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

CONTROL OF *LISTERIA MONOCYTOGENES* IN THE FOOD INDUSTRY

Riina Tolvanen

ACADEMIC DISSERTATION

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Supervising Professor
Professor Hannu Korkeala, DVM, Ph.D., M.Soc.Sc.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Supervisors
Professor Hannu Korkeala, DVM, Ph.D., M.Soc.Sc.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Docent Janne Lundén, DVM, Ph.D.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Reviewed by
Laura Raaska, Ph.D.
Docent, University of Helsinki
Director, Biosciences and Environment Research Unit
Academy of Finland
Helsinki, Finland

Associate professor Marcello Trevisani
Department of Veterinary Medical Sciences
Faculty of Veterinary Medicine
University of Bologna
Bologna, Italy

Opponent
Riitta Maijala, DVM, PhD
Docent, University of Helsinki
Vice President for Research
Academy of Finland
Helsinki, Finland

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ABSTRACT

Contamination routes of *Listeria monocytogenes* were examined during an 8-year period in a chilled food-processing establishment that produced ready-to-eat meals using amplified fragment length polymorphism (AFLP) analysis. The three compartments (I to III) of the establishment exhibited significantly different contamination statuses. Compartment I, producing cooked meals, was heavily contaminated with three persistent AFLP types, and compartment II, producing uncooked chilled foods, was contaminated with persistent and non-persistent AFLP types. Only one environmental sample was positive for *L. monocytogenes* in compartment III, producing heat-treated ready-to-eat products. The persistent contamination appears to be influenced by the cleaning routines, product types and lack of compartmentalisation in facilities producing cooked meals. The reconstruction of the production line in compartment II resulted in the elimination of two persistent AFLP types.

The survival of five *L. monocytogenes* strains was studied in dry-fermented sausages prepared using two different starter cultures with or without a bacteriocin-producing *Lactobacillus plantarum* DDEN 2205 strain. *L. monocytogenes* was detected throughout the ripening process in sausages containing no bacteriocin-producing strain. The use of one starter with a high concentration or another starter with a low concentration of bacteriocin-producing culture resulted in *L. monocytogenes*-negative sausages after 17 days of ripening. Differences in survival were found among the *L. monocytogenes* strains. Two of the strains survived in sausages with bacteriocin-producing cultures better than the other strains, whereas two other strains were inhibited by all the cultures used. Bacteriocin-producing strains provide an appealing hurdle in dry sausage processing, but differences in survival of *L. monocytogenes* strains require the use of other hurdles as well.

The acid and heat tolerance of 17 persistent and 23 non-persistent *L. monocytogenes* strains were studied. *L. monocytogenes* strains exhibited large variation in both acid and heat tolerance. The persistent strains exhibited higher tolerance to acidic conditions than the non-persistent strains, but significant differences in heat tolerance between persistent and non-persistent strains were not detected. Acid tolerance may have an effect on the persistence of *L. monocytogenes* contamination. Due to the great differences in acid and heat tolerances between *L. monocytogenes* strains, preventive measures should be designed to be effective against the most tolerant strains.

Ultrasonic cleaning was tested on three conveyor belt materials: polypropylene, acetal and stainless steel at two temperatures and two cleaning times with two cleaning detergents. Conveyor belt materials were soiled with milk-based soil and three *L. monocytogenes* strains. The ultrasonic cleaning was efficient for cleaning conveyor belt materials, but the reduction of *L. monocytogenes* was significantly greater in stainless steel than in plastic materials. Cleaning treatments with potassium hydroxide detergent reduced *L. monocytogenes* more than combined

potassium and sodium hydroxide detergent. The ultrasonic cleaning of a conveyor belt was further studied by building a pilot-scale conveyor with an ultrasonic cleaning bath. A piece of the stainless steel conveyor belt was contaminated with meat-based soil and three *L. monocytogenes* strains. The detachment of *L. monocytogenes* from the conveyor belt caused by the ultrasonic treatment was significantly greater than without ultrasound. Ultrasonic cleaning efficiency was tested with different cleaning durations and temperatures. In both studies, lengthening of the treatment time did not significantly increase the detachment of *L. monocytogenes*. However, an increase in temperature improved the effect of the ultrasonic treatment significantly. Ultrasonic cleaning for 10 s at 50 °C reduced *L. monocytogenes* counts by more than 5 log units. These results indicate that the ultrasonic cleaning of conveyor belts is effective even with short treatment times.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Keto-Timonen, R., Tolvanen, R., Lundén, J., and Korkeala, H. 2007. An 8-year surveillance of diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. J. Food Prot. 70: 1866-1873.

II Tolvanen, R., Hellström, S., Elsser, D., Morgenstern, H., Björkroth, J., and Korkeala, H. 2008. Survival of *Listeria monocytogenes* strains in a dry sausage model. J. Food Prot. 71: 1550-1555.

III Lundén, J., Tolvanen, R., and Korkeala, H. Acid and heat tolerance of persistent and nonpersistent *Listeria monocytogenes* food plant strains. 2008. Lett. Appl. Microbiol. 46: 276–280.

IV Tolvanen, R., Lundén, J., Korkeala, H. and Wirtanen, G. Ultrasonic cleaning of conveyor belt materials using *Listeria monocytogenes* as a model organism. 2007. J. Food Prot. 70: 758-761.

V Tolvanen, R., Lundén, J, Hörman, A., and Korkeala, H. Pilot-scale continuous ultrasonic cleaning equipment reduces *Listeria monocytogenes* levels on conveyor belts. 2009. J. Food Prot. 72: 408-411.

ABBREVIATIONS

a_w	water activity
AC	acriflavine, ceftazidime (agar)
AFLP	amplified fragment length polymorphism
ALOA	agar *Listeria* according to Ottaviani and Agosti
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCM	Biosynth chromogenic medium
CAMP	Christie-Atkins-Munch-Petersen-test
CDC	Centers for Disease Control and Prevention
cfu	colony-forming units
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EHA	enhanced haemolytic agar
ESP	EDTA-sodium lauroyl sarcosine proteinase K solution
EURL	European Union Reference Laboratory
FDA	United States Food and Drug Administration
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
HCLA	haemolytic ceftazidime lithium chloride agar
ISO	International Standard Organization
IU	international unit
K	Kelvin
kHz	kilohertz
LCA	*Listeria* chromogenic agar
LEB	*Listeria* enrichment broth
log	logarithm
L-PALCAMY	polymyxin B, acriflavine, lithium chloride, ceftazidime, aesculin, mannitol, yeast extract (broth)
LPM	lithium chloride, phenylethanol, moxolactam (agar)
LMBA	*Listeria monocytogenes* blood agar
MEE	multilocus enzyme electrophoresis
MHz	megahertz
MLST	multilocus sequence typing
MPa	megapascal
MPN	most probable number
NCFA	Nordic Committee on Food Analysis
PALCAM	polymyxin B, acriflavine, lithium chloride, ceftazidime, aesculin, mannitol (agar)
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis

ppm	parts per million
RAPD	randomly amplified polymorphic DNA
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
TE	Tris-HCl, EDTA buffer
USDA	United States Department of Agriculture
UVM	University of Vermont (broth)
W	watt
WGS	whole-genome sequence

1 INTRODUCTION

The importance of *Listeria monocytogenes* as a foodborne pathogen is not based on the amount of people contracting the disease annually. The incidence of listeriosis in Europe is relatively low, 0.33–0.44 cases per 100 000 people, compared to other foodborne diseases such as salmonellosis or campylobacteriosis with incidences of 20.4 and 64.8 cases per 100 000 people, respectively (Anonymous, 2015b; Anonymous, 2015c). The importance of listeriosis lies in the severity of the disease and high mortality (reaching rates up to 20–30%) in immunocompromised people (Swaminathan & Gerner-Schmidt, 2007; Goulet *et al.*, 2012).

L. monocytogenes is ubiquitous in nature, and can be found in soil, water and vegetation (Weis & Seeliger, 1975; Lyautey *et al.*, 2007). The silage and other forage may thus be contaminated with *L. monocytogenes* (Ryser *et al.*, 1997). Animals, especially cattle shed *L. monocytogenes* to their faeces (Nightingale *et al.*, 2004). Fresh vegetables may be contaminated from manure or soil before harvest (Heisick *et al.*, 1989; Strawn *et al.*, 2013).

To control the prevalence of *L. monocytogenes* in processed foods, it is important to trace the contamination routes in food-processing environments. Investigation of *L. monocytogenes* contamination routes in the food-processing industry with DNA-based typing methods, such as pulsed-field gel electrophoresis (PFGE) (Autio *et al.*, 1999; Senczek *et al.*, 2000; Lundén *et al.,* 2003a), has clarified the nature of *L. monocytogenes* contamination. *L. monocytogenes* contamination in processed foods originates mainly from the processing environment, rather than directly from the raw materials (Autio *et al.*, 1999; Alessandria *et al.*, 2010; Chen *et al.*, 2010; Spanu *et al.*, 2015). In addition to transient *L. monocytogenes* strains found in the food-processing environment, *L. monocytogenes* may contaminate the food establishment by persistent *L. monocytogenes* strains, even for years (Miettinen *et al.*, 1999b; Senczek *et al.*, 2000; Lundén *et al.,* 2003a; Lappi *et al.*, 2004). These strains are typically isolated from hard-to-clean equipment, such as conveyors, packaging machines or slicing machines, causing post-processing contamination to processed foods (Autio *et al.*, 1999; Miettinen *et al.*, 1999b; Chasseignaux *et al.*, 2002; Lundén *et al.*, 2002). The persistence of certain *L. monocytogenes* strains has led to studies investigating the properties of these strains, including their attachment to surfaces and their ability to form biofilms, along with the efficiency of disinfectants to destroy *L. monocytogenes* strains (Frank & Koffi, 1990;Lundén *et al.*, 2000; Wang *et al.*, 2015).

In this study the contamination routes of a ready-to-eat food processing establishment were investigated using AFLP analysis, and the effectiveness of different control methods in controlling *L. monocytogenes* in the food processing and food industry were studied.

2 REVIEW OF THE LITERATURE

2.1 *Listeria* spp. and *Listeria monocytogenes*

The genus *Listeria* belongs to the family *Listeriaceace,* order *Bacillales,* class *Bacilli* and phylum *Firmicute*. *Listeria* spp. are facultatively anaerobic, small rods. Capsules and endospores are not produced (Seeliger & Jones 1986). The genus *Listeria* includes the following species: *Listeria monocytogenes* (Pirie, 1940)*, L. innocua* (Seeliger, 1984a), *L. ivanovii* (Seeliger *et al.*, 1984b)*, L. seeligeri, L. welshimeri* (Rocourt & Grimont, 1983), *L. grayi* (Rocourt *et al.*, 1992)*, L. marthii,* (Graves *et al.*, 2010) *L. rocourtiae,* (Leclercq *et al.*, 2010) *L. fleischmannii* (Bertsch *et al.*, 2013) and *L. weihenstephanensis* (Lang Halter *et al.*, 2013).

In addition, subspecies *L. ivanovii* subsp. *ivanovii* and subsp. *londoniensis* have been found within *L. ivanovii* (Boerlin *et al.*, 1992) and subspecies *L. fleischmannii* subsp. *fleischmannii* and subsp. *coloradonensis* within *L. fleischmannii* (den Bakker *et al.*, 2013).

Den Bakker *et al.* (2014) recently isolated several novel species from water, namely *Listeria floridensis* sp. nov., *Listeria aquatica* sp. nov., *Listeria cornellensis* sp. nov., *Listeria riparia* sp. nov. and *Listeria grandensis* sp. nov. Novel species *Listeria booriae* sp. nov. and *Listeria newyorkensis* sp. nov. were also recently isolated from food-processing environments (Weller *et al.*, 2015).

