Fecal (1695)	A-9-94	5/1/2006	4	4	J	4b
L1-092	2313	5/10/06-1P 29	Fecal (1478)	A-9-25	5/1/2006	4	4	J	4b
L1-139	2362	04/24/07-7P #20	Fecal (1492)	A-13-344	4/16/2007	4	4.2	AB	4b

Table 1A. (continued)

QMP ID	CC#	Isolate #	Source	EMSL ID	Date	PFGE Type		C.E	Serotype
						AscI	ApaI	BN	Complex
L1-246	2613	4/21/08-4p #44	Fecal (1946)	A-17-216	4/14/2008	4	4	J	4b
L1-247	2614	4/21/08-5p #20	Fecal (1768)	A-17-333	4/14/2008	4	4	J	4b
L1-240	2607	4/21/08-4p #4	Composite (pen1)	A-17-21	4/14/2008	4	4	J	4b
L1-249	2616	4/28/08-1p #18	MF	A-MS-209	4/22/2008	4	4	J	4b
L1-097	2319	5/10/06-3P 31	Fecal (1411)	A-9-149	5/1/2006	4.1	4.1	L	4b
L1-074	2293	02/26/04-1p # 2	Water (barn water1)	A-1-313	2/17/2004	5	5	S	1/2a
L1-075	2294	02/26/04-1p # 10	Composite (calf man)	A-1-309	2/17/2004	5	5	S	1/2a
L1-077	2296	02/27/04-1p #11	Water (barn water)	A-1-314	2/17/2004	5	5	T	1/2a
L1-078	2297	06/28/04-1p #20	Composite (feral poop)	A-2-27	6/17/2004	5	5	T	1/2a
L1-089	2309	04/19/05-2p # 25	Fecal (1145)	A-5-290	4/12/2005	5	5	T	1/2a
L1-084	2304	04/21/05-1p #38	Fecal (1282)	A-5-85	4/12/2005	5	5	T	1/2a
L1-189	2385	1/30/06-1P #4	MF	A-MS-97	1/25/2006	5	5	T	1/2a
L1-190	2386	2/6/06-1P #1	MF	A-MS-98	1/31/2006	5	5	T	1/2a
L1-276	2391	3/20/06-1p #6	MF	A-MS-104	3/15/2006	5	5	T	1/2a
L1-014	338	4/3/06-1P 1	MF	A-MS-106	3/29/2006	5	5	T	1/2a
L1-196	2396	5/1/06-1P #27	MF	A-MS-110	4/25/2006	5	5	T	1/2a
L1-005	329	5/22/06-1P 1	BTM	A-M-113	5/16/2006	5	5	T	1/2a
L1-200	2400	6/12/06-1P #1	MF	A-MS-116	6/6/2006	5	5	T	1/2a
L1-218	2470	6/14/06-1P #1	BTM	A-M-116	6/6/2006	5	5	T	1/2a
L1-208	2401	6/21/06-1P #1	MF	A-MS-117	6/13/2006	5	5	T	1/2a
L1-201	2402	7/3/06-1P #1	MF	A-MS-119	6/27/2006	5	5	T	1/2a
L1-202	2403	7/10/06-1P #13	MF	A-MS-120	7/6/2006	5	5	T	1/2a
L1-006	330	7/10/06-1P 1	BTM	A-M-120	7/6/2006	5	5	T	1/2a
L1-015	339	7/17/06-1P 9	MF	A-MS-121	7/11/2006	5	5	T	1/2a
L1-203	2405	7/27/06-1P #1	MF	A-MS-122	7/18/2006	5	5	T	1/2a
L1-206	2408	8/14/06-1P #17	MF	A-MS-125	8/9/2006	5	5	T	1/2a
