Occurrence and growth of *Listeria monocytogenes* in packaged raw milk

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Hanna Castro, Marjo Ruusunen and Miia Lindström*

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

* Corresponding author:

Miia Lindström

Department of Food Hygiene and Environmental Health

Faculty of Veterinary Medicine

P. O. Box 66

FI-00014 University of Helsinki

FINLAND

Tel. +358-9-191 57107

Fax +358-9-191 57101

Email: miia.lindstrom@helsinki.fi
ABSTRACT

The increased availability of packaged raw drinking milk necessitates the investigation of the occurrence and growth of *Listeria monocytogenes* in raw milk during distribution and storage. The occurrence of *L. monocytogenes* in 105 retailed raw milk bottles, 115 bulk tank milk samples, 23 in-line milk filter socks and in 50 environmental samples collected from an on-farm dairy establishment were investigated. Growth of inoculated low-level *L. monocytogenes* contamination was also investigated in two types of raw milk packaging, namely in 1-litre plastic bottles and 3-litre bag-in-boxes, both stored at three different storage temperatures of 6, 8 and 10 °C. The occurrence of *L. monocytogenes* was higher (4.8%) in bottled raw milk stored until the use-by-date of the package compared to fresh bulk tank milk (1.7%). *L. monocytogenes* counts were ≤13 CFU/ml in bottled raw milk and ≤1 CFU/ml in bulk tank milk. *L. monocytogenes* was not detected in the packaging facility, but occurred very frequently (39%) in the milk filter socks. Subtyping of *L. monocytogenes* isolates using pulsed-field gel-electrophoresis revealed seven pulsotypes, of which two occurred in multiple samples. Targeted inoculum levels of 1-2 CFU/ml yielded *L. monocytogenes* counts ≥100 CFU/ml within seven days of storage in 22% of the raw milk packages stored at 6 °C, and in all of the raw milk packages stored at 8 °C. °C. The frequent occurrence of *L. monocytogenes* in raw milk and the ability of a low-level *L. monocytogenes* contamination to grow at refrigeration temperatures highlights the importance of consumer education regarding the appropriate raw milk storage and handling.
HIGHLIGHTS

- *L. monocytogenes* occurred frequently in packaged raw milk with counts of ≤1–13 CFU/ml
- 1 CFU/ml of *L. monocytogenes* in raw milk can yield 100 CFU/ml in 7 days at 6 °C
- 1 CFU/ml of *L. monocytogenes* in raw milk can yield 100 CFU/ml in 5 days at 10 °C
- Consumer education on appropriate handling and storage of raw milk is warranted

KEYWORDS

Unpasteurized milk; ready-to-eat food; shelf-life; growth modelling; growth rate; lag time; refrigeration; food safety
1. Introduction

The practice of pasteurising milk on a commercial scale began in Europe in the 1880’s. More than a century later, the commercial sale of raw milk remains a controversial issue. Regulation (EC) No 853/2004 defines raw milk as “milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect”. Many European countries allow the direct sale of raw milk from farms to consumers, provided that the operation complies with the hygienic criteria in Regulation (EC) No 853/2004 and the General Food Law (Regulation [EC] No. 178/2002). In addition, Regulation (EC) No. 2073/2005 constitutes the microbiological criteria for foodstuffs, which include the microbiological food safety criteria for *Listeria monocytogenes* in ready-to-eat foods. Specifically, producers must demonstrate that *L. monocytogenes* counts in products placed on the market (n=5) will not exceed 100 CFU/g at any point within the shelf-life of the product. Furthermore, if the producer is unable to demonstrate to the competent authority that *L. monocytogenes* counts will not exceed 100 CFU/g during the shelf-life the product, the producer must demonstrate the absence of *L. monocytogenes* in 25 g of the product (n=5) before it has left the immediate control of the producer. Approved dairy establishments in Finland can package raw milk and distribute it to retail outlet stores in compliance with the Finnish Ministry for Agriculture and Forestry Act 699/2013. Packaged raw milk is currently available in 1-litre plastic bottles and in 3-litre bag-in-boxes. The bag-in-box package comprises a double-layered flexible film bag that is held inside a paperboard carton. Milk is dispensed through a valve, which prevents the uptake of air during dispensing, thus limiting the product exposure to oxygen.

The consumer demand for raw milk arises from perceptions of better sensory and nutritional qualities of raw milk over those of pasteurised milk, and also from a desire of many consumers to support local and small-scale agriculture (Perkiömäki *et al.*, 2012; Rahn *et al.*, 2016). Additionally, raw milk consumption is anecdotally attributed as having various health benefits, yet these assertions fall short of scientific validity (Claeys *et al.*, 2013). In contrast, epidemiological data
clearly demonstrate microbiological health risks associated with raw milk consumption. Langer et al. (2012) showed that per unit of dairy product consumed, unpasteurised dairy products were associated with a 150-fold greater incidence of infectious disease outbreaks than pasteurised dairy products. Furthermore, outbreaks involving unpasteurised dairy products had a higher hospitalisation rate and involved a greater portion of underage individuals than outbreaks caused by pasteurised products. The number of outbreaks linked to raw milk consumption during the 2007–2012 period, totalled 27 and affected 304 individuals in Europe (EFSA BIOHAZ, 2015). Corresponding numbers for the United States were 81 outbreaks and 979 individuals for the same period (Mungai et al., 2015). Moreover, sporadic cases of raw milk-associated illness vastly outnumber the cases linked to outbreaks (Robinson et al., 2014). In both Europe and in the United States, Campylobacter spp., Salmonella spp. and shiga toxin-producing Escherichia coli (STEC) were responsible for the majority of raw milk-mediated outbreaks and cases of sporadic illness. Consumption of raw milk contaminated by L. monocytogenes in 2014 caused two hospitalisations and one mortality in the United States (CDC, 2016). The incident demonstrated that liquid raw milk, among other ready-to-eat products, can act as a vehicle for listeriosis. Listeriosis is a rare but serious foodborne illness that primarily affects immunodeficient individuals (Bertrand et al., 2016; Goulet et al., 2012; Lundén et al., 2004). Listeriosis may also lead to abortion and life-threatening infection of the foetus. Europe has witnessed a significantly increasing trend of listeriosis over the 2008–2014 period (EFSA and ECDC, 2015). Of the 2161 confirmed listeriosis cases in 2014, 99% led to hospitalisation and 15% to death. The hospitalisation and mortality rates for listeriosis were the highest among all foodborne pathogens (EFSA and ECDC, 2015).

Cattle frequently shed Listeria in their faeces and the farm environment is a rich reservoir for L. monocytogenes (Haley et al., 2015; Ho et al., 2007; Nightingale et al., 2004; Rocha et al., 2013). Subsequently, L. monocytogenes is a common contaminant of raw milk. Several studies of European bulk tank milk samples reported a 4.9–6.1% prevalence range for L. monocytogenes (De Reu et al., 2004; Desmasures et al., 1997; Fenlon et al., 1995; O’Donnell, 1995; Rea et al., 1992; Ruusunen et al., 2013; Vilar et al., 2007). Three studies describe a lower prevalence of 0.4–1.5%
(Bachmann and Spahr, 1995; Botsaris et al., 2016; Waak et al., 2002), whereas a recent Estonian study reported a prevalence as high as 29% for *L. monocytogenes* in the bulk tank milk of farms that distribute raw milk to vending machines (Kalmus et al., 2015).

