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

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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 cm^2 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 *Apa*I and *Asc*I 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

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 dataproduced 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

568 milk used for the production of packaged raw milk is free of *L. monocytogenes* is exceedingly
569 difficult, since *L. monocytogenes* is ubiquitous on dairy farms (Fox *et al*., 2009; Nightingale *et al*.,
570 2004) and low-level contamination of bulk tank milk occurs frequently (Ruusunen *et al.* 2013). The
571 Finnish Act 699/2013 stipulates that those producers in Finland that sell more than 2500 kg of raw
572 milk annually must test bulk tank milk for the presence of *L. monocytogenes* using a minimum
573 sampling scheme of 5 bulk tank milk samples per year. If the raw milk is packaged in a dairy
574 establishment, the samples (n=5) must be taken from the end-product leaving the dairy
575 establishment. The Finnish Food Safety Authority recommends additional sampling (n=5) with
576 increasing frequency when >5000 kg of raw milk is sold annually. In the present study, 1/23 (4%)
577 of the bulk tank milk sample sets (n=5) tested positive for *L. monocytogenes*, which exemplifies the
578 difficulty of detecting *L. monocytogenes* contamination with microbial testing of raw materials.
579
580 Dairy operators are obliged to adjust the shelf-life of raw milk so that the 100 CFU/g food safety
581 criterion is not exceeded during the product shelf-life. In the present study, *L. monocytogenes*
582 counts in 3/18 raw milk packages inoculated to the targeted inoculum level 2 CFU/ml exceeded
583 100 CFU/ml within 7 days of storage at 6 °C. Therefore, 7 days from packaging is not a suitable
584 use-by-date for raw milk packaged in bottles or bag-in-boxes, as contamination levels <3 CFU/ml
585 in bulk tank milk are likely to occur even on farms with good hygienic practices (Meyer-Broseta *et*
586 *al*., 2003). The growth of *L. monocytogenes* in raw milk inoculated to target levels 2 and 20 CFU/ml
587 was slightly faster in milk packaged in bag-in-boxes than in bottles. While the differences in *L.*
588 *monocytogenes* growth between package types were small, the large size of the bag-in-box (3
589 litres) might prompt consumers to store and consume the product over a longer period, potentially
590 increasing the listeriosis risk associated with raw milk packaged in bag-in-boxes.
591
592 Besides shortening the shelf-life, dairy operators can attempt to reduce *L. monocytogenes* risk by
593 stipulating a lower storage temperature for raw milk for consumers, but this strategy requires
594 consumer education and compliance. Additionally, Act 699/2013 legislates that raw milk
595 consumers must be provided with written instructions about storage temperature and the use-by-

596 date of raw milk. Consumers must also be provided with a written warning notifying that the
597 product may contain pathogenic microbes and that high-risk groups should not consume the
598 product without prior heat treatment. Finally, the warning must specify that “high-risk groups
599 include children, elderly and pregnant individuals, and individuals with severe underlying health
600 conditions.
601
602 It is important to note that the methodology used in the present study did not include a period of
603 cold adaptation before inoculation of the *L. monocytogenes* strains into milk. Pre-adaptation of the
604 strains to the raw milk storage temperature would probably shorten the lag time, which should
605 result in faster initiation of the exponential phase and maximum growth (Beaufort *et al*., 2014;
606 Walker *et al*., 1990). *L. monocytogenes* is able to adapt to cold stress in 3–5 days (Bolton & Frank,
607 1999; Notermans *et al.*, 1991), which is in agreement with the 3-day lag time observed in the
608 present study. The investigated on-farm dairy stored raw milk in the bulk tank ≤16 hours before
609 packaging. Therefore, it is unlikely that *L. monocytogenes* contamination in the bulk tank milk
610 would have adequate time to adapt to the temperature of chilled milk before packaging.
611 Nevertheless, dairy operators should account for the time spent between milking and packaging
612 when assigning a use-by-date for raw milk.
613
614 Beaufort *et al*. (2014) recommend the use of inoculum levels of 100 CFU/g in *L. monocytogenes*
615 growth studies to minimise the effect of measurement uncertainty. Indeed, *L. monocytogenes*
616 counts on day 0 were more varied in raw milk packages with a targeted inoculum level of 2 CFU/ml
617 (SD=0.3 log CFU/ml) than in packages with targeted inoculum levels of 20 CFU/ml or 200 CFU/ml
618 (SD=0.1 log CFU/ml). However, variance of the colony counts increased throughout the storage
619 period, and by day 14 colony counts were highly variable regardless of the targeted inoculum level
620 (SD>0.5 log CFU/ml). The increase in colony count variability towards the end of the storage
621 period may result from the potentiation of initial differences in cell counts during exponential
622 growth, as well as from inter-batch variability of the packaged raw milk. The physicochemical
623 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