The genus *Listeria* is commonly found in the environment, in soil, water, decaying vegetation and the animal faeces (Weis & Seeliger, 1975; MacGowan, 1994). Only *L. monocytogenes* and *L. ivanovii* are recognised to cause illness to humans and animals. *L. ivanovii* predominantly causes disease in animals, especially sheep and cattle (Sergeant *et al.*, 1991; Alexander *et al.*, 1992; Gill *et al.*, 1997; Chand & Sadana, 1999), but few cases of listeriosis caused by *L. ivanovii* have been reported in humans. These cases of septicaemia have been diagnosed from immunocompromised people (Cummins *et al.*, 1994; Lessing *et al.*, 1994; Snapir *et al.*, 2006; Guillet *et al.*, 2010).

2.1.1 Listeria monocytogenes

Listeria monocytogenes was described by Murray *et al.* in 1926 and named *Bacterium monocytogenes*. The bacterium was isolated from infected laboratory guinea pigs and rabbits, and it caused monocytosis in animals. After an outbreak in wild gerbils in South Africa, Pirie (1927) isolated the bacterium he named *Listerella hepatolytica*. In 1940 the present name, *Listeria monocytogenes*, was established (Pirie, 1940). The earliest plausible description of *L. monocytogenes* was reported by Hülphers in 1911. He isolated the bacterium from the liver of a rabbit, and called it *Bacterium hepatis*. Unfortunately the isolate was not permanently conserved, and thus further comparisons with later studies were not possible (Hülphers, 1911; McLauchlin, 2004).

L. monocytogenes is a gram-positive, small rod, 0.5 μm wide and 1–2 μm long, facultatively anaerobic, β-haemolytic and produces catalase, but not oxidase. *L.* monocytogenes ferments rhamnose, but not xylose (Seeliger & Jones, 1986). Although *L. monocytogenes* is actively motile by means of peritrichous flagella at 20–25°C, it does not synthesise flagella at body temperatures (37 °C) (Peel *et al.*, 1988).

Growth occurs in temperatures ranging from -1.5 °C to 45 °C (Seeliger, 1961; Junttila *et al.*, 1988; Hudson *et al.*, 1994), in pH range 4.3–9.6 (George *et al.*, 1988; Cole *et al.*, 1990) and in a_w as low as 0.90 (Nolan *et al.*, 1992; Lado & Yousef, 2007).

Sporadic clinical cases of listeriosis were increasingly reported in both animals and humans from the 1920s onwards, and listeriosis was later recognised as an important cause of meningitis, septicaemia, abortions and stillbirth in humans. Listeriosis cases were associated e.g. with working with farm animals, but the epidemiology and role of food was not well established (Nyfeldt, 1929; Stenius, 1941; Kaplan, 1945; Gray & Killinger, 1966). In the 1950s drinking raw milk was associated with several perinatal listeriosis cases in Germany and Czechoslovakia (Gray, 1963). However, the importance of *L. monocytogenes* as a cause of foodborne illness was realised in 1981, when an outbreak of listeriosis in Halifax, Nova Scotia, involving 41 cases and 18 deaths, mostly in pregnant women and neonates, was epidemiologically linked to the consumption of coleslaw. The cabbage used to produce the coleslaw had been fertilised with sheep manure contaminated with *L. monocytogenes* (Schlech *et al.*, 1983).

2.1.2 Detection and characterisation

Detection

The isolation of *L. monocytogenes* has developed over the years. Cold enrichment at 4 °C was initially used to enhance the isolation of *L. monocytogenes* from samples that did not grow after direct plating. Cold enrichment could take several months (Gray *et al.*, 1948). The use of selective enrichment broths has shortened the time needed in enrichment, enabling the growth of *Listeria* while inhibiting competing organisms from samples such as food. Selective substances, such as acriflavine, are commonly used to inhibit gram-positive coccoid bacteria and nalidixic acid to inhibit gram-negative bacteria (Curtis & Lee, 1995). *Listeria* can tolerate certain antibiotics such as polymyxin B, used to inhibit gram-negative bacteria, and cefalosporins, which are also used in enrichment. Several broths have been developed, including Fraser broth containing lithium chloride, acriflavine and nalidixic acid (Fraser & Sperber, 1988), University of Vermont (UVM) broth containing acriflavine and nalidixic acid (McClain & Lee, 1988), *Listeria* enrichment broth (LEB) containing acriflavine, nalidixic acid and cycloheximide (Lovett *et al.*, 1987) and L-PALCAMY broth containing polymyxin B, acriflavine, lithium chloride and ceftazidime (van Netten *et al.*, 1989). Indicators such as esculin with Fe^{3+} ions are used to indicate esculin hydrolysis. Esculin hydrolysis forms esculetin, which reacts with Fe^{3+} ions changing broth colour to black. These selective enrichment broths are

in use in the form of two-step enrichment, where the first enrichment is performed in broth including less-selective substances than the second broth.

L. monocytogenes pure cultures grow well in ordinary agar media, such as tryptose or blood agar, incubated in 37 °C for 24 to 48 h. Selective media were developed to ease the growth and identification of *L. monocytogenes*. These media include AC (acriflavine, ceftazidime) media (Bannerman & Bille, 1988) LPM (lithium chloride, phenylethanol, moxolactam) media (Lee & McClain, 1986), Oxford (lithium chloride, acriflavine, colistin, cycloheximide, cefotetan, phosphomycin) media (Curtis *et al.*, 1989), and modified Oxford (lithium chloride, ceftazidime, colistin) media (McClain & Lee, 1989). PALCAM media has the same selective ingredients as L-PALCAMY broth and esculin and phenol red with mannitol as indicators (van Netten *et al.*, 1989).

Fluorogenic and chromogenic agar media have been developed to further facilitate the identification of *L. monocytogenes* from other *Listeria* sp. Differentiation is based on the detection of phosphatidylinositol phospholipase C enzyme activity and fermentation of certain sugars, mostly xylose and rhamnose. These agar media include fluorogenic EHA (enhanced haemolytic agar) (Cox *et al.*, 1991) and chromogenic BCM *L. monocytogenes* plating media (Restaino *et al.*, 1999), Rapid'*L. mono*- media (Foret & Dorey, 1997, Karpíšková *et al.*, 2000) and CHROMagar Listeria (Allerberger, 2003). In addition to phosphatidylinositol phospholipase C, chromogenic agar *Listeria* according to Ottaviani and Agosti (ALOA) media is based on X-glucoside that reacts to the β-glucosidase enzyme (Ottaviani *et al.*, 1997).

To simplify the differentiation of *L. monocytogenes* from the possible overgrowth of other *Listeria* species in foods, especially *L. innocua*, selective media with blood, such as haemolytic ceftazidime lithium chloride agar (HCLA) (Poysky *et al.*, 1993) and *Listeria monocytogenes* blood agar (LMBA) (Johansson, 1998) have been developed to detect β-haemolysis.

Standardised qualitative methods used in accredited food laboratories in Europe (ISO, NCFA) and in the Unites States (FDA, USDA) direct the use of selective media. Oxford medium is used in ISO and FDA methods. Modified Oxford medium is used in FDA and USDA methods. PALCAM medium is used in ISO and FDA methods. ALOA medium is used in the NCFA method and trial use is recommended in the FDA method. Several plating media, LCA, LMBA or chromogenic *Listeria* Agar, can also be used in the NCFA method. The FDA method uses LPM plates with esculin, and recommends the trial use of BCM, Rapid'*L. mono*, and CHROMagar *Listeria* (Anonymous, 1997; Anonymous, 2006; Anonymous, 2010; Hitchins & Jinneman, 2011).

Various tests are performed to identify the isolates, including gram staining, haemolysis on blood agar, motility, the CAMP (Christie-Atkins-Munch-Petersen) test, and tests to establish the production of catalase and oxidase, and the fermentation of sugars (Seeliger & Jones, 1986; Fraser, 1964, McKellar, 1994).

Commercial tests, such as API *Listeria* (Bio-Merieux, France) and MICRO ID Listeria (Organon-Teknika Corp., Durham, N.C.) are widely used for testing the

fermentation of different sugars and enzymatic reactions because of their ease of use and rapidity (Bille *et al.*, 1992; Bannerman *et al.*, 1992).

Characterisation

Serotyping was the first method used to differentiate *L. monocytogenes* strains from each other (Paterson, 1939; Paterson, 1940). Strains were initially divided into four serotypes, but *L. monocytogenes* is currently divided into 13 serotypes, of which 1/2a, 1/2b and 4b comprise the majority of human isolates. Serotypes consist of flagellar (H) and somatic cell wall (O) antigens (Donker-Voet, 1966; Seeliger & Höhne, 1979). Phage typing was developed to distinguish strains of the same serotype. Phage typing had the advantage of being able to process relatively large numbers of cultures; however it is not able to analyse all strains, untypable strains varying between 20–51% in different studies (Sword & Pickett, 1961; McLauchlin *et al.*, 1986; McLauchlin *et al.*, 1996).

The development of molecular biological methods has enabled far more efficient tools for epidemiological studies, and the phylogenetic grouping of strains. A number of genotyping methods have been used in the characterisation of *L. monocytogenes*, such as ribotyping (Grimont & Grimont, 1986), restriction endonuclease analysis (REA) (Nocera *et al.*, 1990; Gerner-Smidt *et al.*, 1996), multilocus enzyme electrophoresis (MEE) (Selander *et al.*, 1986; Piffaretti *et al.*, 1989), restriction fragment length polymorphism (RFLP) (Saunders *et al.*, 1989; Swaminathan *et al.*, 1996), randomly amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990; Wernars *et al.*, 1996), PFGE (Brosch *et al.*, 1991; Autio *et al.*, 1999; Miettinen *et al.*, 1999a), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995; Aarts *et al.*, 1999; Keto-Timonen *et al.*, 2003; Autio *et al.*, 2003), and multilocus sequence typing (MLST) (Maiden *et al.*, 1998; Salcedo *et al.*, 2003; Haase *et al.*, 2014). Whole-genome sequence (WGS)-based typing methods, such as core genomic MLST, have recently been developed for *L. monocytogenes*. The advantage of these methods is that they are highly discriminatory (Schmid *et al.*, 2014).

Piffaretti *et al.* (1989) identified two *L. monocytogenes* phylogenetic lineages using MLST, and other researchers have confirmed the existence of these lineages using other methods such as PFGE (Brosch *et al.*, 1994). Further studies brought forward a third phylogenetic lineage, lineage III, based on analyses of partial DNA sequences for *flaA*, *iap* and *hly* (Rasmussen *et al.*, 1995) and confirmed further by later studies (Wiedmann *et al.*, 1997; Ward *et al.*, 2004). Roberts *et al.* (2006) found that strains in lineage III could be further divided into subgroups III A, III B and III C. Strains in subgroups III B and IIIC do not ferment rhamnose and several strains of lineage III do not have certain genes typical to other strains of *L. monocytogenes*, which may cause difficulties in classifying these strains correctly as *L. monocytogenes* (Roberts *et al.*, 2006). Subsequent studies showed that *L. monocytogenes* isolates can be classified into four genetic lineages, so that lineage III B was classified as lineage IV (Liu *et al.*, 2006; Ward *et al.*, 2008; Orsi *et al.*, 2008; den Bakker *et al.*, 2012). Strains from all lineages have beeen associated with human listeriosis, however strains from lineages I and II are more common in human isolates and lineage II strains also in food and environmental isolates.

Lineages II and III have been associated with animal listeriosis (Lukinmaa *et al.,* 2003; Gray *et al.*, 2004; Orsi *et al.*, 2008; Ward *et al.*,2008; Haase *et al.*, 2014).