Contamination of the bovine udder surface from faeces and the barn environment is the predominant source of *L. monocytogenes* contamination in bulk tank milk (Nightingale et al., 2004; Sanaa et al., 1993; Vilar et al., 2007). In addition, *L. monocytogenes* nested in biofilms on the milking equipment may exfoliate cells into bulk tank milk (Latorre et al., 2010). Regardless of the contamination source, *L. monocytogenes* disperses into the entire volume of milk collected in the bulk tank, and subsequent contamination levels in bulk tank milk are generally low. Levels described in literature fall in the range of ≤1–60 CFU/ml (Fenlon et al., 1995; Meyer-Broseta et al., 2003; O’Donnell, 1995; Ruusunen et al., 2013; Waak et al., 2002). *L. monocytogenes* infection of the udder (mastitis) is an infrequent source of raw milk contamination. A Danish study of 1 million dairy cows revealed a 0.04% incidence for listerial mastitis, which nearly always presented in a single udder quarter (Jensen et al., 1996). Milk from an infected quarter is often visually unchanged (Hunt et al., 2012) but can contain *L. monocytogenes* counts as high as 10 000–60 000 CFU/ml (Bourry et al., 1995; Farber et al., 1990; Jensen et al., 1996). Consequently, listerial mastitis could theoretically result in high (>100 CFU/ml) *L. monocytogenes* counts in the bulk tank milk (Bourry et al., 1995).

*L. monocytogenes* is a psychrotroph, capable of growing in refrigerated milk (Donnelly and Briggs, 1986; Rosenow and Marth, 1987; Walker et al., 1990). However, the availability of growth data for *L. monocytogenes* in refrigerated raw milk is limited, and published studies often involve short storage times of <3 days (Gay and Amgar, 2005), or high initial counts of ≥10 000 CFU/ml (Farber et al., 1990; Gaya et al., 1991). Consequently, the growth potential of the frequently observed low *L. monocytogenes* counts in raw milk remains poorly understood. Latorre et al. (2011) used a quantitative risk assessment procedure to demonstrate that the consumer’s refrigerator temperature was the most important single parameter that affected the listeriosis risk associated
with raw milk consumption. A survey of 267 Finnish raw milk consumers found that the refrigerator temperatures in households varied between 1–10 °C with a mean of 6 °C. Raw milk storage times varied from 0–14 days from purchase, with a mean of 5 days (Perkiömäki et al., 2012). Among the respondents were individuals that were susceptible to listeriosis, including pregnant women (3%), and individuals with an immunity debilitating disorder (2%). Only 2% of consumers reported that they heated the raw milk before consumption.

The overall objective of this study was to elucidate the occurrence and growth potential of low-level L. monocytogenes contamination during the distribution and storage of packaged raw milk. The occurrence of L. monocytogenes in retailed raw milk bottles, bulk tank milk samples, in-line milk filter socks and in environmental samples from an on-farm dairy establishment were investigated, and the naturally occurring L. monocytogenes counts present in retailed raw milk bottles were compared with those found in fresh bulk tank milk. A further objective was to investigate the growth of inoculated low-level L. monocytogenes contamination in two types of raw milk packaging, namely in 1-litre plastic bottles and 3-litre bag-in-boxes stored at three different storage temperatures of 6, 8 and 10 °C.

2. Materials and Methods

2.1 Occurrence of L. monocytogenes in bottled raw milk, bulk tank milk, milk filter socks and the environment of an on-farm dairy establishment

Between November 2013 and September 2015, the occurrence of L. monocytogenes in bottled raw milk, bulk tank milk, in-line milk filter-socks, and in the environment of a Finnish on-farm dairy establishment was investigated. All of the raw milk packaged by the on-farm dairy establishment (<50 000 kg per year) was produced on that farm.
2.1.1 Sample collection

Totals of 105 bottles of raw milk, 115 bulk tank milk samples, 23 in-line milk filter socks and 50 environmental samples from an on-farm dairy establishment were collected between November 2013 and September 2015 (Figs. 1 and 2). The milk and filter sock samples were collected in 23 samplings. At each sampling, one in-line milk filter sock and five 50-ml samples of bulk tank milk were obtained from the on-farm dairy establishment and three to five 1-litre bottles of the dairy’s raw milk were purchased from a retail store. The packaging date of the purchased raw milk bottles was either the same as the date of bulk tank milk sampling, or three days after the date of bulk tank milk sampling. Bulk tank milk and milk filter sock samples were always collected on the same date after morning milking, so that a portion of the milk sampled from the bulk tank had passed through the collected filter sock. Bulk tank milk samples were collected into Falcon™ Polypropylene Centrifuge Tubes and the milk filter socks were collected and placed into Minigrip® bags, and the samples were delivered to the laboratory within 24 hours in packages containing ice packs. The environmental surface swab samples of the raw milk packaging facility were collected in 10 independent samplings between January 2014 and September 2014 from milk filler heads (19 samples) and milk inlet valves of milk fillers (18 samples), hoses used for conveying milk (8 samples) and the floor of the dairy (5 samples). Environmental samples were collected after routine cleaning of the equipment and premises. Milk fillers were sampled from the inner surface of the milk filler outlet (through which milk is dispensed into packages) using a sterilized cotton swab stick. After swabbing, the swab was placed into a tube and immersed into 1 ml of buffered peptone water (Thermo Fisher Scientific, Waltham, Massachusetts). The remaining environmental samples were collected using sterile sponge swabs (VWR, Radnor, Pennsylvania) that had been moistened with 5 ml of buffered peptone water. Samples were taken from the inner surface of the outlet of the hose and floor samples were collected by swabbing a 900 cm² floor area under the milk filler. The environmental samples of the bulk tank milk and milk filter socks were analysed immediately upon arrival at the laboratory. Raw milk bottles were purchased from a retail store approximately 24
hours after packaging. The bottles were transported to the laboratory in coolers, stored at 6 °C and analysed on the use-by-date of the milk (7 days from packaging).

2.1.2 Isolation and detection of *L. monocytogenes* and other *Listeria* spp.

*L. monocytogenes* and other *Listeria* spp. were isolated from the samples according to the NMKL 136:2010 standard, which is comparable to the ISO11290-1:1996 and ISO 11290-2:1998 standards with Amendment 1:2004. The method involves two-step enrichment, where the 25-ml sample was first enriched in 225 ml of half-Fraser broth at 30 °C for 24 hours, after which 100 µl of the cultivated half-Fraser broth was enriched in 10 ml of Fraser broth (Lab M Limited, Bury, United Kingdom) at 37 °C for 48 h. After each enrichment step, 100 µl of the cultivated enrichment broth was plated on a Harlequin™ chromogenic *Listeria* agar (Lab M Limited) plate and a *Listeria monocytogenes* blood agar (Lab M Limited) plate. Entire filter socks, sponge swabs and swab sticks, and 25-ml aliquots of the milk samples were used for the enrichment. The enumeration of *L. monocytogenes* in milk samples was carried out by dividing 1 ml of each milk sample onto three separate Harlequin™ chromogenic *Listeria* agar plates without prior enrichment. Colonies with morphology representative of *L. monocytogenes* or other *Listeria* spp. detected on the selective agar plates were cultivated on Columbia blood agar plates (Lab M Limited) with 5% bovine blood and identified as *L. monocytogenes* and other *Listeria* species using a multiplex PCR method (Bansal et al., 1996).