652 with raw milk consumption. Nevertheless, heat treatment of raw milk prior to consumption remains
653 the most effective risk management strategy.
654
655 **5. Conclusions**
656
657 The present study demonstrates that low-level *L. monocytogenes* contamination (≤13 CFU/ml)
658 occurs frequently in bulk tank milk and in bottled raw milk, and that the low-level contamination
659 leads to growth in raw milk stored at typical consumer storage temperatures. These findings
660 highlight the importance of consumer education regarding appropriate raw milk storage and
661 handling. Susceptible individuals, for whom even low-level *L. monocytogenes* contamination can
662 present a health risk, should avoid the consumption of raw milk without prior heating.
663
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665
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671
672 **7. References**
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838 **FIGURE CAPTIONS**

839

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

845

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

853

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

860

861 **Fig. 4. Growth of *L. monocytogenes* in raw milk packaged in bottles and bag-in-boxes.**
862 Three *L. monocytogenes* strains (ATCC 19115, S1 and S2) were inoculated individually into raw
863 milk bottles and bag-in-boxes to targeted inoculum levels of 200 CFU/ml (A), 20 CFU/ml (B), and 2
864 CFU/ml (C). Inoculated milk packages were stored at 6 ^{0}C and *L. monocytogenes* were
865 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

Strain name	Source	Pulsotype[a]	Serogroup
S1	bottled raw milk	I	1/2a
S2	dairy cattle farm	VII	1/2a
ATCC 19115	human clinical isolate	VIII	4b

[a]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.

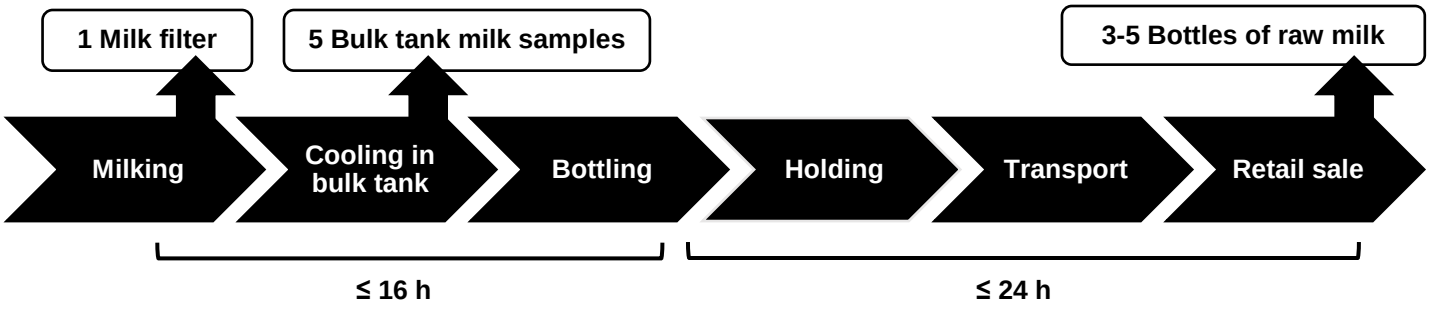
Mean growth of L. monocytogenes experimental replicates (N=54) in raw milk packaged in bottles and in bag-in-boxes inoculated to targeted levels of 2, 20 and 200 CFU/ml. Raw milk was stored at 6 °C and sampled 0, 3, 5, 7 and 14 days from the inoculation. Nine experimental replicates were performed for each package type and inoculation level.