Table 1. *Division of serotypes to lineages (Liu et al., 2006; Ward et al.,2004; Ward et al., 2008)*

	Lineages			
	I	II	IIIA/C	IV
Serotypes	1/2b	1/2a	4a	4a
	3b	1/2c	4b	4b
	4b	3a	4c	4c
	4d	3c		7
	4e			

2.1.3 Listeriosis

Listeriosis is a relatively uncommon disease in humans. The severity of the disease in immunocompromised people renders listeriosis a significant foodborne infection. Mortality from listeriosis in immunocompromised people can be as high as 20–30%. People vulnerable to listeriosis include the elderly, infants and people with underlying conditions such as cancer, autoimmune disease, organ transplantation, diabetes or pregnancy (McLauchlin, 1990; Swaminathan & Gerner-Schmidt, 2007; Goulet *et al.*, 2012). Predisposition to listeriosis is related to impaired cell-mediated immunity, especially dysfunction in T-cell-mediated immunity. Macrophages, i.e. natural killer cells, and neutrophils are important in the early stages of infection for restricting the growth of *L. monocytogenes* (Unanue, 1997; Vázquez-Boland *et al.*, 2001). Listeriosis is an untypical foodborne infection, since the infection seldom occurs as typical gastroenteritis. In vulnerable people listeriosis exhibits as meningitis, meningoencephalitis, septicaemia, as abortions or stillbirths in pregnant women, and less frequently as endocarditis, pneumonia, arthritis, pleuritis or local infection. Listeriosis causes flu-like symptoms such as fever, chills, headache and myalgia in pregnant women (Ericsson *et al.*, 1997; Goulet *et al.*, 1998; Vázquez-Boland *et al.*, 2001; Swaminathan & Gerner-Smidt, 2007; Goulet *et al.*, 2012; McCollum *et al.*, 2013).

The infective dose is assumed to be small in vulnerable people, and the regulation of microbiological criteria in the European Union (EU) requires the absence of *L. monocytogenes* in 25 grams of certain ready-to-eat foods that support the growth of *L. monocytogenes*. The absence in 25 g is required at the end of production for ready-to-eat foods able to support growth, if the manufacturer is unable to demonstrate that the product will not exceed the limit of 100 colony-forming units (cfu) per gram throughout the shelf life. The amount of *L. monocytogenes* shall not exceed 100 cfu per gram during the entire shelf life of foods that cannot support *L. monocytogenes* growth (EU, 2005).

In healthy adults listeriosis can cause febrile gastroenteritis with symptoms including fever, diarrhoea, vomiting, arthralgia, headache and body pain. However, in these cases the amount of *L. monocytogenes* in food has been high (10^5–10^9 cfu

per gram) (Dalton *et al.*, 1997; Miettinen *et al.*, 1999a; Frye *et al.*, 2002). Febrile gastroenteritis cases have shorter incubation times than invasive listeriosis, ranging from six to 51 hours, sometimes up to 240 h, with a median incubation time of 18 to 31 h (Dalton *et al.*, 1997; Aureli *et al.*, 2000; Frye *et al.*, 2002; Carrique-Mas *et al.*, 2003; Miettinen *et al.*, 1999a; Pichler *et al.*, 2009; Salamina *et al.*, 1996)

Most human cases of listeriosis are sporadic, and cannot be associated with a certain food. Epidemiological studies are hindered by the long incubation period of invasive listeriosis, which is typically a few weeks, but can be up to 70 days (Linnan *et al.*, 1988; Goulet *et al.*, 2013). The incubation period depends on the clinical type of listeriosis. The incubation periods of bacteraemia, central nervous system and pregnancy-associated cases range from 1 to 12 days, 1 to 14 days and 17 to 67 days, respectively (Goulet *et al.*, 2013). Adequate information on food consumption from patients and tracing of foods for microbiological analyses is therefore demanding. However, development of continuous surveillance and typing of isolates from human listeriosis cases and isolates from food have revealed possible common origins in seemingly sporadic listeriosis cases. Development of the European Union Reference Laboratory (EURL) *Lm* Database enables comparing the strains isolated from food, the environment and animals in Europe (Lukinmaa *et al.*, 2003; Goulet *et al.*, 2006; Lyytikäinen *et al.*, 2006; Swaminathan *et al.*, 2006; CDC, 2013; Félix *et al.*, 2014). The European Centre for Disease Prevention and Control has recently published scientific advice on the introduction of new typing methods for food- and waterborne diseases in the EU in order to facilitate the use of whole genomic sequencing methods in surveillance (Anonymous, 2015a).

Several outbreaks have been reported since the 1980s that have been connected to specific foods. Most outbreaks have been associated with dairy, meat and fish products. Vegetables and fruits, such as cantaloupes have also been less frequently associated with listeriosis outbreaks (Table 2). The ready-to-eat food served in hospitals and ready-made meals delivered to vulnerable people at home have recently been associated with outbreaks. Sandwiches have been connected to eight hospital-acquired outbreaks in the United Kingdom between 1999 and 2011 (Little *et al.*, 2012). A hospital-acquired outbreak in the United States was connected to the celery used in chicken salads (Gaul *et al.*, 2013) and to camembert cheese in Norway (Johnsen *et al.*, 2010). The hospitals in these reported outbreaks did not have specific instructions on serving ready-to-eat foods to high-risk patient groups (Johnsen *et al.*, 2010; Little *et al.*, 2012; Gaul *et al.*, 2013). A survey regarding food safety practices was performed in New York City hospitals, and it was discovered that the majority of hospitals served high-risk ready-to-eat foods to immunocompromised patients prone to listeriosis (Cokes *et al.*, 2011). Smith *et al.* (2011) reported an outbreak caused by precooked beef meal delivered by the Meals on Wheels service in Denmark, which also emphasizes the importance of responsibility of food business operators preparing and serving meals to vulnerable people.

Table 2. *Listeriosis outbreaks associated with different foods.*

Year	Food type	Number of cases (deaths)	Country	Reference
1949–1957	Raw milk	About 100	Germany	Seeliger, 1961
1981	Coleslaw	41 (18)	Canada	Schlech *et al*., 1983
1983	Pasteurised milk	49 (14)	United States	Fleming *et al*., 1985
1983–1987	Soft cheese	122 (33)	Switzerland	Büla *et al*, 1995
1985	Soft cheese	142 (48)	United States	Linnan *et al*., 1988
1986	Raw milk/vegetables	28 (5)	Austria	Allerberger & Guggenbichler, 1989
1987–1989	Pâté	366 (NR)	United Kingdom	McLauchlin *et al*., 1991
1989	Shrimp	2 /10 GE	United States	Riedo *et al*., 1994
1989–1990	Cheese	26 (6)	Denmark	Jensen *et al*.,1994
1992	Pork tongue	279 (85)	France	Goulet *et al*., 1993; Jacquet *et al*., 1995
1993	Rice salad	18 GE	Italy	Salamina *et al*., 1996
1993	Rillettes	38 (11)	France	Goulet *et al*., 1998
1994	Chocolate milk	48 GE	United States	Dalton *et al*., 1997
1994–1995	Gravad rainbow trout	8 (2)	Sweden	Ericsson *et al*., 1997
1995	Soft cheese	37 (11)	France	Goulet *et al*., 1995
1996	Imitation crab meat	2 GE	Canada	Farber *et al*., 2000
1997	Corn	1566 GE	Italy	Aureli *et al*., 2000
1997	Soft cheese	14	France	Jacquet *et al*., 1998
1997	Rainbow trout	5 GE	Finland	Miettinen *et al*., 1999a
1998–1999	Butter	25 (6)	Finland	Lyytikäinen *et al*., 2000; Maijala *et al*., 2001
1998–1999	Hot dog	108 (14)	United States	Graves *et al*., 2005; Mead *et al*., 2006
1999–2000	Rillettes	10 (3)	France	De Valk *et al.,* 2001
1999–2000	Pork tongue	32 (10)	France	De Valk *et al.,* 2001
2000	Ready-to-eat turkey	30 (7)	United States	Olsen *et al*., 2005
2000	Soft cheese	13 (5)	United States	MacDonald *et al*., 2005
2000	Ready-to-eat meat	9 GE	New Zealand	Sim *et al*., 2002
2000	Ready-to-eat meat	21 GE	New Zealand	Sim *et al*., 2002
2001	Soft cheese	48 GE	Sweden	Carrique-Mas *et al*., 2003; Danielsson-Tham *et al*., 2004
2001	Cheese	38 GE	Japan	Makino *et al*., 2005

Table 2. *Continued*

Year	Food type	Number of cases (deaths)	Country	Reference
2001	Ready-to-eat turkey	16 GE	United States	Frye *et al*., 2002
2002	Ready-to-eat turkey	54 (8)	United States	Gottlieb *et al*., 2006
2003	Sandwiches	2 (0)	United Kingdom	Shetty *et al*., 2009
2003	Sandwiches	4 (0)	United Kingdom	Dawson *et al*., 2006
2006–2007	Acid curd cheese	ca. 189/ 26[a]	Germany	Koch *et al*., 2010
2007	Soft cheese	17 (3)	Norway	Johnsen *et al*., 2010
2008	Jellied pork	12 GE	Austria	Pichler *et al*., 2009
2008	Tuna salad	5	United States	Cokes *et al*., 2011
2008–2009	Soft cheese	8 (2 stillbirth)	United States	Jackson *et al*., 2011
2009	Ready-made beef meal	8 (2)	Denmark	Smith *et al*., 2011
2009–2010	Soft cheese	34 (8)	Austria /Germany	Pichler *et al*., 2011
2011	Hard cheese	12 (2)	Belgium	Yde *et al*., 2012
2010	Celery	10 (3)	United States	Gaul *et al*., 2013
2011	Cantaloupe	147 (33)	United States	McCollum *et al*., 2013
2012	Fresh cheese	2 (0)	Spain	de Castro et al., 2012
2013–2014	Ready-to-eat salad	32 (4)	Switzerland	Stephan *et al*., 2015

NR= not reported, GE= gastroenteritis, [a]= all reported listeriosis cases during the outbreak, includes non-outbreak cases.

2.2 Transmission of *Listeria monocytogenes*

2.2.1 Sources

L. monocytogenes is a common bacterium in the environment and the raw materials of food processing. *L. monocytogenes* is found in a variety of natural environments, and the transmission to food-processing environments and subsequently to humans has been the subject of several studies. Soil and water are suggested to represent niches for the transmission of *L. monocytogenes* to plant materials and animals, and soil may serve as a source of animal feed contamination by *L. monocytogenes* (Botzler *et al*., 1974; Nightingale *et al*., 2004). Natural water, especially near urban areas, crop farming, dairy farms and wastewater sources harbour *L. monocytogenes* (Lyautey *et al*., 2007; Linke *et al*., 2014). Ruminants are more likely to be infected by *L. monocytogenes* than other farm animals. Nightingale *et al*. (2004) discovered that the epidemiology and transmission of *L. monocytogenes* differ between small-ruminant and cattle farms. The prevalence of *L. monocytogenes* in cattle farm environments was higher than in sheep and goat farms, and healthy cattle shed more *L. monocytogenes* in their faeces than sheep and goats. Low levels of *L. monocytogenes* in feeds apperar to multiply in cattle, thus maintaining the high contamination levels on cattle farms The variety of *L. monocytogenes* strains was also found to be greater on bovine farms than on

small-ruminant farms (Nightingale *et al.*, 2004). Most listeriosis cases in ruminants are associated with feeding on improperly fermented, poor quality silage, but good quality silage may also be contaminated with *L. monocytogenes* (Gray, 1960; Fenlon *et al.*, 1996; Ryser *et al.*, 1997). Husu *et al.* (1990a) analysed 225 silage samples collected from 80 Finnish dairy farms. *Listeria* spp. were detected in 19% of silages treated with acid additives, in 23% of untreated silages and in 44% of lactic acid bacteria-inoculated silages. The pH of silage was found to be strongly related to the occurrence of *L. monocytogenes* in raw milk (Sanaa *et al.*, 1993). Animals may be asymptomatic carriers as well and shed listeria with their faeces to the farm environment. Contaminated sheep manure used to fertilise cabbages resulted in an outbreak in Canada (Schlech *et al.*, 1983). Farm environments and animal faeces are likely to be a source of listeria that is introduced further into the food chain. Direct contact with contaminated materials, such as manure from infected or shedding animals may attribute to the occurrence of *L. monocytogenes* in food products that are not processed before consumption e.g. raw milk (Husu *et al.*, 1990b; Husu, 1990). The contamination of root vegetables may be due to increased contact with soil (Heisick *et al.*, 1989). The preharvest contamination risk of vegetables is associated with manure application to fields, the appearance of wildlife in fields, recent irrigation of vegetables and soil cultivation (Strawn *et al.*, 2013).