2.1.3 Molecular characterisation of *L. monocytogenes* isolates

One *L. monocytogenes* isolate from each positive sample was subtyped using pulsed-field gel electrophoresis (PFGE) with *Apa* I and *Asc* I (New England Biolabs, Ipswich, Massachusetts) restriction (Autio et al. 1999). The DNA fragments were separated by size by electrophoresing the samples through a 1.0% (w/v) agarose gel (SeaKem Gold, FMC Bioproducts, Rockland, Maine) at 200 V and 8 °C in the Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala,
Sweden) with switch times of 1 to 35 s over an 18 h period. DNA fragment size was determined using a low-range pulsed-field gel marker (New England Biolabs). PFGE profiles were analysed using the BioNumerics software version 5.10 (Applied Maths, Austin, Texas). Bands were assigned automatically and adjusted manually after visual assessment. Automated cluster analysis of the combined Apal and Ascl fingerprint profiles was done by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient with a 1.5% position tolerance limit and 1% optimization. Serogroups of the subtyped isolates were determined by a multiplex PCR method described by Doumith et al. (2004). The method enables the differentiation of four *L. monocytogenes* PCR serogroups: IIa (serovars 1/2a and 3a); IIC (serovars 1/2c and 3c), IIb (serovars 1/2b, 3b and 7); and IVb (serovars 4b, 4d and 4e).

### 2.2 The growth of *L. monocytogenes* in differently packaged raw milk

To investigate *L. monocytogenes* growth in packaged raw milk, 33 1-litre plastic bottles and 33 3-litre bag-in-boxes from a single producer were purchased from a retail store approximately 24 hours after packaging of the milk. Packages were transported to the laboratory in coolers and utilized immediately in the growth study. Prior to the inoculation of *L. monocytogenes* into the raw milk packages, negative control samples were collected from the packages to ensure that they were initially *Listeria* free. Inoculation was performed immediately after the collection of the control samples. The volume of the negative control samples was 109 ml for bottles and 327 ml for bag-in-boxes. Control samples were analysed using the method described in section 2.1.2.

#### 2.2.1 Preparation of the inocula

The growth studies were conducted for three *L. monocytogenes* strains (Table 1), one of which (S1) was isolated from bottled raw milk that was produced by the on-farm dairy described above (section 2.1). The growth of each strain was investigated individually in separate bottles and bag-in-boxes. Strains were stored in TS/80-MX Cryobeads (TSC Technical Service Consultants Ltd,
Lancashire, United Kingdom) at -70 °C. To calculate the amount of inocula needed to reach the targeted levels, overnight growth of each L. monocytogenes strain was first investigated. In brief, the strains were extracted from Cryobeads onto blood agar plates and cultivated at 37 °C for 24 hours. Single colonies were transferred to 10 ml of brain heart infusion broth (BHI; Lab M Limited) and incubated at 37 °C for 24 hours with agitation at 100 rpm. The cultivated BHI broths were diluted into isotonic saline in a series of dilutions from $10^{-1}$ to $10^{-10}$. From the dilutions $10^{-5}$ to $10^{-10}$, 100 µl of each dilution was cultivated onto Harlequin™ Chromogenic Listeria agar plates for the enumeration of L. monocytogenes. As all three strains grew to 9 log CFU/ml, the same protocol for the preparation of the inocula was used for each strain.

The inocula were prepared by extracting the strains from cryogenic tubes onto blood agar plates and cultivated at 37 °C for 24 hours. Single colonies were selected and grown in 10 ml of BHI broth at 37 °C for 24 hours with shaking at 100 rpm. The cultures were diluted in isotonic saline in a series of dilutions from $10^{-1}$ to $10^{-6}$. The inocula for the targeted inoculum levels of 200 CFU/ml, 20 CFU/ml and 2 CFU/ml were prepared by pipetting 10 ml of the $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions, respectively, into bottles containing 40 ml of isotonic saline. From the bottles containing the appropriately diluted inocula, 9 ml of dilution was inoculated into a raw milk bottle and 27 ml was inoculated into a bag-in-box.

2.2.2 Inoculation and enumeration of L. monocytogenes

The growth study was performed in triplicate for each strain, package type, and targeted inoculum level (18 experimental replicates for each targeted inoculum level). Each bag-in-box was inoculated with a sterile needle and syringe, after which the puncture hole was closed aseptically with adhesive tape. Bottles were inoculated by pipetting. The inoculated packages were stored at 6 °C and sampled 0, 3, 5, 7 and 14 days after inoculation to determine viable L. monocytogenes counts on selective agar plates (Harlequin™ Chromogenic Listeria agar). The packages were mixed with 30 gentle inversions at each sampling, after which 10-ml samples were collected.
through the mouth of the bottles and 30-ml samples were collected through the nozzle of the bag-in-boxes. A 2-ml volume of milk was divided between 6 agar plates to enumerate *L. monocytogenes* counts ≤100 CFU/ml. A 200 μl volume of each dilution of a 10-fold dilution series was divided and pipetted onto two agar plates for the enumeration of counts >100 CFU/ml. The plates were then incubated at 37 °C for 48 h after which they were enumerated. Additionally, the pH of each milk sample was measured using the inoLab® pH 7110 (Xylem Analytics, Beverly, Massachusetts) pH meter, which was calibrated with technical buffers (Xylem Analytics) on each sampling day.

### 2.2.3 pH and aerobic bacteria in uninoculated packages

Six of the purchased raw milk packages (three bottles and three bag-in-boxes) were left uninoculated. The uninoculated packages were stored at 6 °C and the milk was sampled on days 0, 3, 5, 7 and 14 to enumerate viable aerobic bacteria and to measure pH. The pH measurements were conducted as described in section 2.2.2. Additionally, total viable aerobic bacterial counts of the uninoculated milk were determined after incubation at 30 °C for 72 hours, as described in the ISO 4822:2003 method, using Plate Count Agars (Thermo Fisher Scientific) with 1 g/l of skimmed milk powder (Lab M Limited).