L1-219	2472	8/14/06-1P #8	BTM	A-M-125	8/9/2006	5	5	T	1/2a
L1-149	2409	8/21/06-1P #1	MF	A-MS-126	8/15/2006	5	5	T	1/2a
L1-150	2410	9/6/06-1P #1	MF	A-MS-128	8/30/2006	5	5	T	1/2a
L1-274	2411	3/11/06-1p #1	MF	A-MS-129	9/6/2006	5	5	T	1/2a
L1-151	2412	9/18/06-1P #1	MF	A-MS-130	9/12/2006	5	5	T	1/2a
L1-007	331	9/20/06-1P 1	BTM	A-M-130	9/12/2006	5	5	T	1/2a
L1-016	340	9/27/06-1P 1	MF	A-MS-131	9/19/2006	5	5	T	1/2a
L1-220	2474	9/27/06/1P #28	BTM	A-M-131	9/19/2006	5	5	T	1/2a
L1-152	2414	10/2/06-1P #1	MF	A-MS-132	9/26/2006	5	5	T	1/2a
L1-017	341	12/18/06-1P 5	MF	A-MS-142	12/12/2006	5	5	T	1/2a
L1-008	332	12/18/06-1P 1	BTM	A-M-142	12/12/2006	5	5	T	1/2a
L1-154	2417	12/26/06-1P #1	MF	A-MS-143	12/19/2006	5	5	T	1/2a
L1-155	2418	01/08/07-1P #1	MF	A-MS-144	1/3/2007	5	5	T	1/2a
L1-105	2327	01/16/07-1P #17	Water (heifer)	A-12-4	1/8/2007	5	5	S	1/2a
L1-106	2328	01/16/07-2P #26	Bedding (calf bedding)	A-12-20	1/8/2007	5	5	T	1/2a
L1-156	2419	01/16/07-2P #33	MF	A-MS-145	1/9/2007	5	5	T	1/2a
L1-157	2420	01/22/07-1P #1	MF	A-MS-146	1/16/2007	5	5	T	1/2a
L1-158	2421	01/29/07-1P #1	MF	A-MS-147	1/23/2007	5	5	T	1/2a
L1-159	2422	02/05/07-1P #1	MF	A-MS-148	1/31/2007	5	5	T	1/2a
L1-025	590	2/19/07-1P #1	BTM	A-M-150	2/14/2007	5	5	T	1/2a
L1-024	588	2/26/07-1P #1	MF	A-MS-151	2/20/2007	5	5	T	1/2a
L1-221	2477	03/05/07-1P #1	BTM	A-M-152	2/27/2007	5	5	T	1/2a
L1-160	2424	03/14/07-1P #1	MF	A-MS-153	3/6/2007	5	5	T	1/2a
L1-161	2425	03/26/07-3P #1	MF	A-MS-154	3/14/2007	5	5	T	1/2a
L1-222	2478	03/19/07-3P #1	BTM	A-M-154	3/14/2007	5	5	T	1/2a
L1-162	2426	03/26/07-3P #5	MF	A-MS-155	3/21/2007	5	5	T	1/2a
L1-223	2479	03/30/07-1P #15	BTM	A-M-155	3/21/2007	5	5	T	1/2a
L1-224	2480	04/02/07-2P #17	BTM	A-M-156	3/27/2007	5	5	T	1/2a
L1-225	2481	04/09/07-1P #1	BTM	A-M-157	4/4/2007	5	5	T	1/2a
L1-163	2428	04/23/07-1P #21	MF	A-MS-158	4/11/2007	5	5	T	1/2a
L1-117	2339	04/23/07-6P #3	Fecal (1385)	A-13-77	4/16/2007	5	5	S	1/2a
L1-164	2429	04/23/07-1P #1	MF	A-MS-159	4/17/2007	5	5	T	1/2a
L1-022	347	4/30/07-2P #36	MF	A-MS-160	4/30/2007	5	5	T	1/2a