2.2.2 Contamination in the food industry

L. monocytogenes occurs commonly in raw materials such as raw fish (Autio *et al.*, 1999; Hoffman *et al.*, 2003; Markkula *et al.*, 2005), raw milk (Waak *et al.,* 2002; Van Kessel *et al.*, 2004; Ruusunen *et al.*, 2013) and raw meat (Chasseignaux *et al.*, 2001; Vitas *et al.,* 2004; Busani *et al.*, 2005; Pesavento *et al.*, 2010). Several studies suggest that raw food materials are a significant source of initial contamination. The *L. monocytogenes* strains isolated from farms and dairies were compared, and the same strains could be identified from farm environments and dairies (Arimi *et al.*, 1997). The same pulsotypes were detected in a pig slaughterhouse from pluck sets, splitting saw and carcasses, indicating that *L. monocytogenes* from pig tongues and tonsils may contaminate equipment and subsequently the carcasses (Autio *et al.*, 2000). Tracing the sources and routes of *L. monocytogenes* has shown that strains isolated from food products, such as cold-smoked salmon or soft cheese, originate from the food-processing environment, especially from equipment, rather than from raw materials (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Alessandria *et al.*, 2010; Chen *et al.*, 2010). On the other hand, raw fish was indicated as a notable contamination source in food-processing equipment and subsequently processed fish (Markkula *et al.*, 2005). Contamination of the food-processing industry may have several sources. In addition to raw food materials, contamination may also enter the food-processing establishment through equipments, personnel or flies carrying *L. monocytogenes* (El-Shenawy, 1998; Lundén *et al.*, 2002, Pava-Ripoll *et al.*, 2012).

Most strains entering the food establishment are destroyed during food processing such as heat treatment. The main cause of *L. monocytogenes* contamination of processed food products is the post-process contamination of a

finished product originating from the food-processing plant environment and equipment with strains able to survive in the food processing environment (Uyttendaele *et al*., 1999; Lundén *et al*,. 2002; Alessandria *et al*., 2010; Spanu *et al*., 2015). Prevention of product recontamination during post-processing handling, such as slicing and packaging, is especially important in the ready-to-eat food industry (Tompkin *et al*., 1999).

L. monocytogenes has several attributes that enable survival in the food-processing environment. The bacterium survives and grows at low temperatures, and tolerates acidity and low water activity. *L. monocytogenes* is able to attach to surfaces and form biofilms in adverse conditions, such as low temperatures, and at a pH range of 4.0–9.0. However, alkaline conditions significantly reduced the attachment rate (Smoot & Pierson, 1998). Adherence to surfaces was slower but not prevented in low temperatures (Smoot & Pierson, 1998; Norwood & Gilmour 2001). *L. monocytogenes* is able to form biofilms on different materials, such as stainless steel, plastic and rubber used in the food industry (Mafu *et al*., 1990; Blackman & Frank, 1996; Beresford *et al*., 2001). Plastic conveyor belts, polyurethane, acetal and polypropylene exhibited stronger bacterial adhesion compared with stainless steel after 48 hours. A lower number of *L. monocytogenes* attached to the stainless steel conveyor belt than to the plastic materials (Veluz *et al*., 2012). The ability to form biofilms enhances the stress tolerance of bacteria and allows the survival of bacteria, and may contribute to the persistence of strains in food establishments (Frank & Koffi, 1990; Sofos & Geornaras, 2010; Hansen & Vogel, 2011). Attached bacteria and biofilms form a source of *L. monocytogenes* contamination on surfaces. The transfer of *L. monocytogenes* from stainless steel surfaces to foods seems to increase with the dryness of the biofilm (Rodríguez *et al*., 2007).

Persistent *L. monocytogenes* strains have been isolated for several month or years from the same food establishments (Lawrence & Gilmour, 1995; Unnerstad *et al*., 1996; Senczek *et al*., 2000; Lundén *et al.,* 2003a; Peccio *et al*., 2003; Lappi *et al*., 2004). One *L. monocytogenes* strain was repeatedly isolated from an ice cream plant over a period of eight years (Miettinen *et al*., 1999b). The properties of strains persisting in the same food establishment have been compared with transient strains. Persistent strains adhere to surfaces considerably better and more rapidly than transient strains (Lundén *et al*., 2000; Wang *et al*., 2015). Persistent strains were shown to genotypically differ from sporadic strains but the persistent strains did not seem to form any specific evolutional lineage (Autio *et al*., 2003). Verghese *et al*. (2011) hypothesised that the *comK* prophage appearing in persistent outbreak-related strains plays a key role in the attachment, growth and biofilm formation abilities of these *L. monocytogenes* strains. On the other hand, Carpentier and Cerf (2011) suggested that the persistence of certain *L. monocytogenes* strains is not caused by distinctive properties of these strains, but conditions in food establishments such as hard-to-clean places in the environment and equipment where *L. monocytogenes* may persist.

2.2.3 *L. monocytogenes* in foods

Several obstacles are used in the food-processing industry to inhibit the growth and survival of bacteria. These include refrigeration, packing technologies, such as vacuum and modified atmosphere packaging, lowering the water activity in foods, and high salt content and acidity of foods among other things. As a psychrotrophic bacterium *L. monocytogenes* grows in refrigeration temperatures (Junttila *et al.*, 1988; Hudson *et al.*, 1994), vacuum and modified atmosphere packaging (Rørvik *et al.*, 1991; Grau & Vanderlinde, 1992; Devlieghere *et al.*, 2001) and survives in relatively acidic and salty foods (McClure *et al.*, 1989; George *et al.*, 1988; Parish & Higgins, 1989), and in low water activity (Nolan *et al.*, 1992; Lado & Yousef, 2007). These properties enable the growth and survival of *L. monocytogenes* in a variety of foods during storage.

Cross-contamination after heating or other food processing renders *L. monocytogenes* a problem especially in ready-to-eat foods with long shelf lives. In 2010–2011 a European Union-wide baseline survey was carried out to estimate the prevalence of *L. monocytogenes* in certain ready-to-eat foods, such as smoked or gravad fish, heat-treated meat products and soft or semi-soft cheeses. A very low percentage of samples contaminated by *L. monocytogenes* at levels exceeding the EU limit of 100 cfu/g were detected in the survey, with fish being the most contaminated category (EFSA, 2013).

The prevalence of *L. monocytogenes* in raw milk varies between 1–6.5% (Waak *et al.,* 2002; Van Kessel *et al.*, 2004; Ruusunen *et al.*, 2013), and prevalence in soft cheese between 0.4% and 5.5% (Busani *et al.*, 2005; Wagner *et al.*, 2007, Lambertz *et al.*, 2012; EFSA, 2013). Prevalence in semi-hard and hard cheeses is lower than in soft cheese, due to low pH and water activity, ranging from 0–4.4% (Cordano & Rocourt, 2001; Rudolf & Scherer, 2001; Little *et al.,* 2009). Prevalence in other dairy products has been reported to be as low: in butter 0% (Little *et al.*, 2009) and 0.3–3.5% in ice cream (Miettinen *et al.*, 1999b; Cordano & Rocourt, 2001; Busani *et al.*, 2005).

The prevalence of *L. monocytogenes* in raw meat varies between animal species. Prevalence has been reported to be 4.6–5.4% in beef, 6.9–33% in pork and 1.9–36% in poultry (Chasseignaux *et al.*, 2001; Vitas *et al.,* 2004; Busani *et al.*, 2005; Pesavento *et al.*, 2010). A 39% prevalence of *L. monocytogenes* was observed in marinated poultry when looking at raw meat preparations (Aarnisalo *et al.*, 2008) and 4.9–11% prevalence in raw sausages (Cordano & Rocourt, 2001; Wagner *et al.*, 2007). The prevalence in precooked ready-to-eat meats was reported as 1.2–2.1% (Cordano & Rocourt, 2001; Lambertz *et al.*, 2012; Meldrum *et al.,* 2010; EFSA, 2013). However, the prevalence was higher (4.6–8.8%) in ready-to-eat meats sliced after heat treatment (Vitas *et al.,* 2004; Little *et al.,* 2009). Other studies showed the prevalence of specific meat products to be 4.5% in cooked sausage, 1.5–15% in fermented sausages and 1.3–6.7% in cured meat products (Jemmi *et al.,* 2002; Vitas *et al.,* 2004; Wagner *et al.*, 2007 Meldrum *et al.,* 2010).

The prevalence of *L. monocytogenes* in raw fish has been reported as 2–15% (Autio *et al.*, 1999; Hoffman *et al.*, 2003; Markkula *et al.*, 2005) and 6.4% in raw fish and fish products (Busani *et al.*, 2005). Prevalence of *L. monocytogenes* in ready-to-

eat smoked fish varies from 4.8% to 20% (Wagner *et al.*, 2007, Meldrum *et al.,* 2010; EFSA, 2013; Gonzáles *et al.*, 2013). Cold-smoked and gravad fish have a higher prevalence than hot smoked fish, 14–38%, 14–33% and 2–12%, respectively (Lyhs *et al.*, 1998; Jemmi *et al.,* 2002; Lambertz *et al.*, 2012).

The prevalence of *L. monocytogenes* in various vegetables has been low, e.g. 0–0.9% in green salad and 0% in sprouts, fruits, seeds and dried fruits (Wagner *et al.*, 2007; Meldrum *et al.,* 2010), but higher in frozen vegetables ranging from 1.8% to 25% (Vitas *et al.,* 2004; Cordano & Jacquet, 2009). The prevalence in ready-to-eat vegetable foods such as mixed salads has been reported to be 0–4.8%, but the prevalence was higher (10%) in fresh salads made in Chilean supermarkets (Little *et al.*, 2007; Christison *et al.*, 2008; Cordano & Jacquet, 2009; Meldrum *et al.,* 2010; Pesavento *et al.*, 2010).

The prevalence of *L. monocytogenes* has been reported as 1.7–7% in other ready-to-eat products, such as sandwiches, and 1% in confectionery products containing fresh cream (Christison *et al.*, 2008; Little *et al.*, 2009; Meldrum *et al.,* 2010; Pesavento *et al.*, 2010).

2.2.4 Incidence in humans

Reliable incidence reporting requires a surveillance system and comparable case definitions, which may differ between countries. The notification of cases to public health authorities is not mandatory in all European countries. A European survey conducted in 2002 showed that surveillance systems were in operation in 16 of the 17 countries surveyed, and that infection was statutorily notifiable in ten of these countries (de Valk *et al.*, 2005). The notification rate in the European Union was 0.33–0.36 cases per 100 000 people in 2010–2012 (Anonymous, 2015b). A total of 1 763 confirmed cases of listeriosis were reported in 2013, and the overall EU notification rate was 0.44 cases per 100 000 people (Anonymous, 2015c). During the 2000s, the annual number of reported listeriosis cases has increased in several European countries (Koch & Stark, 2006; Gillespie *et al.*, 2006), which was not explained by common-source outbreak clusters (Goulet *et al.*, 2008). The incidences in France, England and Wales increased mostly in over 60-year-olds, regardless of whether they had a recognised underlying medical condition (Gillespie *et al.*, 2006; Goulet *et al.*, 2008). The overall annual incidence of listeriosis in the United States in 2004–2011 varied from 0.25 to 0.32 cases per 100 000 people. The incidence in pregnant women was substantially higher each year from 2007 through 2009 than in any other year since 2001. The average incidence rate of pregnancy-associated listeriosis was 4.5 cases from 2004 through 2009 and 3.0 from 2009 through 2011 per 100 000 pregnant women (Silk *et al.*, 2012; CDC, 2013).