### 2.3 *L. monocytogenes* growth in raw and pasteurised milk at inordinate consumer storage temperatures

#### 2.3.1 The growth of *L. monocytogenes* in raw milk stored at 6, 8 and 10 °C

Raw milk was obtained from a nearby dairy cattle farm and was collected into sterilised 1-litre laboratory bottles and transported in coolers to the laboratory. Control samples were taken from each bottle and analysed as described above (section 2.1.2) to ensure that the milk was initially free of *Listeria*. The milk was then divided into 99-ml aliquots in 250 ml bottles and the growth
13 study was initiated immediately. *L. monocytogenes* strains ATCC 19115, S1 and S2 (Table 1) were
extracted from Cryobeads onto blood agar plates and cultivated in 10 ml of BHI broth at 37 °C for
24 hours, as described above (section 2.2.1). The cultivated BHI broths were diluted in isotonic
saline to dilutions $10^{-1}$ to $10^{-6}$. Dilutions $10^{-5}$ and $10^{-6}$ were used for the inocula of targeted inoculum
levels 10 and 1 CFU/ml, respectively. Cocktails containing equal portions of the three strains were
prepared by pipetting 3 ml of the cultivated BHI broth dilution of each strain into bottles containing
81 ml of isotonic saline. From the bottles, 1 ml of the cocktail was inoculated into bottles containing
99 ml of raw milk. The study was performed in triplicate for each targeted inoculum level and
storage temperature. The storage temperatures were 6, 8 and 10 °C, which represent the mean to
maximum range of consumer storage temperatures for raw milk, as reported by Perkiömäki et al.
(2012). *L. monocytogenes* growth was determined on storage days 0, 5, 7 and 14 days as
described above (section 2.2.2).

2.3.2 The growth of *L. monocytogenes* in pasteurised milk stored at 6 and 10 °C

Raw milk was obtained and controlled for the presence of *Listeria* as described in section 2.3, to
compare the growth of *L. monocytogenes* in pasteurised milk to that of its growth in raw milk. Raw
milk was divided into sterilized bottles in 99-ml aliquots and pasteurised by immersing the bottles in
a hot water bath (75 °C) with a shaker stirring the milk at 80 rpm, until the temperature inside a
control milk bottle reached 72 °C for 15 seconds, after which the milk was cooled to 6 °C. The
same cocktail containing three *L. monocytogenes* strains described in section 2.3.1 was used to
inoculate the bottles with *L. monocytogenes* to a targeted inoculum level of 10 CFU/ml. Inoculated
pasteurised milk bottles were stored at either 6 or 10 °C and sampled 5 and 14 days after
inoculation. Three replicates were performed for both storage temperatures. *L. monocytogenes*
counts were determined as described in section 2.2.2, and the results were compared with those
obtained from raw milk in section 2.3.1.

2.4 Data analyses
The Baranyi and Roberts model (Baranyi and Roberts, 1994) was fitted to the experimental growth data (mean colony counts) of *L. monocytogenes* inocula in packaged raw milk using the Combase DMFit software (http://www.combase.cc/tools/). Growth parameters (maximum growth rate and lag-time) were derived from the modelled growth. Statistical analyses were run on the IBM SPSS Statistics 23 software. Standard deviations and standard errors of the mean were calculated from log-transformed colony count data. If no colonies were detected in a given sample, -0.3 log CFU/ml was used as the log-transformed value for the calculation. An independent-samples two-tailed t-test without assumption of equal variances was used to compare the mean *L. monocytogenes* colony counts between bottles and bag-in-boxes, and between raw and pasteurised milk. The mean colony counts between *L. monocytogenes* strains were compared using an independent-samples Kruskal-Wallis test. The correlation between pH and colony counts was determined using bivariate Pearson correlation.
3. Results

3.1 Occurrence of *L. monocytogenes* in bottled raw milk, bulk tank milk, milk filter socks and the environment of an on-farm dairy establishment

The occurrence of *L. monocytogenes* in 105 retailed raw milk bottles, 115 bulk tank milk samples, 23 in-line milk filter socks and in 50 environmental samples of the packaging facility were investigated (Fig. 2). All of the sampled raw milk bottles, bulk tank milk, filter socks and environmental samples originated from the same on-farm dairy establishment. The overall occurrence of all *Listeria* spp. was 6.7% for bottled raw milk, 3.5% for bulk tank milk, 57% for in-line milk filter socks, and 8.0% for environmental samples of the packaging facility. Of the 105 raw milk bottles examined, five (4.8%) were positive for *L. monocytogenes*. Two raw milk bottles, both from the August 2014 sample set, contained *L. monocytogenes* counts of 1 and 13 CFU/ml on direct plating. Although the two bottles contained milk of the same batch, two different *L. monocytogenes* pulsotypes (II and III) were isolated from them (Fig. 3). Bulk tank milk samples of the August 2014 sample set were negative for *L. monocytogenes*, and the milk filter sock of the same sample set contained a *L. monocytogenes* pulsotype (IV) that differed from those of the bottled raw milk.

*L. monocytogenes* was detected less frequently in bulk tank milk samples than in raw milk bottles as only two of the 115 bulk tank milk samples (1.7%) were positive for *L. monocytogenes*. One of the two positive bulk tank milk samples contained a *L. monocytogenes* count of 1 CFU/ml with direct plating. Both positive bulk tank milk samples belonged to the December 2013 sample set, in which one of the raw milk bottles and the milk filter sock were also positive for *L. monocytogenes*. Furthermore, all samples positive for *L. monocytogenes* in the December 2013 sample set contained the same pulsotype (I).
L. monocytogenes occurred more frequently in milk filter socks than in bulk tank milk or in bottled raw milk, with 9/23 (39%) filter socks being positive for L. monocytogenes. Subtyping of filter sock isolates revealed two reoccurring pulsotypes (I and IV) and one sporadically occurring pulsotype (V). All of the sampled milk filter socks from November 2013 to February 2014 were positive for L. monocytogenes pulsotype I. From March 2013 to July 2015, L. monocytogenes pulsotype IV occurred intermittently in four milk filter socks. When a bulk tank milk sample was positive for L. monocytogenes or other Listeria spp., the milk filter sock of the respective sample set was also found to be positive. However, L. monocytogenes positive bottled raw milk samples also occurred in sample sets with negative milk filter socks. All L. monocytogenes isolates from raw milk and filter socks belonged to PCR serogroup IIa.

L. monocytogenes was not detected in any of the 50 samples collected from the environment of the packaging facility. Four (8.0%) environmental samples were, however, positive for Listeria spp. other than L. monocytogenes. One of these samples was collected from the inner surface of the milk filler head, through which milk is dispensed into packages, whereas the remaining three samples were obtained from the floor underneath the milk filler.

3.2 The growth of L. monocytogenes in differently packaged raw milk

The growth of L. monocytogenes strains ATCC 19115, S1 and S2 was investigated in bottles and bag-in-boxes stored at 6 °C at three targeted inoculum levels: 200 CFU/ml, 20 CFU/ml and 2 CFU/ml. Additionally, the pH of the milk in the inoculated packages was measured through the 14-day storage period. When the L. monocytogenes strains were inoculated individually into separate raw milk bottles and bag-in-boxes to a targeted inoculum level of 2.3 log CFU/ml (200 CFU/ml) in milk, no statistically significant differences in colony counts were observed between the three strains on storage days 0–14 (p>0.05). The strains grew in bottles from a mean initial colony count of 2.3 log CFU/ml (SD=0.1 log CFU/ml) on day 0 to a mean final colony count of 4.0 log CFU/ml (SD=0.5 log CFU/ml) on day 14 (Fig. 4). Colony counts in bag-in-boxes did not differ significantly
from colony counts in bottles (p>0.05). In bag-in-boxes, the strains grew from a mean initial colony
count of 2.3 CFU/ml (SD=0.1 log CFU/ml) on day 0 to a mean final colony count of 4.0 log CFU/ml
(SD=0.6 log CFU/ml) on day 14. Fitting the Baranyi and Roberts model to the mean colony counts
in bottles and bag-in-boxes produced growth curves with standard errors (SE) of fit equal to 0.01 in
bottles and 0.09 in bag-in-boxes. The maximum growth rates of the fitted growth curves were 0.4
log CFU/ml/day for both package types and the lag time for growth was approximately three days
for both package types.