Table 1A. (continued)

QMP ID	CC#	Isolate #	Source	EMSL ID	Date	PFGE Type		C.E	Serotype
						AscI	ApaI	BN	Complex
L1-165	2431	05/14/07-1P #1	MF	A-MS-162	5/16/2007	5	5	T	1/2a
L1-226	2483	05/16/07-2P #1	BTM	A-M-162	5/10/2007	5	5	T	1/2a
L1-028	596	5/21/07-4P #21	BTM	A-M-163	5/15/2007	5	5	T	1/2a
L1-029	598	5/21/07-4P #1	BT Outlet	A-S2-51	5/16/2007	5	5	T	1/2a
L1-018	342	5/21/07-1P #16	MM (2-R)	A-S2-4	5/16/2007	5	5	T	1/2a
L1-046	712	06/04/07-1p#8	MF	A-MS-165	5/30/2007	5	5	T	1/2a
L1-050	716	06/18/07-1p #19	BTM	A-M-167	6/12/2007	5	5	T	1/2a
L1-051	717	06/25/07-1p #23	MF	A-MS-168	6/19/2007	5	5	T	1/2a
L1-054	720	07/12/07-1p #5	BTM	A-M-170	7/5/2007	5	5	T	1/2a
L1-058	724	07/23/07-1p #25	MF	A-MS-172	7/18/2007	5	5	T	1/2a
L1-060	726	08/06/07-1p#17	MF	A-MS-174	7/31/2007	5	5	T	1/2a
L1-062	728	08/13/07-1p#3	MF	A-MS-175	8/8/2007	5	5	T	1/2a
L1-061	727	08/13/07-1p#1	BTM	A-M-175	8/8/2007	5	5	T	1/2a
L1-169	2442	8/22/07-1p #1	MF	A-MS-176	8/15/2007	5	5	T	1/2a
L1-063	729	08/20/07-1p#5	BTM	A-M-176	8/15/2007	5	5	T	1/2a
L1-171	2444	9/4/07-1p #36	MF	A-MS-178	8/28/2007	5	5	T	1/2a
L1-233	2499	10/1/07-1p #1	BTM	A-M-182	9/25/2007	5	5	T	1/2a
L1-067	N/A[1]	1/14/08-2p#17	MM (3-L)	A-S3-68	1/7/2008	5	5	T	1/2a
L1-064	N/A	1/14/08-1p#1	MM (4-L)	A-S3-4	1/7/2008	5	5	T	1/2a
L1-066	N/A	1/14/08-1p#38	MM (2-R)	A-S3-43	1/7/2008	5	5	T	1/2a
L1-070	N/A	3/3/08-1p#1	MM (3-L)	A-S4-8	2/25/2008	5	5	T	1/2a
L1-087	2307	04/19/05-2p # 11	Fecal (1106)	A-5-155	4/12/2005	5.1	5.1	U	1/2a
L1-082	2302	04/19/05-4p # 20	Water (dry cow)	A-5-5	4/15/2005	5.1	5.1	U	1/2a
L1-100	2322	5/10/06-7P 21	Fecal (1006)	A-9-277	5/1/2006	5.1	5.1	U	1/2a
L1-096	2318	5/10/06-3P 21	Fecal (1550)	A-9-143	5/1/2006	5.1	5.1	U	1/2a
L1-098	2320	5/10/06-4P 25	Fecal (1713)	A-9-203	5/1/2006	5.1	5.1	U	1/2a
L1-119	2341	04/23/07-5P #38	Fecal (1810)	A-13-83	4/16/2007	5.1	5.1	U	1/2a
L1-125	2347	04/23/07-7P #21	Fecal (1547)	A-13-167	4/16/2007	5.1	5.1	U	1/2a
L1-065	N/A	1/14/08-1p#16	MM (8-L)	A-S3-13	1/7/2008	5.1	5.1	U	1/2a
L1-003	327	5/8/06-2P 41	Fecal (1612)	A-9-76	5/1/2006	6	6	A	1/2b