Between 2000 and 2014, 18–71 cases were reported annually to the National Infectious Disease Register of the National Institute of Health and Welfare in Finland, with incidence varying from 0.35 to 1.3 per 100 000 people (THL, 2015). A marked increase in listeriosis cases was detected in 2010, when 71 cases were reported with an incidence of 1.3 per 100 000 people. Of these cases, 13 had the same pulsotype, which was also isolated from fish products. This pulsotype was the most

common pulsotype found in foods in 2010 and was particularly associated with gravad and cold-smoked salmon (Nakari *et al.*, 2014). In previous years some clusters of cases have also been associated with gravad and cold-smoked fish (Lyytikäinen *et al.*, 2006).

2.3 Control of *L. monocytogenes* in the food industry

The ability of *L. monocytogenes* to survive at low temperatures, tolerate acidity, low water activity and to form biofilms in food-processing environments poses challenges in controlling *L. monocytogenes* in the food industry. Several aspects of good hygiene practices can help food business operators cope with *L. monocytogenes*. The foundation of food safety lies in familiarity of food business operators with food safety, and in developing and maintaining knowledge of food safety risks associated with the products and processes in the food business in question.

2.3.1 Factory and equipment design

The factory and equipment design play an important role in the prevention of cross-contamination in food establishments. Improper designs create niches both in the environment of the establishment and in the equipment enabling the survival of *L. monocytogenes*. Factory design should allow the manufacturing process to proceed consistently and manufacturing lines should be linear to avoid transferring raw materials and half-finished products back and forth during the process. The crossing of material flows and personnel permits cross-contamination between raw and finished products. Equipment layout should allow for hygienic working practices, efficient cleaning and maintenance (Tompkin *et al.*, 1999; Rørvik 2000; Van Donk & Gaalman, 2004).

Compartmentalisation of the processing line into separate hygiene areas was shown to be a significant factor in preventing cross-contamination in a poultry-processing establishment (Lundén *et al.*, 2003a). Similar results were shown in shrimp factories, stressing the importance of separating high- and low-risk areas, and implementing strict rules involving the movement of equipment and staff (Gudmundsdóttir *et al.*, 2006). The high levels of contamination in trolleys and containers, which were moved through the different hygienic zones, were likely to be an important factor in cross-contamination between raw and cooked products in ready-to-eat food-processing plants (Salvat *et al.*, 1995).

The cavities, crevices and dead ends in pipelines may harbour *L. monocytogenes*. The problematic equipment contributing to the post-process contamination in the food industry includes dicing and slicing machines that usually have a complex design including cutter blades, brining equipment (Autio *et al.*, 1999; Bērziņš *et al.*, 2010), conveyors (Lyytikäinen *et* al., 2000), and packaging machines (Ericsson *et al.*, 1997; Miettinen *et al.*, 1999b; Lyytikäinen *et al.*, 2000). The location of the *L. monocytogenes* contamination in an establishment can be limited to a specific packaging line of ready-to-eat foods (Tompkin, 2002). However, Lundén *et al.*

(2003a) found that persistent PFGE type *L. monocytogenes* were often widely spread in the processing plants, contaminating several sites and more than one processing line.

Dicing equipment was found to harbour a persistent *L. monocytogenes* contamination, and despite the dismantling and cleaning of the machinery, the same persistent *L. monocytogenes* strain was later isolated from the dicing line of another establishment where the machine was transferred (Lundén *et al.*, 2002). Salting and slicing equipment were found to harbour *L. monocytogenes* contamination in a cold-smoked and cold-salted fish processing plant, mainly due to difficulties in cleaning the equipment (Johansson *et al.*, 1999). The feeding teeth of a brining machine were found to harbour *L. monocytogenes* before production in a cold-smoked pork-processing plant, and several other contamination sites were found on the equipment during production (Bērziņš *et al.*, 2010). Miettinen *et al* (2001) studied *L. monocytogenes* contamination in two broiler slaughterhouses with attached meat-processing plants, where *L. monocytogenes* was found from the skin-removing machine at both slaughterhouses. Contamination of broiler carcasses probably occurred during chilling or in the skin-removing machine, because *L. monocytogenes* was not found prior to these steps (Miettinen *et al.*, 2001).

Not only equipment design but also neglected maintenance may cause problems with *L. monocytogenes* contamination. Worn and frayed conveyer belts are particularly difficult to clean and disinfect properly. In a study of pork and poultry establishments, *L. monocytogenes* was not detected on smooth surfaces, but granular, stripped or damaged surfaces were contaminated with the bacterium (Chasseignaux *et al.*, 2002). A brine pasteuriser, with cracks in the exchange plates, was found to recontaminate the brine after heat treatment (Alessandria *et al.*, 2010).

2.3.2 Cleaning and disinfection

Cleaning and disinfection play a significant role in the effort of controlling *L. monocytogenes* in food establishments. The possibility of transferring *L. monocytogenes* from a contaminated product to other delicatessen meat products through a slicing machine in a retail shop stresses the importance of thorough cleaning (Vorst *et al.*, 2006). Different cleaning methods are based on the use of mechanical, chemical and heat energy (Gibson *et al.*, 1999). A study comparing low- and high-pressure cleaning did not observe enhanced removal of *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms with increasing water spray pressure. The use of lower pressure may also limit the potential spread of contamination by aerosols (Gibson *et al.*, 1999). Mechanical scrubbing was found to be more efficient than applying extra disinfectants when cleaning *L. monocytogenes* biofilms (Jessen & Lammert, 2003).

Persistent equipment contamination may require enhanced cleaning to eradicate *L. monocytogenes*. Enhanced cleaning includes dismantling equipment when possible, the application of hot steam, heating small pieces in an oven or in hot water, and alkali-acid-alkali detergent cleaning (Autio *et al.,* 1999; Miettinen *et al.*, 1999b; Lundén *et al.*, 2002).

All disinfectants are efficient in destroying *L. monocytogenes* if the concentration of disinfectant and treatment duration are sufficient and prior cleaning has removed the organic soil (Jessen & Lammert, 2003). However, *L. monocytogenes* has been shown to adapt to disinfectants when exposed to sublethal concentrations (Aase *et al.*, 2000; Lundén *et al.*, 2003b). Lundén *et al.* (2003b) demonstrated that *L. monocytogenes* strains adapted to quaternary ammonium compounds and tertiary alkylamine after sublethal exposure, but adaptation to potassium persulphate and sodium hypochlorite was not observed. Adaptation of *L. monocytogenes* cells to progressively increasing disinfectant concentrations at 10 °C and 37 °C was observed with all disinfectants except potassium sulphate (Lundén *et al.*, 2003b). In a study of 200 *L. monocytogenes* strains, 10% were determined as tolerant to benzalkonium chloride, but the serial subcultivation of initially sensitive and tolerant strains in sublethal concentrations resulted in approximately equal tolerance of all strains to the disinfectant (Aase *et al.*, 2000). Adaptation to a disinfectant may lead to cross-adaptation to other disinfectants, enhancing the survival of the bacteria (Lundén *et al.*, 2003b). Despite the adaptation observed in the above-mentioned studies, the concentrations used in commercial solutions in the food industry were not exceeded. The need to use appropriate concentrations of commercially available disinfectants, especially in the case of quaternary ammonium compounds and hypochlorite was emphasised in a study by Aarnisalo *et al.* (2007). The microbicidal efficacy of eight disinfectants to *L. monocytogenes* strains grown on stainless steel and polyethylene discs was studied at refrigerated temperatures. The reduction of *L. monocytogenes* on stainless-steel surfaces and on polyethylene surfaces was found to be similar. The quaternary ammonium compound and peracetic acid disinfectant were not effective against all tested *L. monocytogenes* strains, which could be explained by the shorter disinfection time used in the study compared to the time recommended by the manufacturer (Aarnisalo *et al.*, 2007).

Persistent *L. monocytogenes* strains isolated from food establishments were initially more tolerant to disinfectants than transient strains. Persistent and transient *L. monocytogenes* strains exhibited similar adaptation to disinfectants at 37 °C, but the persistent strain exhibited increased tolerance to a quaternary ammonium compound and the tertiary alkylamine at 10 °C compared with the transient strain (Lundén *et al.*, 2003b). Holah *et al.* (2002) found that persistent strains were not more tolerant to disinfectants than an *L. monocytogenes* laboratory strain and concluded that persistence in food establishments is not related to increased tolerance of persistent strains to the most commonly used disinfectants but may be due to physical adaptation to the environmental conditions in food establishments.

The mechanisms of the adaptation or innate tolerance to disinfectants and the genetic basis for tolerance have been studied. Efflux pumps have been identified as a mechanism of benzalkonium chloride and other quaternary ammonium compound tolerance (Aase *et al.*, 2000). The adaptation of sensitive strains of *L. monocytogenes* to benzalkonium chloride resulted in significant increases in the expression of the *mdrL* gene, which indicates that the efflux pump MdrL is at least partly responsible for the adaptation to benzalkonium chloride (Romanova *et al.,* 2006). A plasmid (pLM80)-associated benzalkonium chloride resistance cassette (*bcrABC*) was

characterised in an outbreak-related strain of *L. monocytogenes*. Transcription of the resistance genes was increased under sublethal exposure to disinfectant and was higher at reduced temperatures than at 37 °C. The *bcrABC* resistance cassette provides increased tolerance to benzalkonium chloride due to the small multidrug resistance protein family transporter (Elhanafi *et al.*, 2010; Dutta *et al.*, 2013). A novel transposon Tn6188 in *L. monocytogenes* strains was recently characterised (Müller *et al.*, 2013). The transposon Tn6188 has been shown to mediate the tolerance to quaternary ammonium compounds, via a quaternary ammonium compound resistance protein QacH, a small multidrug resistance protein family transporter (Müller *et al.*, 2013; Müller *et al.*, 2014).

2.3.3 Use of low pH

L. monocytogenes is able to adapt to acidic environments. *L. monocytogenes* cells grown at pH 5·0 survived significantly better at low pH levels compared with cells grown at a neutral pH (Kroll & Patchett, 1992). A short exposure to an acidic environment induces a stress response, which enhances survival in more acidic conditions (O'Driscoll *et al.,* 1996). *L. monocytogenes* cells in a stationary growth phase are more tolerant to acidity than cells in an exponential growth phase (O'Driscoll *et al.,* 1996). *L. monocytogenes* has weaker tolerance of organic volatile acids, such as acetic or lactic acid, than inorganic acids, such as hydrogen chloride, because weak acids permeate the cell membrane in their undissociated form and cause a lower intracytoplasmic pH (Vasseur *et al.*, 1999; Phan-Thanh *et al.*, 2000).

The genetic response of many bacteria to changes in environmental conditions results from the sensing and regulatory activities of two-component signal transduction systems. A two-component system consists of a membrane-bound histidine kinase, which senses a certain environmental variable, and a cytoplasmic response regulator, which enables the cell to respond to environmental alterations, often by regulating target genes (Kofoid & Parkinson, 1988; Stock *et al.*, 1989). The *L. monocytogenes* genome encodes 15 histidine kinases and 16 response regulators constituting two-component regulatory systems (Glaser *et al.,* 2001), several of which, including lisRK, are associated with heat and acid tolerance (Cotter *et al.*, 1999; Kallipolitis & Ingmer, 2001). Tolerance of acidic and alkaline conditions has been observed to be dependent on alternative sigma factors, of which the sigma factor σB is an important regulator, redirecting the RNA polymerase action to the transcription of genes whose products enable the adaptation to changing conditions (Wiedmann *et* al., 1998; Ferreira *et al.*, 2003; Giotis *et al.*, 2008).