When the three *L. monocytogenes* strains were inoculated individually into separate raw milk
bottles and bag-in-boxes to a targeted inoculum level of 1.3 log CFU/ml (20 CFU/ml) in milk, no
statistically significant differences in colony counts were observed between the three strains on
storage days 0–7 (p>0.05). On day 14, ATCC 19115 reached higher colony counts (mean 3.8 log
CFU/ml, SD=0.4 log CFU/ml) than S1 (mean 3.6 log CFU/ml, SD=0.6 log CFU/ml) and the
difference was significant with an independent-samples Kruskal-Wallis post hoc test (p=0.02,
df=2). The three *L. monocytogenes* strains grew in bottles from a mean initial colony count of 1.3
log CFU/ml (SD=0.1 log CFU/ml) on day 0 to a mean final colony count of 3.7 log CFU/ml, (SD=0.7
log CFU/ml) on day 14 (Fig. 4). In bag-in-boxes, the strains grew from a mean initial count of 1.4
log CFU/ml (SD=0.1 log CFU/ml) on day 0 to a mean final colony count of 3.7 log CFU/ml (SD=0.5
log CFU/ml) on day 14. On day 5, colony counts were significantly higher (p=0.02) in bag-in-boxes
(mean 2.9 log CFU/ml, SD=0.3 log CFU/ml) than in bottles (mean 2.4 log CFU/ml, SD=0.1 log
CFU/ml). Although not statistically significant (p>0.05), colony counts on day 7 were also notably
higher and more varied in bag-in-boxes (mean 3.5 log CFU/ml, SD=0.4 log CFU/ml) than in bottles
(mean 3.2 log CFU/ml, SD=0.2 log CFU/ml). The lag time was approximately three days in both
package types. Fitting the Baranyi and Roberts model to the experimental growth data produced
growth curves with SE of fit equal to 0.06 for bottles and 0.10 for bag-in-boxes. The maximum
growth rates of the fitted growth curves were 0.7 log CFU/ml/day for bag-in-boxes and 0.5 log
CFU/ml/day for bottles. The fitted growth curves exceeded the 100 CFU/g EU food safety criterion
for ready-to-eat foods within four days in bag-in-boxes and within four days and a half in bottles.
When the three *L. monocytogenes* strains were inoculated individually into separate raw milk bottles and bag-in-boxes to a targeted inoculum level of 0.3 log CFU/ml (2 CFU/ml) in milk, no statistically significant differences in colony counts were observed between the three strains on storage days 0–14 (p>0.05). Measured *L. monocytogenes* counts in milk on day 0 were slightly below the targeted inoculum level in both package types. In bottles, the strains grew from a mean initial colony count of 0.0 log CFU/ml (SD=0.3 log CFU/ml) on day 0 to a mean final colony count of 2.0 log CFU/ml (SD=0.7 log CFU/ml) on day 14 (Fig. 4). In bag-in-boxes, the strains grew from a mean initial count of 0.1 log CFU/ml (SD=0.3 log CFU/ml) on day 0 to a mean final colony count of 2.1 log CFU/ml (SD=0.5 log CFU/ml) on day 14. On day 5, the mean colony counts were notably higher in bag-in-boxes (mean 1.0 log CFU/ml, SD=0.5 log CFU/ml) than in bottles (mean 0.6 log CFU/ml, SD=0.4 log CFU/ml), although differences in colony counts between the two package types were not statistically significant on any sampling date. Fitting the Baranyi and Roberts model to the experimental growth data produced growth curves with SE of fit equal to 0.11 in bottles and 0.10 in bag-in-boxes. The maximum growth rates of the fitted growth curves were 0.4 log CFU/ml/day for the bag-in-boxes and 0.6 log CFU/ml/day for the bottles. Despite the greater maximum growth rate of listeria in bottles, the colony counts in bottles were lower on days 3–5 due to a longer lag time (over four days) in contrast to those found for the bag-in-boxes (three days). The fitted growth curve of *L. monocytogenes* in bag-in-boxes exceeded the 100 CFU/g EU food safety criterion within nine days. Although the fitted growth curve of *L. monocytogenes* in bottles did not exceed 100 CFU/g criterion within the 14-day sampling period, four of the nine experimental replicates of bottles had final *L. monocytogenes* counts above 100 CFU/ml. Moreover, two experimental replicates of bottles and one of bag-in-box exceeded 100 CFU/ml by day 7.

Milk in all packages at the beginning of the experiment had a pH typical of normal fresh milk (pH 6.6–6.8). Milk that was inoculated with *L. monocytogenes* to a targeted inoculum level of 200 CFU/ml became sour (pH<6.6) by storage day 5. In contrast, milk that was inoculated with *L.
monocytogenes to targeted inoculum levels of 20 and 2 CFU/ml maintained normal pH (6.6–6.8) for storage days 0-7. However, the milk in all inoculated packages was sour by storage day 14. All inoculated raw milk packages considered, there was a weak but significant negative correlation between final L. monocytogenes counts and milk pH on day 14 (r = -0.32, p=0.02). Moreover, milk pH on storage day 14 was significantly lower in bottles than in bag-in-boxes inoculated to targeted inoculum levels of 200 CFU/ml (p=0.01) and 20 CFU/ml (p=0.02). The pH difference between bottles and bag-in-boxes was independent of final L. monocytogenes counts, which did not significantly differ between package types (p>0.05). Milk in the uninoculated packages maintained a normal pH (6.6–6.8) for the first 7 days of storage, but turned sour (pH<6.6) by storage day 14.

Total aerobic bacterial counts of the milk in the uninoculated packages were 0.1–0.5 log CFU/ml higher in bottles than in bag-in-boxes throughout the experiment. In bottles, total aerobic bacterial counts grew from a mean count of 3.4 log CFU/ml (SD=0.1 log CFU/ml) on day 0 to a mean final count of 8.6 log CFU/ml (SD=0.3 log CFU/ml) on day 14. In bag-in-boxes, total aerobic bacterial counts grew from a mean count of 3.3 log CFU/ml on day 0 (SD=0.1 log CFU/ml) to a mean final count of 8.2 log CFU/ml (SD=0.1 log CFU/ml) on day 14.