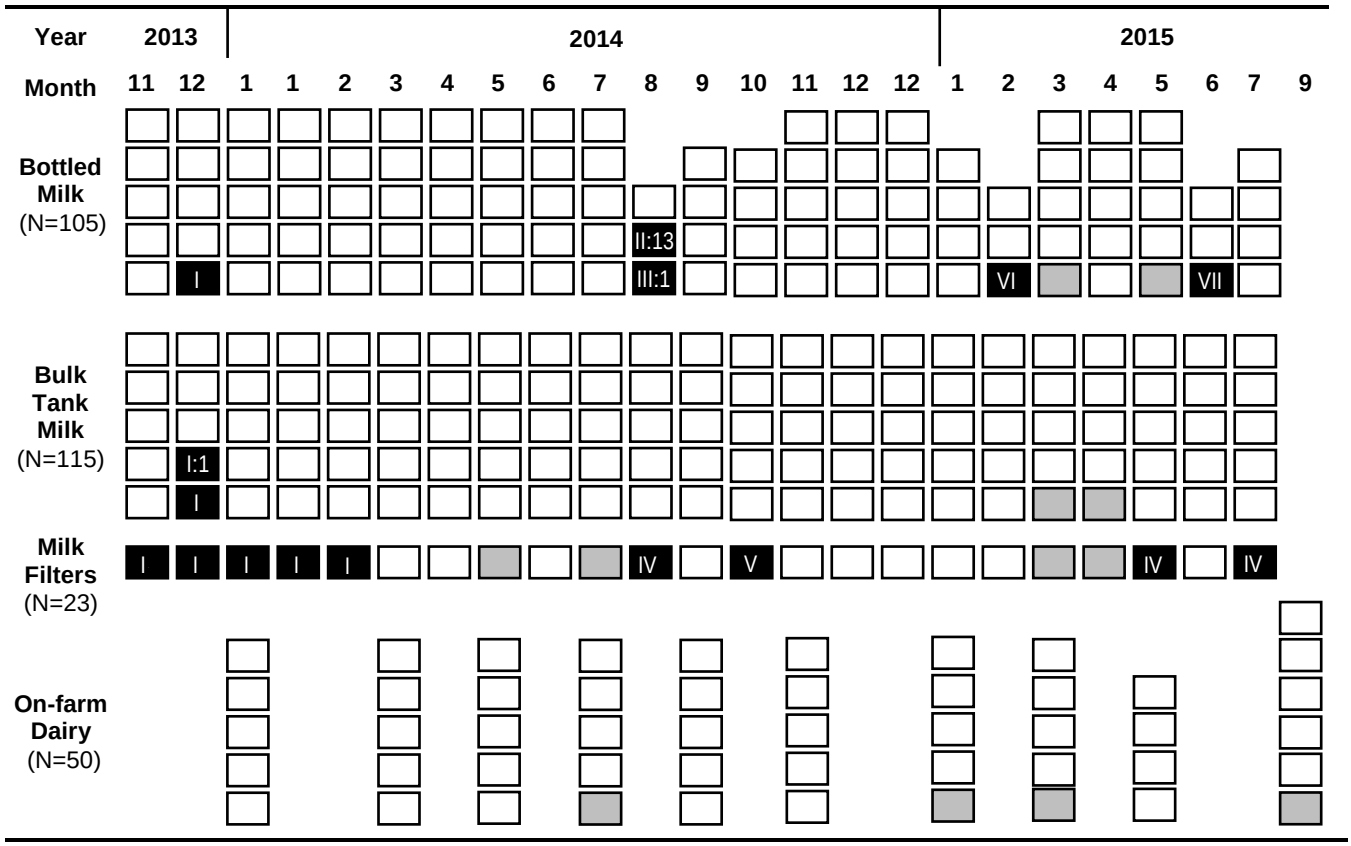
Inoculum (cfu/ml)	Package type	Storage day	M[b]	SD[c]	SEM[d]
1	Bottle	0	0.0	0.301	0.100
		3	0.2	0.337	0.112
		5	0.6	0.440	0.147
		7	1.7	0.837	0.279
		14	2.0	0.688	0.229
1	Bag-in-box	0	0.1	0.312	0.106
		3	0.2	0.186	0.062
		5	1.0	0.493	0.164
		7	1.7	0.797	0.266
		14	2.1	0.513	0.171
20	Bottle	0	1.3	0.136	0.045
		3	1.5	0.143	0.048
		5	2.4	0.347	0.116
		7	3.2	0.507	0.169
		14	3.7	0.662	0.221
20	Bag-in-box	0	1.4	0.095	0.032
		3	1.6	0.125	0.042
		5	2.9	0.323	0.108
		7	3.5	0.408	0.136
		14	3.7	0.537	0.179
200	Bottle	0	2.3	0.068	0.023
		3	2.4	0.075	0.025
		5	3.1	0.231	0.077
		7	3.8	0.419	0.140
		14	4.0	0.548	0.183
200	Bag-in-box	0	2.3	0.066	0.022
		3	2.5	0.156	0.052
		5	3.2	0.338	0.113
		7	3.7	0.335	0.112
		14	4.0	0.601	0.200