L1-093	2314	5/10/06-3P 1	Fecal (1602)	A-9-59	5/1/2006	6	6	A	1/2b
L1-136	2359	04/24/07-6P #21	Fecal (1628)	A-13-290	4/16/2007	6	6	A	1/2b
L1-294	4804	2/11/08-1P#1	MF	A-MS-198	2/4/2008	6	6	A	1/2b
L1-122	2344	04/23/07-6P #42	Fecal (1670)	A-13-113	4/16/2007	6.1	14	Q	1/2b
L1-115	2337	04/23/07-4P #50	Fecal (1589)	A-13-50	4/16/2007	6.1	14	Q	1/2b
L1-120	2342	04/25/07-2P #35	Fecal (1426)	A-13-98	4/16/2007	6.1	14.1	R	1/2b
L1-126	2348	04/23/07-6P #10	Fecal (1659)	A-13-179	4/16/2007	6.1	14	Q	1/2b
L1-127	2349	04/23/07-7P #37	Fecal (1683)	A-13-185	4/16/2007	6.1	14	Q	1/2b
L1-133	2355	04/24/07-5P #36	Fecal (1602)	A-13-254	4/16/2007	6.1	14	Q	1/2b
L1-110	2332	04/20/07-2P #49	Water (dry cow)	A-13-5	4/16/2007	6.1	14	Q	1/2b
L1-111	2333	04/23/07-3P #39	Pit (pit grab)	A-13-7	4/16/2007	6.1	14	Q	1/2b
L1-112	2334	04/23/07-4P #21	Composite (pen 2)	A-13-16	4/16/2007	6.1	14.1	R	1/2b
L1-138	2361	04/25/07-4P #42	Fecal (1495)	A-13-305	4/16/2007	6.2	14	AC	1/2b
L1-128	2350	04/23/07-7P #44	Fecal (1250)	A-13-200	4/16/2007	6.3	14.2	X	1/2b
L1-108	2330	04/20/07-3P #5	Water (pen 3+4)	A-13-3	4/16/2007	6.3	14.2	X	1/2b
L1-132	2354	04/23/07-8P #29	Fecal (1827)	A-13-239	4/16/2007	6.3	14.2	X	1/2b
L1-140	2364	05/21/07-1P #30	Floor Parlor	A-S2-9	5/16/2007	6.3	14.2	X	1/2b
L1-124	2346	04/23/07-7P #13	Fecal (1445)	A-13-164	4/16/2007	6.4	14.3	AD	1/2b
L1-116	2338	04/23/07-5P #16	Fecal (1697)	A-13-68	4/16/2007	6.5	6.1	AE	1/2b
L1-081	2301	04/19/05-4p # 4	Water (heifer)	A-5-4	4/12/2005	7	7	D	1/2a
L1-107	2329	04/20/07-1P #1	Water (pen 1+2)	A-13-2	4/16/2007	7	7	D	1/2a
L1-118	2340	04/23/07-5P #33	Fecal (1703)	A-13-80	4/16/2007	7	7	D	1/2a
L1-129	2351	04/23/07-6P #14	Fecal (1375)	A-13-203	4/16/2007	7	7	D	1/2a
L1-131	2353	04/24/07-5P #11	Fecal (1406)	A-13-233	4/16/2007	7	7	D	1/2a
L1-135	2358	04/24/07-6P #12	Fecal (1471)	A-13-284	4/16/2007	7	7	D	1/2a
L1-021	345	5/8/07-1P #37	BTM	A-M-161	5/2/2007	7	7	D	1/2a
L1-027	594	5/21/07-3P #1	MM (5-L)	A-S2-32	5/16/2007	7	7	D	1/2a
L1-026	592	5/29/07-2P #31	MF	A-MS-164	5/22/2007	7	7	D	1/2a
L1-045	711	06/04/07-1p #1	BTM	A-M-165	5/30/2007	7	7	D	1/2a