3.3 L. monocytogenes growth in raw and pasteurised milk at inordinate consumer storage temperatures

To appreciate the risk posed by low-level L. monocytogenes contamination in raw milk stored at inordinate consumer storage temperatures, growth studies utilising a cocktail of three L. monocytogenes strains as inocula were performed in raw milk stored at 6, 8, and 10 °C. To compare the growth of L. monocytogenes in raw milk to growth in pasteurised milk, the cocktail containing three L. monocytogenes strains was also inoculated into pasteurised milk bottles to a targeted inoculum level of 10 CFU/ml, and the bottles were stored for 14 days in 6 °C and 10 °C.

When the targeted inoculum level was 1 log CFU/ml (10 CFU/ml), L. monocytogenes grew from initial colony counts of 0.9-1.2 log CFU/ml to a mean final colony count of 4.5 log CFU/ml (SD=0.8
log CFU/ml) at 6 °C, 4.2 log CFU/ml (SD=0.4 log CFU/ml) at 8 °C, and 4.3 log CFU/ml (SD=0.4 log CFU/ml) at 10 °C (Fig. 5). The growth of *L. monocytogenes* was expectedly faster in raw milk stored at 8 or 10 °C, than at 6 °C. Fitting the Baranyi and Roberts model to the experimental growth data produced growth curves with SE of fit equal to 1.22 for growth at 6 °C, 0.40 at 8 °C, and 0.31 at 10 °C. The maximum growth rates of the fitted growth curves were 0.3 log CFU/ml/day at 6 °C, 0.4 log CFU/ml/day at 8 °C, and 0.6 log CFU/ml/day at 10 °C. The EU food safety criterion 100 CFU/g was exceeded by all experimental replicates in <5 days at 8 and 10 °C and in <7 days at 6 °C.

When the targeted inoculum level was 0 log CFU/ml (1 CFU/ml), *L. monocytogenes* grew from initial colony counts of ≤0.2 log CFU/ml to a mean final colony count of 3.0 log CFU/ml (SD=0.2 log CFU/ml) at 6 °C, 3.1 log CFU/ml (SD=0.3 log CFU/ml) at 8 °C, and 4.2 log CFU/ml (SD=0.7 log CFU/ml) at 10 °C (Fig. 5). Fitting the Baranyi and Roberts model to the experimental growth data produced growth curves with SE of fit equal to 0.53 for growth at 6 °C, 0.20 at 8 °C, and 0.24 at 10 °C. The maximum growth rates of the fitted growth curves were 0.3 log CFU/ml/day at 6 °C, 0.4 log CFU/ml/day at 8 °C, and 0.5 log CFU/ml/day at 10 °C. The EU food safety criterion 100 CFU/g was exceeded by all experimental replicates in <5 days at 10 °C, in <7 days at 8 °C and in <14 days at 6 °C. Furthermore, one experimental replicate at 6°C exceeded 100 CFU/g in <7 days.

The growth of *L. monocytogenes* in pasteurised milk at 6 °C was consistently faster in pasteurised whole milk than in raw milk (Fig. 6). *L. monocytogenes* counts in pasteurised milk were on average 1.1 log CFU/ml higher than in raw milk after five days of storage, and 2.7 log CFU/ml higher after 14 days of storage. The difference in *L. monocytogenes* growth between raw and pasteurised milk was even more pronounced at 10 °C, at which counts in pasteurised milk were on average 2.7 log CFU/ml higher than in raw milk after five days, and 4.3 log CFU/ml higher than in raw milk after 14 days of storage.

4. Discussion
The frequent isolation of *L. monocytogenes* from in-line milk filter socks demonstrates that *L. monocytogenes* was prevalent at the on-farm dairy investigated. *L. monocytogenes* was remarkably more prevalent in milk filter socks (39%) than in sample sets composed of five aliquots of bulk tank milk (4%). *L. monocytogenes* contamination is difficult to detect in bulk tank milk samples, because counts in bulk tank milk are typically very low, <3 CFU/ml (Meyer-Broseta et al., 2003). Sampling in-line milk filter socks instead of bulk tank milk improves the sensitivity of *L. monocytogenes* detection (Borucki et al., 2005; Latorre et al., 2009; Van Kessel et al., 2011). As *L. monocytogenes* was not detected in the premises used for raw milk packaging, contaminated bulk tank milk was the probable source of *L. monocytogenes* contamination in raw milk bottles.

However, *Listeria* spp. other than *L. monocytogenes* were detected in the packaging premises on the inner surface of a milk filler head in July 2014, representing a potential contamination risk. Previous findings of *L. monocytogenes* contamination in milk fillers (Kells & Gilmour, 2004; Pritchard et al., 1995) support the notion that dairy operators should be vigilant at maintaining or enhancing the hygienic design and sanitation of the filling units.

All *L. monocytogenes* counts in naturally contaminated bottled milk were below the 100 CFU/g EU food safety criterion set for ready-to-eat foods at the end of their shelf life. The occurrence of *L. monocytogenes* in bottled raw milk sampled on the use-by-date of the package was nearly three-fold the occurrence in fresh bulk tank milk samples. *L. monocytogenes* contamination levels initially below the detection limit in the bulk tank may subsequently grow to detectable levels during the seven-day shelf-life of the raw milk package, resulting in a higher occurrence in bottled milk than in bulk tank milk samples. Additionally, higher direct plate counts of *L. monocytogenes* were detected in bottled raw milk (≤13 CFU/ml) than in bulk tank milk (≤1 CFU/ml). These findings appear to support the hypothesis that low initial levels of naturally occurring *L. monocytogenes* contamination in milk result in growth during the distribution and storage of packaged raw milk. Alternatively, the apparently elevated *L. monocytogenes* counts in packaged raw milk may have resulted from the separation of clumped cells during storage (Hunt et al., 2017).
Subtyping of *L. monocytogenes* isolates collected from bottled raw milk, bulk tank milk and milk filter socks revealed seven different PFGE pulsotypes. Two of the pulsotypes reoccurred in milk filter socks in a continuous (pulsotype I) or intermittent (pulsotype IV) pattern. The remaining five pulsotypes occurred sporadically in single milk or filter sock samples. These findings are consistent with those of earlier studies on *L. monocytogenes* epidemiology in dairy farms (Borucki *et al.*, 2005; Haley *et al.*, 2015; Ho *et al.*, 2007; Latorre *et al.*, 2009) and in dairy processing plants (Fox *et al.*, 2011; Miettinen *et al.*, 1999; Leong *et al.*, 2014), where persistent *L. monocytogenes* subtypes occurred in conjunction with several sporadically occurring subtypes. It is possible that some *L. monocytogenes* positive samples contained two or more different pulsotypes; however, these were not detected as only one isolate per sample was subtyped.

The Finnish Ministry of Agriculture and Forestry Act 699/2013 legislates that raw milk must be maintained at ≤6 °C and sold from the dairy farm within two days from milking. Furthermore, the Finnish Food Safety Authority Evira recommends that the use-by-date of raw milk is set to no more than two days from the date of sale from the dairy. In the present study, *L. monocytogenes* growth was negligible for three days of storage at 6 °C, suggesting that a three-day shelf-life for raw milk stored at ≤6 °C does not markedly increase the *Listeria* risk. Currently, raw milk packages sold in Finland have use-by-dates 5–7 days from packaging. The Finnish national legislation maintains that dairy operators can determine a longer use-by-date for raw milk than two days from sale, provided that the longer durability the raw milk can be demonstrated using shelf-life studies.