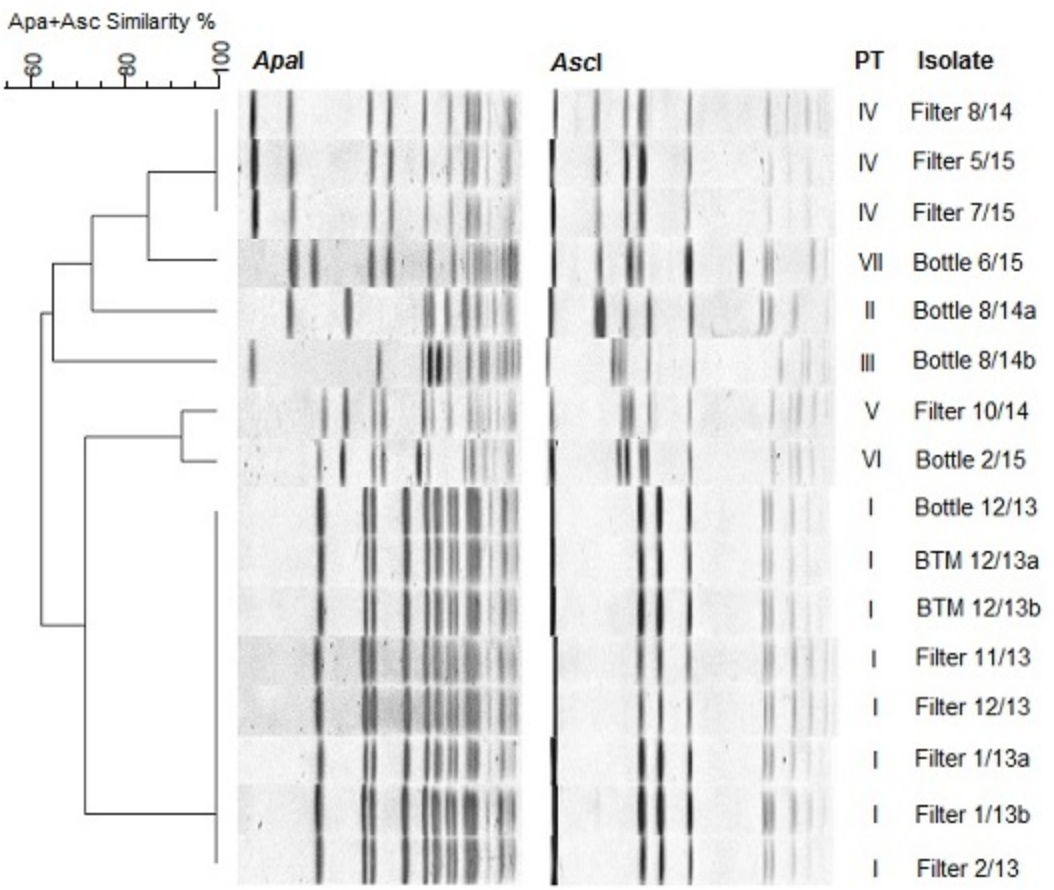
[a]M: arithmetic mean (in log CFU/ml) of the *L. monocytogenes* colony counts of experimental replicates

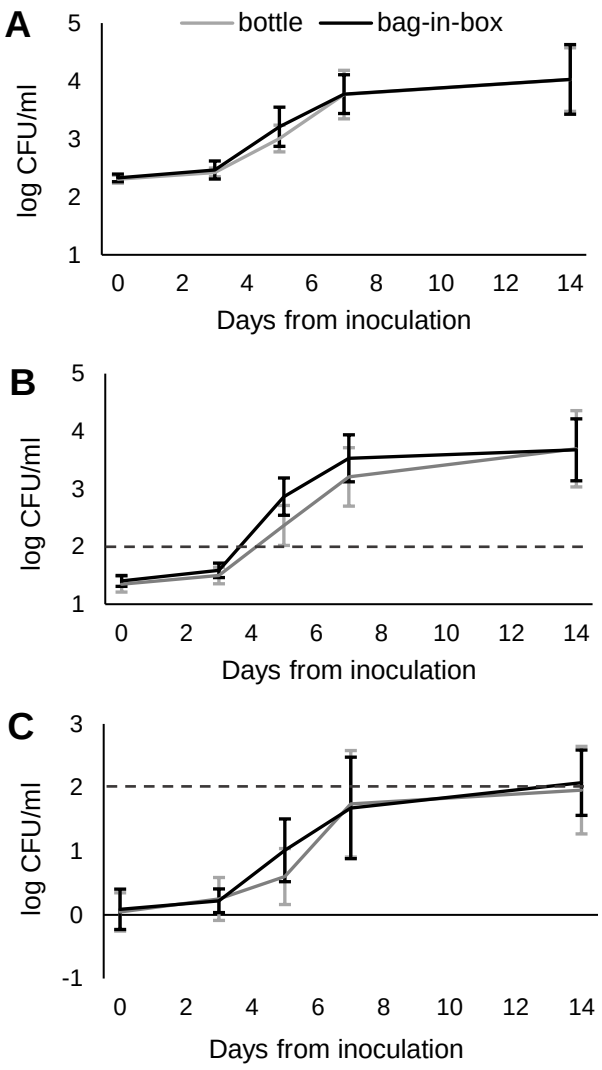
[b]SD: standard deviation (in log CFU/ml) of the log transformed *L. monocytogenes* colony counts