Table 1A. (continued)

QMP ID	CC#	Isolate #	Source	EMSL ID	Date	PFGE Type		C.E	Serotype
						AscI	ApaI	BN	Complex
L1-047	713	06/18/07-1p #1	MF	A-MS-166	6/6/2007	7	7	D	1/2a
L1-048	714	06/13/07-1p #16	BTM	A-M-166	6/6/2007	7	7	D	1/2a
L1-049	715	06/18/07-1p #15	MF	A-MS-167	6/12/2007	7	7	D	1/2a
L1-052	718	07/02/07-2p #21	BTM	A-M-169	6/27/2007	7	7	D	1/2a
L1-053	719	07/09/07-1p #9	MF	A-MS-170	7/5/2007	7	7	D	1/2a
L1-056	722	07/16/07-1p #13	MF	A-MS-171	7/10/2007	7	7	D	1/2a
L1-055	721	07/16/07-1p #3	BTM	A-M-171	7/10/2007	7	7	D	1/2a
L1-057	723	07/23/07-1p #1	BTM	A-M-172	7/18/2007	7	7	D	1/2a
L1-059	725	07/30/07-1p #20	BTM	A-M-173	7/25/2007	7	7	D	1/2a
L1-170	2443	8/27/07-1p #1	MF	A-MS-177	8/23/2007	7	7	D	1/2a
L1-229	2495	8/29/07-1p #5	BTM	A-M-177	8/23/2007	7	7	D	1/2a
L1-230	2496	9/4/07-1p #25	BTM	A-M-178	8/28/2007	7	7	D	1/2a
L1-172	2445	9/10/07-1p #1	MF	A-MS-179	9/5/2007	7	7	D	1/2a
L1-173	2446	9/17/07-1p #9	MF	A-MS-180	9/12/2007	7	7	D	1/2a
L1-231	2497	9/17/07-1p #1	BTM	A-M-180	9/12/2007	7	7	D	1/2a
L1-174	2447	9/24/07-1p #12	MF	A-MS-181	9/19/2007	7	7	D	1/2a
L1-232	2498	9/24/07-1p #1	BTM	A-M-181	9/19/2007	7	7	D	1/2a
L1-234	2500	10/9/07-1p #1	BTM	A-M-183	10/3/2007	7	7	D	1/2a
L1-175	2448	10/15/07-1p #7	MF	A-MS-184	10/9/2007	7	7	D	1/2a
L1-176	2449	10/29/07-1p #33	MF	A-MS-186	10/23/2007	7	7	D	1/2a
L1-235	2501	10/29/07-1p #41	BTM	A-M-186	10/23/2007	7	7	D	1/2a
L1-177	2450	11/5/07-1p #17	MF	A-MS-187	10/31/2007	7	7	D	1/2a
L1-236	2502	11/5/07-1p #1	BTM	A-M-187	10/31/2007	7	7	D	1/2a
L1-289	4799	11/13/07-1P#13	MF	A-MS-188	11/7/2007	7	7	D	1/2a
L1-301	4811	11/13/07-1P#1	BTM	A-M-188	11/7/2007	7	7	D	1/2a
L1-290	4800	11/19/07-1P#34	MF	A-MS-189	11/14/2007	7	7	D	1/2a
L1-302	4812	11/26/07-1P#6	BTM	A-M-189	11/14/2007	7	7	D	1/2a
L1-291	4801	12/4/07-1P#19	MF	A-MS-190	11/27/2007	7	7	D	1/2a
L1-303	4813	12/4/07-1P#1	BTM	A-M-190	11/27/2007	7	7	D	1/2a
L1-292	4802	12/10/07-3P#1	MF	A-MS-191	12/4/2007	7	7	D	1/2a
L1-293	4803	12/17/07-1P#2	MF	A-MS-192	12/12/2007	7	7	D	1/2a
L1-295	4805	2/25/08-1P#6	MF	A-MS-200	2/18/2008	7	7	D	1/2a
L1-296	4806	3/3/08-3P#35	MF	A-MS-201	2/25/2008	7	7	D	1/2a
L1-304	4814	3/6/08-1P#11	BTM	A-M-201	2/25/2008	7	7	D	1/2a
L1-305	4815	3/10/08-1P#1	BTM	A-M-202	3/4/2008	7	7	D	1/2a
L1-309	4819	3/17/08-1P#11	MF	A-MS-203	3/11/2008	7	7	D	1/2a
L1-306	4816	3/19/08-1P#1	BTM	A-M-203	3/11/2008	7	7	D	1/2a
L1-297	4807	3/25/08-1P#1	MF	A-MS-204	3/18/2008	7	7	D	1/2a
L1-307	4817	3/27/08-1P#1	BTM	A-M-204	3/18/2008	7	7	D	1/2a
L1-298	4808	3/31/08-1P#1	MF	A-MS-205	3/25/2008	7	7	D	1/2a
L1-308	4818	4/7/08-1P#1	BTM	A-M-206	3/31/2008	7	7	D	1/2a
L1-299	4809	4/21/08-2P#13	MF	A-MS-208	4/14/2008	7	7	D	1/2a
L1-310	2632	5/5/08-6P#39	MF	A-MS-210	4/29/2008	7	7	D	1/2a
L1-253	2635	5/27/08-1p #3	MF	A-MS-213	5/21/2008	7	7	D	1/2a
L1-254	2636	6/2/08-1p #5	MF	A-MS-314	5/28/2008	7	7	D	1/2a
L1-255	2637	6/11/08-1p #8	MF	A-MS-215	6/4/2008	7	7	D	1/2a
L1-130	2352	04/23/07-8P #3	Fecal (1756)	A-13-209	4/16/2007	7.1	7	E	1/2a
L1-020	344	5/21/07-2P #11	TC (7-R)	A-S2-17	5/16/2007	7.1	7	E	1/2a
L1-073	2292	03/03/04-1p #16	Fecal (1378)	A-1-156	2/17/2004	8	8	P	1/2b
L1-076	2295	03/03/04-1p # 2	Fecal (1326)	A-1-303	2/17/2004	8	8	P	1/2b
L1-080	2300	1/18/05-1p#19	Water (heifer)	A-4-4	1/11/2005	9	9	B	1/2a
L1-083	2303	04/19/05-1p # 40	Fecal (1480)	A-5-75	4/12/2005	10	10	G	1/2a
L1-099	2321	5/10/06-5P 39	Fecal (727)	A-9-257	5/1/2006	10	10	G	1/2a
L1-101	2323	5/10/06-10P 13	Fecal (1359)	A-9-304	5/1/2006	10	10	G	1/2a
L1-085	2305	04/19/05-1p # 43	Fecal (1257)	A-5-110	4/12/2005	10.1	10.1	H	1/2a
L1-113	2335	04/23/07-4P #34	Feed (TMR pens 1-4)	A-13-18	4/16/2007	10.1	10.1	H	1/2a
L1-102	2324	7/17/06-1P 21	Flies (no info~source)	A-10-22	7/17/2006	11	11	W	1/2a
L1-103	2325	01/16/07-1P #3	Water (pen 1+2)	A-12-2	1/8/2007	12	12	V	1/2a