The present study demonstrated that low initial counts of *L. monocytogenes* have growth potential in refrigerated raw milk. Raw milk packages with use-by-dates of ≥5 days from packaging must be classified as Food Category 1.2 of Regulation (EC) No 2073/2005, namely as “Ready-to-eat foods able to support the growth of *L. monocytogenes* other than those intended for infants and special medical purposes” (Beaufort *et al.*, 2014). The producer must ensure that raw materials and the food production environment are absent of *L. monocytogenes*. However, ensuring that bulk tank
milk used for the production of packaged raw milk is free of _L. monocytogenes_ is exceedingly
difficult, since _L. monocytogenes_ is ubiquitous on dairy farms (Fox _et al._, 2009; Nightingale _et al._,
2004) and low-level contamination of bulk tank milk occurs frequently (Ruusunen _et al._ 2013). The
Finnish Act 699/2013 stipulates that those producers in Finland that sell more than 2500 kg of raw
milk annually must test bulk tank milk for the presence of _L. monocytogenes_ using a minimum
sampling scheme of 5 bulk tank milk samples per year. If the raw milk is packaged in a dairy
establishment, the samples (n=5) must be taken from the end-product leaving the dairy
establishment. The Finnish Food Safety Authority recommends additional sampling (n=5) with
increasing frequency when >5000 kg of raw milk is sold annually. In the present study, 1/23 (4%)
of the bulk tank milk sample sets (n=5) tested positive for _L. monocytogenes_, which exemplifies the
difficulty of detecting _L. monocytogenes_ contamination with microbial testing of raw materials.

Dairy operators are obliged to adjust the shelf-life of raw milk so that the 100 CFU/g food safety
criterion is not exceeded during the product shelf-life. In the present study, _L. monocytogenes_
counts in 3/18 raw milk packages inoculated to the targeted inoculum level 2 CFU/ml exceeded
100 CFU/ml within 7 days of storage at 6 °C. Therefore, 7 days from packaging is not a suitable
use-by-date for raw milk packaged in bottles or bag-in-boxes, as contamination levels <3 CFU/ml
in bulk tank milk are likely to occur even on farms with good hygienic practices (Meyer-Broseta _et al._,
2003). The growth of _L. monocytogenes_ in raw milk inoculated to target levels 2 and 20 CFU/ml
was slightly faster in milk packaged in bag-in-boxes than in bottles. While the differences in _L._
monocytogenes_ growth between package types were small, the large size of the bag-in-box (3
litres) might prompt consumers to store and consume the product over a longer period, potentially
increasing the listeriosis risk associated with raw milk packaged in bag-in-boxes.

Besides shortening the shelf-life, dairy operators can attempt to reduce _L. monocytogenes_ risk by
stipulating a lower storage temperature for raw milk for consumers, but this strategy requires
consumer education and compliance. Additionally, Act 699/2013 legislates that raw milk
consumers must be provided with written instructions about storage temperature and the use-by-
date of raw milk. Consumers must also be provided with a written warning notifying that the product may contain pathogenic microbes and that high-risk groups should not consume the product without prior heat treatment. Finally, the warning must specify that “high-risk groups include children, elderly and pregnant individuals, and individuals with severe underlying health conditions.

It is important to note that the methodology used in the present study did not include a period of cold adaptation before inoculation of the *L. monocytogenes* strains into milk. Pre-adaptation of the strains to the raw milk storage temperature would probably shorten the lag time, which should result in faster initiation of the exponential phase and maximum growth (Beaufort *et al.*, 2014; Walker *et al.*, 1990). *L. monocytogenes* is able to adapt to cold stress in 3–5 days (Bolton & Frank, 1999; Notermans *et al.*, 1991), which is in agreement with the 3-day lag time observed in the present study. The investigated on-farm dairy stored raw milk in the bulk tank ≤16 hours before packaging. Therefore, it is unlikely that *L. monocytogenes* contamination in the bulk tank milk would have adequate time to adapt to the temperature of chilled milk before packaging.

Nevertheless, dairy operators should account for the time spent between milking and packaging when assigning a use-by-date for raw milk.

Beaufort *et al.* (2014) recommend the use of inoculum levels of 100 CFU/g in *L. monocytogenes* growth studies to minimise the effect of measurement uncertainty. Indeed, *L. monocytogenes* counts on day 0 were more varied in raw milk packages with a targeted inoculum level of 2 CFU/ml (SD=0.3 log CFU/ml) than in packages with targeted inoculum levels of 20 CFU/ml or 200 CFU/ml (SD=0.1 log CFU/ml). However, variance of the colony counts increased throughout the storage period, and by day 14 colony counts were highly variable regardless of the targeted inoculum level (SD>0.5 log CFU/ml). The increase in colony count variability towards the end of the storage period may result from the potentiation of initial differences in cell counts during exponential growth, as well as from inter-batch variability of the packaged raw milk. The physicochemical composition and microbial quality of raw milk is affected by multiple factors, including season, herd
size, and management practices (Elmoslemany et al., 2010). Variability caused by the
aforementioned factors may mask potential strain-specific differences in growth, which were not
significant in the present study. Furthermore, the adaptation of L. monocytogenes to environmental
stressors is prone to phenotypic heterogeneity between individual cells, which leads to a dynamic
stress response (Metselaar et al., 2015). L. monocytogenes grew markedly better in pasteurised
milk than in raw milk, which indicated that results of L. monocytogenes growth studies in heat-
treated milk should not be extrapolated to growth predictions in raw milk.

Total aerobic bacterial counts in the uninoculated packages on day 0 were in the range of 1500-
2500 CFU/ml. This range is slightly smaller than that of the 5000 CFU/ml national geometric mean
for total aerobic bacteria counts that were detected in Finnish bulk tank milk in 2015 (85% of all
Finnish dairy cattle farms represented; Finnish Association for Milk Hygiene, 2016). Furthermore,
the total aerobic bacteria counts of the uninoculated raw milk packages on day 0 were in
compliance with the levels stipulated by the Finnish Ministry of Agriculture and Forestry Act
699/2013, which decrees that total aerobic bacteria counts at 30 °C must not exceed 50 000
CFU/ml in any individual raw milk sample intended for human consumption without pasteurisation
(rolling geometric mean is not used).

Storage temperatures have a significant impact on L. monocytogenes growth in refrigerated milk.
After 5 days of storage, L. monocytogenes counts in raw milk stored at 8 °C were approximately 1
log CFU/ml higher, and at 10 °C approximately 2 log CFU/ml higher, than the counts in milk stored
at 6 °C. It is concerning that over 20% of Finnish raw milk consumers reported to have stored raw
milk at temperatures above 6 °C (Perkiömäki et al., 2012). Moreover, consumer responses may
underestimate actual milk temperatures, as storage temperatures can vary 1–2 °C depending on
location inside the refrigerator and only 24% of consumers store milk in the coldest area of the
refrigerator (Koutsoumanis et al., 2010; Marklinder et al., 2004). Promoting consumer awareness
of refrigerator temperature monitoring and appropriate placement of raw milk inside the refrigerator
(the middle shelves) are important strategies for reducing the L. monocytogenes risk associated
with raw milk consumption. Nevertheless, heat treatment of raw milk prior to consumption remains the most effective risk management strategy.