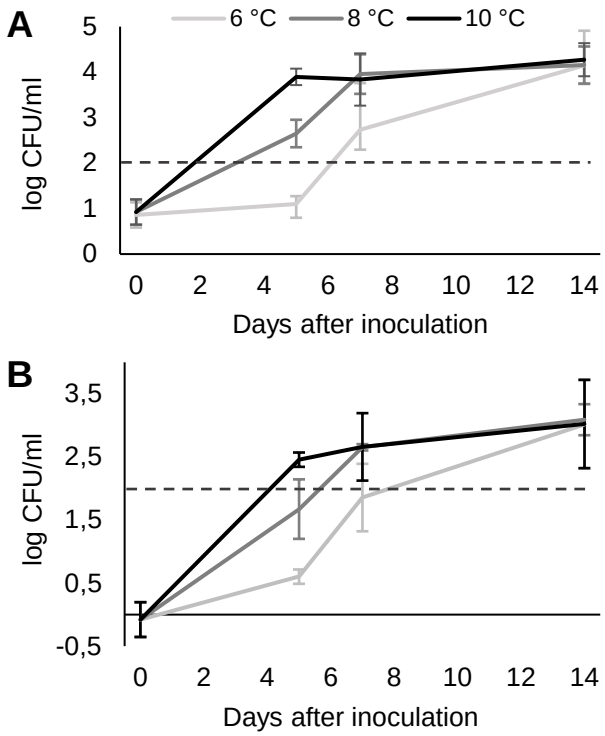
[c]SEM: standard error of the mean (in log CFU/ml) of the log transformed *L. monocytogenes* colony counts


1 Milk filter
5 Bulk tank milk samples
3-5 Bottles of raw milk
Milking
Cooling in bulk tank
Bottling
Holding
Transport
Retail sale
≤ 16 h
≤ 24 h

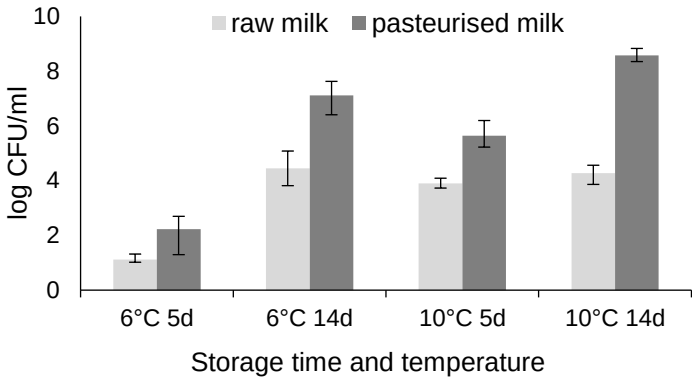

Year
2013
2014
2015
Month
11 12 1 1 2 3 4 5 6 7 8 9 10 11 12 12 1 2 3 4 5 6 7 9
Bottled Milk (N=105)
II:13
III:1
I
VI
VII
Bulk Tank Milk (N=115)
I:1
I
Milk Filters (N=23)
I I I I I IV V IV IV
On-farm Dairy (N=50)


Apa+Asc Similarity %
60
80
100
ApaI
AscI
PT
Isolate
IV Filter 8/14
IV Filter 5/15
IV Filter 7/15
VII Bottle 6/15
II Bottle 8/14a
III Bottle 8/14b
V Filter 10/14
VI Bottle 2/15
I Bottle 12/13
I BTM 12/13a
I BTM 12/13b
I Filter 11/13
I Filter 12/13
I Filter 1/13a
I Filter 1/13b
I Filter 2/13