Table 1A. (continued)

QMP ID	CC#	Isolate #	Source	EMSL ID	Date	PFGE Type		C.E	Serotype
						AscI	ApaI	BN	Complex
L1-256	2640	6/23/08-4p #3	MF	A-MS-211	5/13/2008	12.1	2.1	AF	1/2a
L1-109	2331	04/23/07-2P #1	Water (heifer)	A-13-4	4/16/2007	13	13	C	1/2a
L1-121	2343	04/23/07-5P #47	Fecal (1165)	A-13-104	4/16/2007	13	13	C	1/2a
L1-086	2306	04/19/05-1p 47	Fecal (1409)	A-5-140	4/12/2005	14	16	AG	1/2a
L1-137	2360	04/24/07-6P #27	Fecal (1682)	A-13-299	4/16/2007	15	17	AH	1/2b
L1-071	N/A	1/14/08-2p#44	Bedding (calf bedding)	A-16-10	1/7/2008	16	22	AI	N/S [3]
L1-068	N/A	1/14/08-3p#23	Water (pen 3+4)	A-16-3	1/7/2008	17	N/T[2]	-	N/S
L1-123	2345	04/23/07-7P #7	Fecal (1696)	A-13-155	4/16/2007	18	15	Y	1/2b
L1-141	2365	05/21/07-1P #47	Floor Storage Rm.	A-S2-11	5/16/2007	18	15	Y	1/2b
L1-241	2608	4/21/08-4p #8	Fecal (1944)	A-17-72	4/14/2008	19	18	AJ	N/S
L1-239	2606	4/21/08-3p #5	Water (pen 1+2)	A-17-2	4/14/2008	19	18	AJ	N/S
L1-244	2611	4/21/08-4p #41	Fecal (1856)	A-17-180	4/14/2008	20	19	AK	1/2a
L1-178	2367	4/24/07-5P #49	Fecal (1258)	A-13-266	4/16/2007	21	8.1	AL	1/2b
L1-153	2415	11/21/06-2P #13	MF	A-MS-139	11/16/2006	22	20	AM	4b
L1-251	2633	5/19/08-1p #5	MF	A-MS-212	5/15/2008	23	21	AN	N/S