5. Conclusions

The present study demonstrates that low-level *L. monocytogenes* contamination (≤13 CFU/ml) occurs frequently in bulk tank milk and in bottled raw milk, and that the low-level contamination leads to growth in raw milk stored at typical consumer storage temperatures. These findings highlight the importance of consumer education regarding appropriate raw milk storage and handling. Susceptible individuals, for whom even low-level *L. monocytogenes* contamination can present a health risk, should avoid the consumption of raw milk without prior heating.

6. Acknowledgements

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7. References


FIGURE CAPTIONS

Fig. 1. Schematic diagram of the bottled raw milk distribution chain and sample collection.
At each sampling, one in-line milk filter sock and 5 bulk tank milk samples were obtained from an on-farm dairy. In addition, 3–5 raw milk containing bottles from the study dairy were purchased from a retail store within 24 h from bottling and 40 h from milking. After purchase, raw milk bottles were stored at 6 °C and analysed on the use-by-date of the product (7 days from packaging).

Fig. 2. Occurrence of *L. monocytogenes* and other *Listeria* spp. in bottled raw milk, bulk tank milk samples, in-line milk filter socks, and in the environment of an on-farm dairy.
Each cell represents one sample: black cells represent samples positive for *L. monocytogenes*, grey cells represent samples positive for *Listeria* spp., and the white cells represent samples negative for *Listeria* spp. Roman numerals indicate different *L. monocytogenes* pulsotypes. When *L. monocytogenes* was present in direct plating, the Roman numeral is followed by a colon and the plate count in CFU/ml.

FIG. 3. Cluster analysis of *L. monocytogenes* isolates obtained from raw milk bottles (bottle, n=5), bulk tank milk samples (BTM, n=2), and milk filter socks (filter, n=9). Isolates were digested with the restriction endonucleases *Ascl* and *Apal*. Automated clustering of the combined PFGE profiles was done by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient to analyze the similarities of the banding pulsotypes with a 1.5% tolerance limit and 1% optimization. Pulsotypes (PT) were numbered I-VII in chronological order.

Fig. 4. Growth of *L. monocytogenes* in raw milk packaged in bottles and bag-in-boxes.
Three *L. monocytogenes* strains (ATCC 19115, S1 and S2) were inoculated individually into raw milk bottles and bag-in-boxes to targeted inoculum levels of 200 CFU/ml (A), 20 CFU/ml (B), and 2 CFU/ml (C). Inoculated milk packages were stored at 6 °C and *L. monocytogenes* were enumerated 0, 3, 5, 7 and 14 days from inoculation. The experiment was performed in triplicate for
each strain, package type and targeted inoculum level. Mean colony counts and the standard deviation of the experimental replicates of all three strains are shown for bottles and bag-in-boxes. The dashed line demarks the EU food safety criterion of 100 CFU/g for *L. monocytogenes* in ready-to-eat foods at the end of shelf-life for products placed on the market.

**Fig. 5. Effect of inordinate storage temperature on the growth of *L. monocytogenes* in raw milk.** A cocktail containing equal quantities of *L. monocytogenes* strains ATCC 19115, S1 and S2 was inoculated into raw milk to targeted inoculum levels of 10 CFU/ml (A) and 1 CFU/ml (B). Inoculated milk samples were stored at 6 °C (n=3), 8 °C (n=3) and 10 °C (n=3), and *L. monocytogenes* were enumerated 0, 5, 7 and 14 days from the inoculation. Mean colony counts and the standard deviation of the experimental replicates are represented. The dashed line demonstrates the EU food safety criterion of 100 CFU/g for *L. monocytogenes* for ready-to-eat foods at the end of shelf-life for products placed on the market.

**Fig. 6. Growth of *L. monocytogenes* in raw milk and in milk pasteurised for 15 s at 72 °C.** A cocktail containing equal quantities of *L. monocytogenes* strains ATCC 19115, S1 and S2 was inoculated into raw and pasteurised milk to a targeted inoculum level of 10 CFU/ml. Milk samples were stored at 6 (n=3) and 10 °C (n=3) and *L. monocytogenes* were enumerated 5 and 14 days from the inoculation. Error bars represent the standard deviation of the experimental replicates.
Table 1. *L. monocytogenes* strains used in raw milk growth studies

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Source</th>
<th>Pulsotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serogroup</th>
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<tr>
<td>S1</td>
<td>bottled raw milk</td>
<td>I</td>
<td>1/2a</td>
</tr>
<tr>
<td>S2</td>
<td>dairy cattle farm</td>
<td>VII</td>
<td>1/2a</td>
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<tr>
<td>ATCC 19115</td>
<td>human clinical isolate</td>
<td>VIII</td>
<td>4b</td>
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</table>

<sup>a</sup>Pulsotypes I-VII were named in the order in which they appeared in the *L. monocytogenes* occurrence study of the on-farm dairy (Fig. 2). Pulsotype VIII was not detected in the occurrence study.
Table S1.
Mean growth of L. monocytogenes experimental replicates (N=54) in raw milk packaged in bottles and in bag-in-boxes inoculated to targeted levels of 2, 20 and 200 CFU/ml. Raw milk was stored at 6 °C and sampled 0, 3, 5, 7 and 14 days from the inoculation. Nine experimental replicates were performed for each package type and inoculation level.

<table>
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<th>Inoculum (cfu/ml)</th>
<th>Package type</th>
<th>Storage day</th>
<th>M&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SEM&lt;sup&gt;d&lt;/sup&gt;</th>
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<sup>a</sup>M: arithmetic mean (in log CFU/ml) of the L. monocytogenes colony counts of experimental replicates

<sup>b</sup>SD: standard deviation (in log CFU/ml) of the log transformed L. monocytogenes colony counts

<sup>c</sup>SEM: standard error of the mean (in log CFU/ml) of the log transformed L. monocytogenes colony counts
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Supplementary Figures

Fig. S1. Milk pH in refrigerated raw milk packages inoculated with *L. monocytogenes*. The pH of raw milk packaged in bottles (grey lines) and bag-in-boxes (black lines) inoculated with *L. monocytogenes* to target inoculum levels of 200 CFU/ml (A), 20 CFU/ml (B) and 2 CFU/ml (C). Milk packages were stored at 6 °C and sampled 0, 3, 5, 7 and 14 days from the inoculation.

Fig. S2. Total aerobic bacterial counts and pH in refrigerated raw milk packages.
Total aerobic bacterial counts (solid lines) and milk pH (dashed lines) of uninoculated raw milk packaged in bottles (grey lines) and bag-in-boxes (black lines) after 0, 3, 5, 7 and 14 days of storage at 6 °C.
Fig. S1.

A

Milk pH vs. Days from inoculation

B

Milk pH vs. Days from inoculation

C

Milk pH vs. Days from inoculation
Fig. S2.

![Graph showing milk pH and total bacteria over days of storage.](image)