A
bottle
bag-in-box
log CFU/ml
5
4
3
2
1
0
2
4
6
8
10
12
14
Days from inoculation
B
log CFU/ml
5
4
3
2
1
0
2
4
6
8
10
12
14
Days from inoculation
C
log CFU/ml
3
2
1
0
-1
0
2
4
6
8
10
12
14
Days from inoculation


A
6 °C
8 °C
10 °C
log CFU/ml
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Days after inoculation

B
log CFU/ml
3,5
2,5
1,5
0,5
-0,5
0
2
4
6
8
10
12
14
Days after inoculation


raw milk
pasteurised milk
log CFU/ml
10
8
6
4
2
0
6°C 5d
6°C 14d
10°C 5d
10°C 14d
Storage time and temperature

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 ^{0}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.

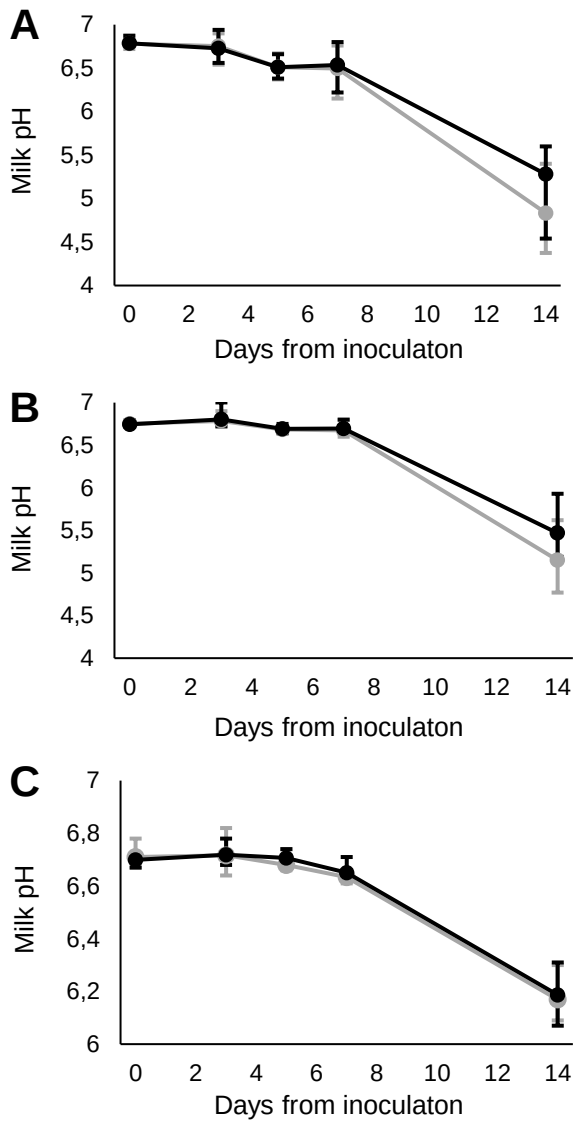
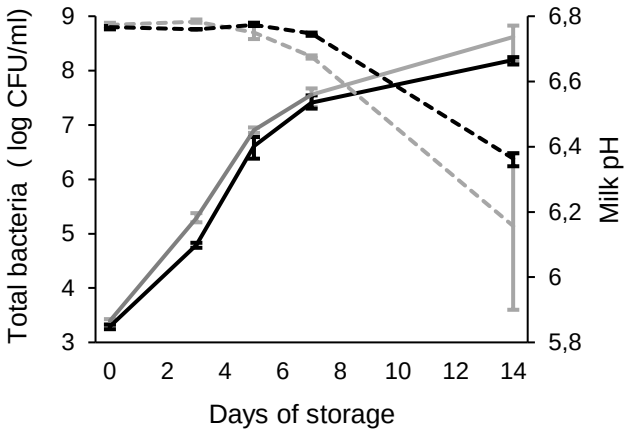

A
Milk pH
7
6,5
6
5,5
5
4,5
4
0 2 4 6 8 10 12 14
Days from inoculaton
B
Milk pH
7
6,5
6
5,5
5
4,5
4
0 2 4 6 8 10 12 14
Days from inoculaton
C
Milk pH
7
6,8
6,6
6,4
6,2
6
0 2 4 6 8 10 12 14
Days from inoculaton

Fig. S2.


Total bacteria (log CFU/ml)
9
8
7
6
5
4
3
0
2
4
6
8
10
12
14
Days of storage
6,8
6,6
6,4
6,2
6
5,8
Milk pH