[1]N/A=Not available

[2]N/T= Non-typeable by ApaI

[3]N/S= Non-serotypeable

Figure 1A. Scanning electron microscopy pictures of a teat-cup rubber liner piece obtained from milking unit 3-left. Panel A shows the presence of cracks in the surface of the liner. Panel B shows the presence of foreign material on the surface of the liner. Scale: Panel A, 50 microns; Panel B, 1500 nm.

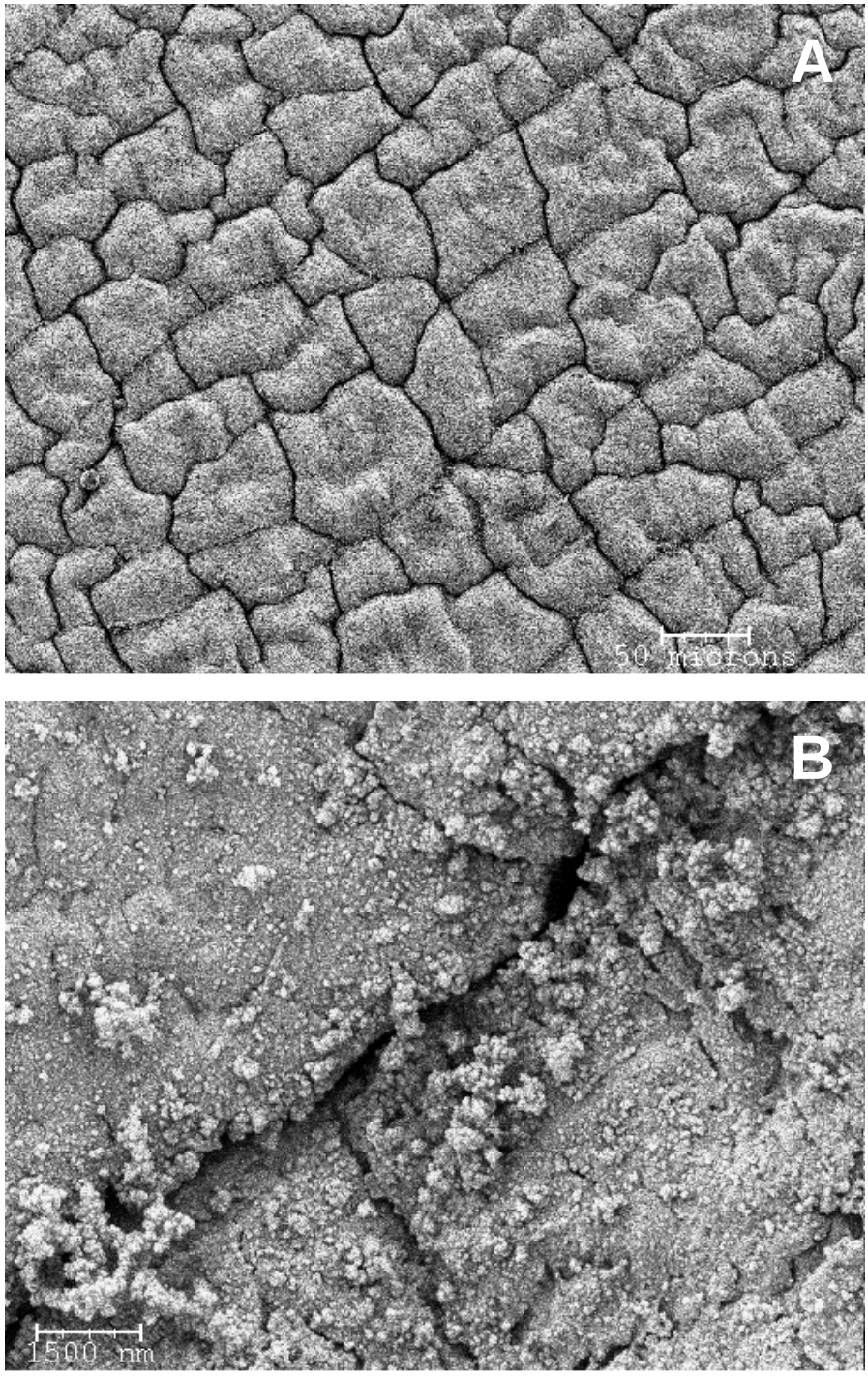