The proton pump systems are mechanisms by which *L. monocytogenes* maintains pH homeostasis in acid environments. The glutamate decarboxylase (GAD) system, which modulates intracellular pH, plays a role in acid tolerance during both the logarithmic and stationary growth phases and is also required for the induction of an optimal acid tolerance response. It is thought to be the key mechanism through which *L. monocytogenes* maintains pH homeostasis. The GAD system operates by converting a molecule of glutamate to aminobutyrate (GABA), using an intracellular proton and decreasing cytoplasmic acidification. Intracellular GABA is then

exchanged for an extracellular glutamate via an antiporter (Cotter & Hill, 2003). Free glutamate enhances the survival of *L. monocytogenes* at mildly acidic conditions (Cotter *et al.*, 2001). The F_0F_1-ATPase, a membranous proton pump, also plays a role in the induction of an acid tolerance response. The F_0F_1-ATPase is a multisubunit enzyme consisting of a catalytic portion (F_1), which may synthesise or hydrolyse ATP and an integral membrane portion (F_0) that functions as a membranous channel for proton translocation (Cotter *et al.*, 2000).

L. monocytogenes strains adapted to acidity show cross-resistance to other stress factors, such as heat, salty environment, ethanol and bacteoricins (O'Driscoll *et al.,* 1996; Lou & Yousef, 1996; van Schaik *et al.*, 1999; Phan-Thanh *et al.,* 2000). Log-phase cultures in broth, adapted to pH 5.4 for several hours, showed a gradual increase in thermotolerance. The maximum increase in thermotolerance was 3.5-fold (Jørgensen *et al.*, 1999). *L. monocytogenes* cells were either subjected to acid shock at pH 4.0 for 1, 2 or 4 hours, or adapted to pH 4.0 gradually, and were afterwards heat-treated to 58 °C in whole milk. Heat resistance was significantly increased both in cells adapted to acidity and in cells subjected to short, 1-hour acid shock (Farber & Pagotto, 1992).

L. monocytogenes exhibit differences in acid tolerance between strains. All tested acids had an inhibitory effect on the growth of all five strains but to varying degrees, two strains had shorter lag times and faster growth than the other three strains (Vasseur *et al.,* 1999). A predictive mathematical model created to estimate growth using 20 *L. monocytogenes* strains isolated from clinical cases, foods and food establishments showed that the estimated minimum growth pH varied between strains from 4.34 to 4.68 (Aryani *et al.*, 2015a).

2.3.4 Heat treatments

Early heat resistance studies of *L. monocytogenes* suspected that a possibility of survival exists in the pasteurisation process (Bearns & Girard, 1958; Fleming *et al.*, 1985). Donnelly *et al.* (1987) claimed that the finding was caused by the test tube method used by Bearns and Girard (1958), and concluded that *L. monocytogenes* did not survive the pasteurisation temperature (Donnelly *et al.*, 1987). Despite the common heating processes utilised in the food industry being sufficient to destroy *L. monocytogenes* in foods, the bacterium is more tolerant of heat than other non-spore-forming bacteria (Mackey & Bratchell, 1989).

Growth phase affects the thermal tolerance of bacteria. Cells in the stationary phase are more tolerant to heat than cells in the exponential growth phase (Lou & Yousef, 1996; Doyle *et al.*, 2001). *L. monocytogenes* cells in logarithmic and stationary phases of growth were subjected to heat at 60 °C with and without heat shock at 46 °C for 30 minutes. The $D_{60\,°C}$-value increased from 0.6 to 1.7 minutes with heat shock in the logarithmic-phase cells and from 2.22 to 4.01 minutes in the stationary-phase cells. The thermotolerance of cells in the logarithmic growth phase after heat shock did not reach the value of stationary-phase cells without heat shock despite the increased thermotolerance (Jørgensen *et al.*, 1999).

A sudden rise in temperature, i.e. heat shock, and adaptation to heat by growing in high temperatures both affect the thermotolerance of *L. monocytogenes*. *L. monocytogenes* cells heat-shocked in 48 °C for 120 minutes exhibited an average 2.4-fold increase in $D_{64\ °C}$-values compared to cells not subjected to heat shock in cured meat (Farber & Brown, 1990). A similar increase in D-values was caused by heat shock at 48 °C for 10 min in trypticase soy broth (Linton *et al.*, 1990). The optimal heat shock temperature for different organisms depends on the range of their growth temperatures. Organisms growing over a broad range of temperatures, such as *L. monocytogenes,* usually achieve the maximum response at 10–15 °C above the optimum growth temperature (Lindquist, 1986; Linton *et al.*, 1990). *L. monocytogenes* cells grown in 43 °C adapted to heat and tolerated heat treatment at 62.8 °C better than cells grown at 37 °C (Knabel *et al.*, 1990). *L. monocytogenes* cells grown at low temperatures (5 °C to 19 °C) were more sensitive to heat treatment at 52 °C and 60 °C compared to cells grown at 37 °C or 42 °C (Smith *et al.*, 1991a; Juneja *et al.*, 1998). Cold shock at 0 °C for three hours reduced the thermal resistance of stationary-, exponential-, and lag-phase *L. monocytogenes* cells by 38%, 15% and 26%, respectively, compared to cells not subjected to cold shock (Miller *et al.*, 2000).

Heat shock induces the production of heat shock proteins, such as class I heat shock proteins DnaK, DnaJ, GrpE, GroEL and GroES, which act as molecular chaperones, protecting essential bacterial proteins from heat denaturation, and transcription repressor HrcA of class I heat-shock genes (Hanawa *et al.*, 2000; Gahan *et al.*, 2001; van der Veen *et al.*, 2007; Selby *et al.*, 2011). Class III heat-shock genes encode chaperones and ATP-dependent Clp proteases, ClpC, ClpE, ClpY, ClpB and ClpP, which degrade damaged or misfolded proteins. Class III heat-shock genes are regulated by transcription repressor CtsR (Rouquette *et al.*, 1996; Nair *et al.*, 2000; van der Veen *et al.*, 2007). The class II stress genes encode several general stress proteins, the expression of which is regulated by the alternative sigma factor σB (Kazmierczak *et al.*, 2003). Genes belonging to class I and III heat-shock regulons exhibited increased expression during heat exposure at 48 °C for 40 minutes. Class III heat-shock genes exhibited a temporary expression, since Clp proteases are required at the early stages of heat shock to remove the initially damaged and misfolded proteins. Class I heat-shock genes exhibited a constant induction in expression levels during heat exposure (van der Veen *et al.*, 2007).

L. monocytogenes strains adapted to heat have been reported to have cross-resistance to other stress factors, such as acidity. *L. monocytogenes* cells grown at 30 °C tolerated acid shock better at pH 2.5 than cells grown at 10 °C (Patchett *et al.*, 1996). However, in a study by Koutsoumanis *et al.* (2003), *L. monocytogenes* cells exposed to 90 minutes at 45 °C or 50 °C did not increase their tolerance to acid shock at pH 3.5 (Koutsoumanis *et al.,* 2003).

Heat tolerance varies between *L. monocytogenes* strains. Twelve *L. monocytogenes* strains were heat-treated at 58 °C, 60 °C and 64 °C. Significant differences in heat resistance were noted between heat sensitive and tolerant strains, D-values being 2- to 4-fold (Sörqvist, 1994). The heat tolerance variation in 25 strains, isolated from foods, animals and human clinical cases, heat-shocked at 55 °C

was shown to be 2-fold at greatest (Lianou *et al.* 2006). Differences between the thermotolerance of twenty *L. monocytogenes* strains is an important source of variability in thermal inactivation kinetics. D-values of the strains varied from 9 to 30 min at 55 °C, from 0.6 to 4 min at 60 °C and from 0.08 to 0.6 min at 65 °C. Differences between strains at all tested conditions were ten times higher than experimental variability and four times higher than biological variability (Aryani *et al.*, 2015b).

2.3.5 Utilisation of bacteriocins (listeriocins) in food manufacturing processes

The starter cultures of lactic acid bacteria have been used for preservation in traditional, fermented foods for centuries. Some lactic acid bacteria produce bacteriocins, protein substances that are bactericidal or bacteriostatic against other bacterial species. Bacteriocins are usually active against species closely related to the producing bacteria, but are shown to be effective against a range of organisms including *L. monocytogenes* (Pucci *et al.*, 1988; Gao *et al.*, 2010; Masuda *et al.*, 2011).

Bacteriocins are categorised in different classes based on the observed characteristics. The original classification of four classes by Klaenhammer (1993) has been proposed to be changed due to the discovery of cyclic peptide bacteriocins, and other bacteriocins that are not adequately classified (Klaenhammer, 1993; Cotter *et al.*, 2005; Franz *et al.*, 2007; Zouhir *et al.*, 2010). Most studied bacteriocins belong to class I (lantibiotics), and class II (small heat-stable non-lanthionine-containing peptides) bacteriocins (Sahl & Bierbaum, 1998). According to Klaenhammer (1993) class III bacteriocins include large, heat-labile proteins and class IV includes protein complexes.

Class I bacteriocins include nisin, discovered in 1928 and subtilin, discovered in 1944 (Rogers, 1928; Jansen & Hirschmann, 1944). Nisin generates pores to the cytoplasmic membrane of gram-positive bacteria, dissipating the membrane potential and inducing an efflux of low-molecular-mass compounds (Garcerá *et al.*, 1993; Héchard & Sahl, 2002).

Many bacteriocins with listericidal effects belong to class IIa bacteriocins, which are small ribosomally synthesised peptides that permeabilise the cell membrane through the formation of pores, causing the release of ions and leakage of molecules, such as amino acids, and dissipate the membrane potential (Chikindas *et al.*, 1993; Héchard & Sahl, 2002). Class IIa bacteriocins, including sakacin P, mundticin, bifidocin B, pediocin PA-1 and AcH, leucocin B-Ta11a, leucocin A, mesentericin Y105, curvacin A, bavaricin A and carnocin 51, are produced by many lactic acid bacteria species, such as *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. and have been isolated from a variety of foods such as vegetables, goat milk, soft cheese and meat (Hastings *et al.*, 1991; Nieto Lozano *et al.* 1992; Héchard *et al.* 1992; Tichaczek *et al.* 1992; Larsen *et al.*, 1993; Ennahar *et al.*, 1996; Herbin *et al.*, 1997; Bennik *et al.*, 1998; Yildirim & Johnson, 1998; Felix *et al.*, 1994; Ennahar *et al.*, 2000a).

Starter cultures containing bacteriocin-producing lactic acid bacteria have been studied in meat and dairy products. *L. monocytogenes*-inoculated semidry sausages were fermented with two starter cultures, with and without the bacteriocin-producing *Pediococcus* strain. The *L. monocytogenes* counts were 1.4 $\log_{10}$ cfu per gram lower in sausages fermented with bacteriocin after 14 h (Berry *et al.*, 1990). Dry sausages were inoculated with 5 $\log_{10}$ cfu per gram of *L. monocytogenes* and fermented with two starter cultures. The culture containing bacteriocin-producing *Pediococcus acidilactici* PA-2 and *Lactobacillus bavaricus* MI-401 resulted in a 3-$\log_{10}$ cfu per gram reduction and the other starter culture in a 0.6–09-$\log_{10}$ cfu per gram reduction of *L. monocytogenes* in 21 days (Lahti *et al.*, 2001). Three different bacteriocin-producing lactic acid bacteria strains and a meat starter culture were used in the dry sausage fermentation. Sausages were inoculated with 3 $\log_{10}$ cfu per gram of *L. monocytogenes*. All three protective strains resulted in *L. monocytogenes*-negative sausages in 21 days, compared to the starter culture, which were *L. monocytogenes* negative after 28 days of ripening (Työppönen *et al.,* 2003). Leroy & de Vuyst (1999) studied a sakacin K-producing *Lactobacillus sake* strain, and concluded that the strain produced sakacin K at conditions suitable for dry sausage fermentation. Temperature and pH were found to affect the sakacin K production, which was best at temperatures ranging from 20 °C to 25 °C and at pH 5.0 to 5.5 (Leroy & de Vuyst, 1999). *Lactobacillus curvatus* 54M16, isolated from traditional Italian fermented sausage, was found to produce three bacteriocins, sakacin P, X and T, and to exhibit inhibition of *L. monocytogenes* at temperatures ranging from 15 °C to 30 °C and at pH 4.5 (Casaburi *et al.,* 2016).

Protective bacteriocin-producing strains have also been studied in vacuum-packaged, cooked meat. *Leuconostoc carnosum* 4010, producing leucocin A and C, and a five-strain mix of *L. monocytogenes* were inoculated to cooked sausage slices, vacuum-packaged and stored at 5 °C. The amount of *L. monocytogenes* was reduced from 4 $\log_{10}$ to <10 cfu per gram in 21 days (Budde *et al.*, 2003). In another study, *Lactobacillus sakei* was sprayed on cooked ham and cervelat sausage before slicing and vacuum-packaging, and the packages were inoculated with three strains of *L. monocytogenes*. The growth of *L. monocytogenes* was inhibited at 4 °C and 8 °C for 28 days (Bredholt *et al.*, 2001).

Fresh Minas cheese was produced using pasteurised milk inoculated with *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch, isolated from yellow cheese. *L. monocytogenes* was later added to curdled milk, and the prepared cheeses were stored at 8 °C. The growth of *L. monocytogenes* was not inhibited by *E. faecium* ST88Ch in 12 days. *E. mundtii* CRL35 inhibited the growth, but did not reduce the amounts of *L. monocytogenes* in 12 days (Pingitore *et al.*, 2012). Pingitore *et al.* (2012) concluded that the inhibitory activity of bacteriocins in culture media is not always reproducible in food systems.

Bacteriocins can also be added as partially purified or purified concentrates, which require specific approval as food additives from the legislative viewpoint. The European Union allows purified nisin as a food additive in dairy products, such as ripened or processed cheese (EU, 2008).

The growth of a five-strain mixture of *L. monocytogenes,* inoculated to a ricotta-type cheese, was inhibited by adding 2.5 mg/l of nisin to whole cow's milk during cheese production. The growth was inhibited up to 55 days of storage at 6–8 °C. When the cheese was made with acetic acid and potassium sorbate, *L. monocytogenes* was completely inhibited for the duration of the study (Davies *et al*., 1997). The addition of 12.5 mg/kg of nisin to fresh Minas cheese did not inhibit the growth of *L. monocytogenes* and resulted in a reduction less than 1 $\log_{10}$ cfu per gram compared to a control cheese group with no added nisin in 12 days (Pingitore *et al*., 2012). Nisin added to whey at 500 IU per gram was more effective at reducing the amount of *L. monocytogenes* than nisin added to whey cheese at an equal concentration. However, the growth of *L. monocytogenes* commenced after ten days of storage, and reached the inoculation level after 30 and 45 days of storage at 4 °C in cheese prepared with natural acidification or acidified with citric acid, respectively. Researches speculated that the reduced antilisterial effectiveness of nisin may have resulted from the higher average moisture content of cheese, which was slightly over the upper limit of traditional Greek whey cheeses (Samelis *et al*., 2003).

Resistance to bacteriocins, such as nisin, leucocin A and pediocin has been observed in *L. monocytogenes* (Modi *et al*., 2000; Gravesen *et al*., 2002). The frequency of pediocin AcH resistance appearing in 12 different *L. monocytogenes* strains was studied and was found to be strain-dependent, varying between $4x10^{-3}$ and $1x10^{-6}$. The pediocin AcH resistance frequency was calculated using the cfu number in the log-phase culture compared to cfu number on pediocin AcH-containing plate count agar plates. The stability of the pediocin-resistant mutant *L. monocytogenes* WSLC 1364R was determined over a period of 16 weeks of cheese ripening. The resistance was found to be stable until the end of the experiment at day 113 (Loessner *et al.,* 2003). Modi *et al*. (2000) studied the heat tolerance of nisin-resistant strains of *L. monocytogenes*, and observed that heat tolerance at 55–65 °C was equal in nisin-sensitive and -resistant strains. However, nisin-resistant strains grown with nisin were more sensitive to heat at 55 °C than nisin-sensitive *L. monocytogenes* strains (Modi *et al*., 2000). The 200- minute exposure to 6% salt was found to significantly increase nisin resistance compared to *L. monocytogenes* strains without salt exposure (Bergholz *et al*., 2013).

Contradictory results have been reported for the cross-resistance between bacteriocins. Two nisin-tolerant *L. monocytogenes* strains, twenty pediocin-resistant strains, and 34 strains with enhanced tolerance to pediocin were characterised among 381 strains of *L. monocytogenes*. Bavaricin sensitivity correlated with the pediocin sensitivity of the strains, but cross-resistance between nisin and pediocin or bavaricin was not detected (Rasch & Knöchel, 1998). The curvaticin 13-tolerant strain of *L. monocytogenes* exhibited cross-resistance to carnocin CP5 and pediocin AcH but remained sensitive to nisin. Nisin-tolerant strains were more sensitive to curvaticin 13 than the parental strain. No cross-resistance between nisin and curvaticin 13 was detected (Bouttefroy & Millière, 2000). Other studies have shown cross-resistance between class IIa bacteriocins and nisin. Nisin resistance in *L. monocytogenes* ATCC 700302 resulted in cross-resistance to pediocin PA-1 and class IV bacteriocin leuconocin S (Crandall & Montville, 1998). The sensitivities of 14

L. monocytogenes strains resistant to nisin A or three class IIa bacteriocins, pediocin PA-1, leucocin A or carnobacteriocin B2, were determined. The strains resistant to a class IIa bacteriocin exhibited cross-resistance to all these bacteriocins. The three nisin-resistant strains also exhibited reduced class IIa bacteriocin sensitivity. However, four class IIa bacteriocin-resistant strains exhibited reduced sensitivity to nisin and seven strains exhibited unaltered nisin sensitivity (Gravesen *et al.*, 2004).

Gravesen *et al.* (2002) suggested that one general mechanism is responsible for the spontaneous development of class IIa resistance in *L. monocytogenes*. This mechanism is irrespective of *L. monocytogenes* strain, class IIa bacteriocin, or the tested environmental conditions, and influences changes in expression of the β-glucoside-specific and the mannose-specific phosphotransferase systems. Prevention of *mpt* (mannose permease two) expression directly confers resistance (Gravesen *et al.*, 2002).

The sensitivity to bacteriocins differs between *L. monocytogenes* strains. The low pH values were found to increase the sensitivity of six *L. monocytogenes* strains to nisin. The sensitivity was compared at pH 5.0, 6.0 and 7.0. Two of the strains were found to be more sensitive and two were more resistant than the other strains (Ukuku & Shelef, 1997). The sensitivity of 14 strains of *L. monocytogenes* to pediocin AcH, divercin V41, enterocin A, mesentericin Y105 and nisin A was determined. The sensitivity to class IIa bacteriocins was variable, one strain was highly sensitive, 11 strains were moderately sensitive; one strain exhibited weak sensitivity and one strain could not be inhibited with the highest bacteriocin concentrations used in the study. On the other hand, all *L. monocytogenes* strains were sensitive to class I bacteriocin nisin A (Ennahar *et al.*, 2000b). The susceptibility of 200 different strains of *L. monocytogenes* was studied, and the differences in sensitivity to nisin, sakacin P, sakacin A and pediocin PA-1 were shown to be manifold (Katla *et al.*, 2003).

2.3.6 Food additives

Food additives are a group of substances added to food to preserve flavour or enhance taste and appearance. Some food additives, i.e. preservatives, are substances that prolong the shelf life of foods by protecting them against deterioration caused by micro-organisms or that protect against the growth of pathogenic micro-organisms. These include lactic acid and lactate, nitrite, nitrate, sorbic acid and sorbate, acetic acid, acetate, benzoic acid and sodium benzoate.

The amount of food additives allowed in foods is regulated by European Union legislation (EU, 2008). Nitrite and nitrate are allowed in meat products in levels up to 150 ppm (mg/kg). Lactic acid, lactate, acetic acid and acetate are allowed in meat preparations. Sorbic acid, sorbates, benzoic acid and benzoates are allowed in the surface treatment of dried meat products and in limited amounts in pâté and aspic. In dairy products sorbic acid and sorbates are allowed up to 1000 ppm in curdled milk, unripened cheese, and pre-packed, sliced and cut whey and ripened cheese, up to 2000 ppm in processed cheeses and quantum satis in the surface treatment of

ripened cheese. Lactic acid, acetic acid and nitrates (150 ppm) are allowed in whey cheeses (EU, 2008).

The effectiveness of different additives in preventing the growth of *L. monocytogenes* has been studied. Cooked, cured ham products were produced with various levels of sodium lactate, sodium diacetate or buffered sodium citrate. *L. monocytogenes* was best inhibited by the addition of 2.5% or 3.3% sodium lactate but also by the addition of 0.2% sodium diacetate. However, the addition of 0.2% sodium diacetate had a negative effect on the odour and taste of the ham product. *L. monocytogenes* was observed to grow to 6 $\log_{10}$ cfu per gram in the ham with buffered sodium citrate (Stekelenburg & Kant-Muermans, 2001). Sodium acetate (0.5%) was found to be most inhibitory against the growth of *L. monocytogenes* in sliced turkey bologna, followed by 2.0% sodium lactate and 0.3% potassium sorbate. However, growth of 1.33 to 2.51 $\log_{10}$ cfu per gram after storage was observed at 70 and 98 days, respectively (Wederquist *et al.*, 1994).

L. monocytogenes was able to survive during the manufacture of fermented sausages with 120 ppm of sodium nitrite during 21 days of fermentation. The amount of *L. monocytogenes* was reduced by 1 $\log_{10}$ cfu per gram (Junttila *et al.*, 1989). Small concentrations of nitrite (30 ppm) in turkey slurry did not prevent the growth of *L. monocytogenes*. The pathogen grew to 8 $\log_{10}$ cfu/ml within 28 days at 4 °C. However, 0.3% and 0.5% sodium diacetate in turkey slurries did prevent the growth of *L. monocytogenes* (Schlyter *et al.*, 1993). Potassium nitrite at a concentration of 140 ppm did not affect the growth of *L. monocytogenes* in beef meat, but 4% lactate extended the lag phase to 1–2 weeks at 5 °C (Shelef & Yang, 1991).

2.3.7 Ultrasound

Ultrasounds are high frequency sound waves with frequencies above the human hearing treshold (> 20 kilohertz (kHz)). Ultrasound is carried in liquid and solid media. It is generated using piezoelectric crystals or magnetostrictive materials that expand or contract when placed in electromagnetic fields (Suslick, 1989).
High (megahertz, MHz) frequency, low intensity ultrasound is mainly used for non-invasive purposes such as the ultrasonic imaging of materials, locating foreign bodies in foods, measuring particle size distribution or in medical applications. Low (kilohertz, kHz) frequency, high intensity, power ultrasound can be used in the ultrasonic welding of plastic materials (Tsujino *et al.*, 1996), several food-processing applications such as emulsification (Behrend *et al.*, 2000), defoaming, degassing of liquids, (Chemat *et al.*, 2011), meat tendering (Smith *et al.*, 1991b; Jayasooriya; *et al.*, 2004), freezing (Delgado *et al.*, 2009), cutting of foods, (Schneider *et al.*, 2008*;* Arnold *et al.*, 2009) and ultrasonic cleaning.

Ultrasound can be used in cleaning different materials with frequencies from 20 to 100 kHz. Lower frequencies (20–40 kHz) are more efficient in microscopic particle cleaning than higher frequencies (Koontz & Amron, 1959; McQueen, 1986). Ultrasonic cleaning systems usually consist of an ultrasound transducer and a bath filled with cleaning solution where the ultrasound is transmitted to. The ultrasound creates waves, alternating regions of compression and expansion in liquid media,

resulting in a phenomenon called cavitation. In transient cavitation, during a few acoustic cycles, small cavitation bubbles, filled with gas or vapour, grow under negative pressure in liquid. Under positive pressure cavitation bubbles collapse in less than a microsecond, and as a result a high amount of energy is locally released (Suslick, 1989; Flint & Suslick, 1991). This energy is released as heat and kinetic energy. The microorganisms in the liquid are exposed to temperatures and pressures of approximately 5 000 K and 100 MPa for a few microseconds (Flint & Suslick, 1991). As the cavitation bubbles implode near the surface of immersed material, it releases a liquid jet hitting the surface equal to high-pressure jetting (Lauterborn & Ohl, 1997). This phenomenon enables the cleaning of the smallest surface crevices. In stable cavitation, on the other hand, bubbles vibrate for many acoustic cycles without collapsing. These bubbles induce microstreaming in the surrounding liquid creating high shear forces, which can also accelerate the dissolution of soluble contaminants (Leighton, 1995; Lamminen *et al.*, 2004; Ashokkumar *et al.*, 2009).