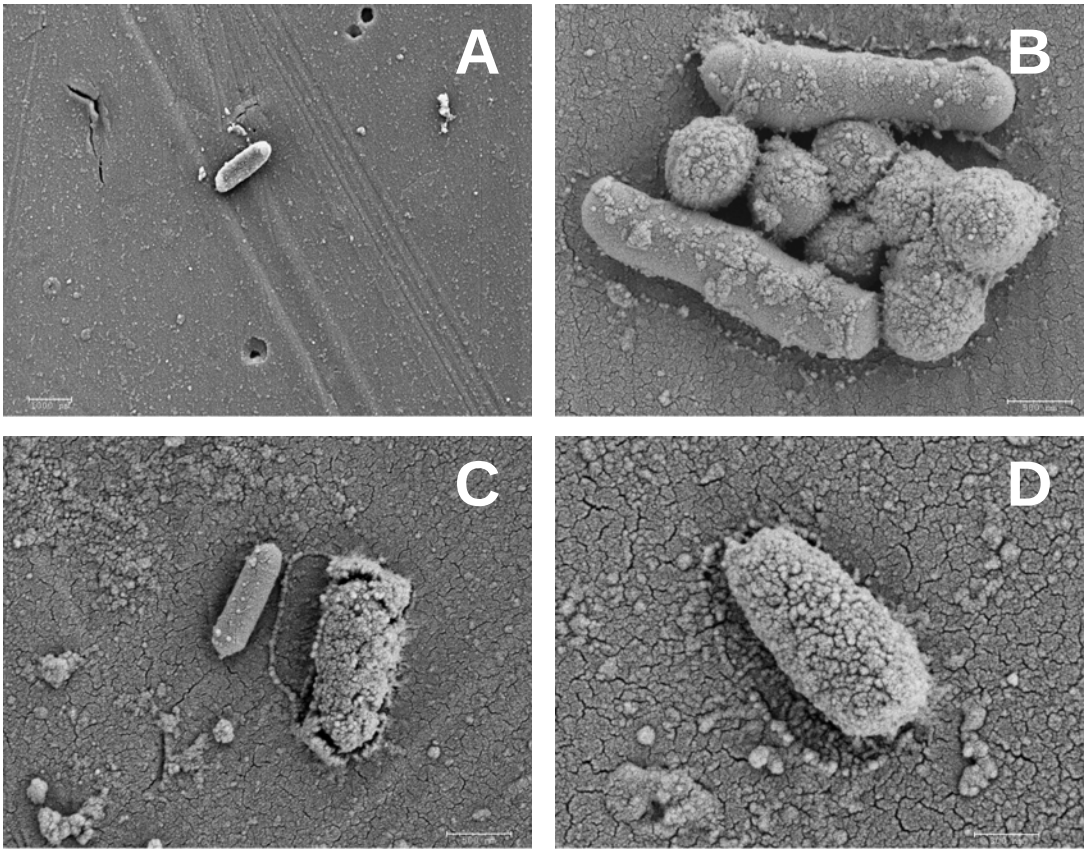

A
50 macrons
B
1500 nm

Figure 2A. Scanning electron microscopy pictures of A) Top cover of milk meter 8-left (scale= 1000 nm); B) Cluster of bacteria attached to the surface of the bottom cover of milk meter 8-left (scale=500 nm); C) and D) Bacteria and bacteria covered in exopolymeric matrix attached to the surface of the bottom cover of milk meter 8-left (scale= 500 nm and 300 nm, respectively).


A
B
C
D