The power of the ultrasonic element must be sufficiently high in relation to the liquid volume used, to generate cavitation in the entire cleaning liquid. Usually the power of an ultrasonic element used in ultrasonic cleaning is 5 to 15 W/l. Volumetric acoustic energy density (W/cm^3) plays an important role in microbial inactivation (Furuta *et al.*, 2004; Ugarte-Romero *et al.*, 2007). The increase of acoustic energy density from 0.49 to 1.43 W/cm^3 resulted in an additional 2-log reduction of *L. monocytogenes* cells in 20 minutes (Ugarte-Romero *et al.*, 2007).

Ultrasonic cleaning is suitable for most hard materials such as metals and hard plastics. Flexible materials, such as rubber, absorb the ultrasonic energy affecting the removal of microbes from surfaces (Jeng *et al.*, 1990). Ultrasonic cleaning has been used for years in the electronics, metal and medical industries. Small-scale ultrasonic cleaning baths are widely used in cleaning dental equipment (Bagg *et al.*, 2007).

Compared to traditional cleaning methods, improved results can be achieved in cleaning challenging objects with crevices and holes. Conveyor belts, which are often constructed with small parts and contain several joints and surfaces, are difficult to clean with traditional cleaning methods. The ultrasonic cleaning of food industry equipment has been used in the dairy industry to clean cheese moulds and in bottling lines. Cheese moulds contain long, narrow channels, which can be cleaned with ultrasonic cleaning (Kivelä, 1996; Salo & Wirtanen, 2007). The ultrasonic cleaning of whole poultry transportation crates at 60 °C reduced *Enterobacteriaceae* spp. counts by $\log_{10}$ 2.3 cfu, to below the detection limit, and the aerobic counts by >2 $\log_{10}$ cfu in three minutes, despite biofilm being present in the crates after commercial cleaning. This biofilm was not totally dislodged with pressure washing or ultrasonic cleaning at 50 °C for one minute (Allen *et al.*, 2008). Ultrasonic pilot-scale cleaning was applied to returnable plastic milk transportation crates. The whole milk crates were found to be cleaned more effectively in the pilot-scale ultrasonic cleaner than in the dairy plant in the cleaning tunnel (Salo & Wirtanen, 2007).

Ultrasound enhances the cleaning effects of heat, pressure and cleaning detergent. Ultrasonic cleaning in combination with these treatments appears to be more effective than heat, pressure or cleaning detergents used separately (Raso *et al.*, 1998; Pagán *et al.*, 1999a; Guerrero *et al.*, 2001). Several studies on the combined

effects of heat, pressure and ultrasound on microbial inactivation have been performed. The reduction in pH did not enhance the effect of ultrasonic treatment on *Saccharomyces cerevisiae* yeast cells, but ultrasound enhanced the effect of temperature at 35 °C and 45 °C. However, at 55 °C the D-values of yeast cells were similar between ultrasound and heat treatment alone (Guerrero *et al.*, 2001). Pasteurisation affects the organoleptic and nutritional quality of apple cider. Ultrasonic treatment of apple cider was found to add 2.3 to 2.4 log reductions of *L. monocytogenes* to heat treatment at 50 °C, 55 °C and 60 °C. Ultrasonic treatment may enable the use of milder heat treatments instead of pasteurisation when reducing the pathogenic bacteria (Baumann *et al.*, 2005). At 35 °C and 50 °C, the ultrasonic treatment of *L. monocytogenes* enhanced the inactivation. However, at 65 °C, the D-values did not differ significantly between heat treatment alone and thermosonication (Ugarte-Romero *et al.*, 2007). The synergism between ultrasound and pressure (i.e. manosonication) was found to decrease the D-values of *L. monocytogenes* at 40 °C from 4.3 minutes without pressure to 1.0 minute with 400 kPa pressure. As the pressure rises, the D-value decreases. However, when a certain pressure value is reached, the D-values remain constant (Pagán *et al.*, 1999b; Mañas *et al.*, 2000). At temperatures below 50 °C, the temperature did not influence the inactivation rate of *L. monocytogenes*. At higher temperatures the inactivating effect of manothermosonication, i.e. ultrasound with heat treatment and pressure, was considerably increased (Pagán *et al.*, 1999b). The similar inactivating effect at higher temperatures was detected with *Yersinia enterocolitica* (Raso *et al.*, 1998).

The surface tension of the liquid affects the energy required for cavitation. The higher the surface tension, the greater the energy needed. The surface tension is reduced by adding cleaning detergent to the liquid used in ultrasonic cleaning baths (O'Donoghue, 1984). The effect that cleaning detergents have on the ultrasonic inactivation of microbes has been studied. Ultrasound with sodium hypochlorite at 20 °C increased the reduction of *Escherchia coli* counts by 2 log cfu compared to sodium hypochlorite solution treatment alone (Duckhouse *et al.*, 2004). The similar synergy of ultrasound and sodium hypochlorite in reducing *L. monocytogenes* was seen on treatments of stainless steel (Lee *et al.*, 2014). The efficacy of *L. monocytogenes* reduction when simultaneously using a sanitiser and ultrasound is dependent on the chemical in question. Peroxide-based sanitiser effectively reduced the numbers of *L. monocytogenes* cells attached to polyvinyl chloride pipes and ultrasound did not increase this reduction. On the other hand, the reduction of attached *L. monocytogenes* cells was less for both chlorine- and quaternary ammonium–based sanitisers, and ultrasonication caused a further reduction to 2.9 and 3.1 cfu/cm^2, respectively (Berrang *et al.*, 2008). The ultrasonic treatment of *L. monocytogenes* biofilm on polystyrene with benzalkonium chloride resulted in a significant decrease in the level of viable cells compared to treatment with benzalkonium chloride alone. Ultrasonic treatment with benzalkonium chloride reduced the number of viable cells by 55% in one minute, compared to a less than 50% reduction with benzalkonium chloride treatment for five minutes (Torlak & Sert, 2013).

The potential to destruct microorganisms with ultrasound was discovered as early as 1928 (Harvey & Loomis, 1929). Microbe shape and size seem to influence the effect of ultrasonic treatment. The coccoid bacteria, *Staphylococcus aureus, Streptococcus lactis, Streptococcus faecalis* and *Streptococcus pyogenes,* were more resistant to ultrasound than the rod-shaped bacteria *Bacterium coli, Lactobacillus casei* and *Microbacterium* sp. (Jacobs & Thornley, 1954). The thicker layer of peptidoglycans on the cell membranes of gram-positive bacteria compared to gram-negative bacteria has been thought to protect the bacteria from the effects of ultrasound. Gram-negative *E. coli* and *Pseudomonas fluorescens* were more sensitive to ultrasonic treatment in milk than *L. monocytogenes* (Cameron *et al.*, 2009). Villamiel and de Jong (2000) reached similar results for the ultrasonic treatment of gram-negative *P. fluorescens* and gram-positive *Streptococcus thermophilus* (Villamiel & de Jong, 2000). However, the germicidal efficacy of ultrasound on *S. aureus, E. coli, Pseudomonas aeruginosa,* and *Bacillus subtilis* did not support this theory, as gram-positive *B. subtilis* was found to be the most sensitive (Scherba *et al.*, 1991). Fluorescence staining of *E. coli* and *Lactobacillus rhamnosus* cells revealed that the germicidal effect of ultrasonic treatment on gram-positive and gram-negative bacteria may not be due to damaged cell membranes, as membrane deterioration was not observed for most of the ultrasound-treated bacteria. Non-membrane-related degradation seemed to cause the lethal effect of high-intensity ultrasound. However, the ultrasound effectively damaged the outer membrane of gram-negative bacteria, allowing the penetration of fluorescent dyes to *E. coli* cells (Ananta *et al.*, 2005).

Ultrasonication has been used to remove biofilms from various surfaces. The frequency and intensity of ultrasonication affects the removal of biofilm. The lower frequency (20 kHz) was found to be more effective at dislodging a *Proteus mirabilis* biofilm than 150- or 350-kHz ultrasound (Mott *et al.*, 1998). Qian et al. (1996) reported that low intensity (0.01 W/cm^2, 500-kHz) ultrasound did not dislodge or change the structure of the biofilm, and thus may be suitable for enhancing the germicidal effect of gentamicin on the biofilms of medical implants. Dislodging the biofilm would release the harmful bacteria to the blood stream (Qian *et al.*, 1996). Significantly higher biofilm removal rates were observed for ultrasonic treatment than for conventional swabbing of stainless steel and polypropylene sheets contaminated with milk biofilms (Oulahal-Lagsir *et al.*, 2000). Power ultrasound significantly reduced the amount of *L. monocytogenes* cells on stainless steel chip biofilms. The lengthening of ultrasonic treatment from 30 to 60 seconds resulted in a reduction of 3.85 log cfu/ml after 30 seconds and 3.78 log cfu/ml after 60 seconds, which was not significantly different (Baumann *et al.*, 2009).

The use of ultrasonic cleaning may be limited by the subharmonic sounds that reach the human hearing range and thus the need to use earmuffs. The erosion of stainless steel due to ultrasound may influence the use of ultrasonic cleaning. The frequency and power must be suitable for the materials and cleaning systems used to avoid the wearing down of materials (Chiu *et al.,* 2005; Nagarajan *et al.*, 2006)

3 AIMS OF THE STUDY

The objective of this work was to assess methods to control *L. monocytogenes* in food processing and to investigate differences in survival between *L. monocytogenes* strains in food processing and food-processing environments.

The specific aims were as follows:

1. to investigate the contamination of a ready-to-eat and ready-to-reheat food-processing establishment using AFLP analysis and to assess the effect of control methods on the prevalence of *L. monocytogenes* (I),

2. to assess the effectiveness of listeriocin-producing protective cultures in dry sausages and the differences in survival of *L. monocytogenes* strains during dry sausage processing (II),

3. to examine the differences in acid and heat tolerance of persistent and non-persistent *L. monocytogenes* strains (III) and

4. to evaluate the applicability of ultrasonic cleaning in controlling *L. monocytogenes* in the food industry (IV,V).

4 MATERIALS AND METHODS

4.1 Bacterial strains (I–V)

4.1.1 Sampling of *L. monocytogenes* strains in food-processing plant (I)

As part of a quality control programme in a chilled food-processing plant, a total of 319 *L. monocytogenes* isolates were obtained by sampling, carried out during an eight-year period. Sampling was focused on the post-heating areas, hard-to-clean-equipment and on sites with prior positive results for *L. monocytogenes*. Basic sampling according to the control programme was carried out on each of the eight processing lines (A–H) at least once weekly, and additional sampling was performed if *L. monocytogenes* was recovered. Samples were collected both during production and after sanitisation. Isolates originated from raw materials (n=18), equipment (n=193), the processing environment (n=77) and the products (n=31). Sampling data were obtained from the records of the food-processing plant. Various raw ingredients, such as meat, fish, vegetables, dairy products and flour, were used in the production of chilled ready-to-eat and ready-to-reheat foods as well as in foods requiring cooking before consumption in the food establishment. The establishment was divided into three compartments with differing degrees of compartmentalisation.

4.1.2 *L. monocytogenes* strains (II–V)

The *L. monocytogenes* strains used in study II were originally isolated from meat and determined to be sensitive to pediocin using the well diffusion test (Tagg & McGiven, 1971). The strains were provided by Danisco, Niebüll, Germany (*L. monocytogenes* DCS 31, DCS 184), and the Department of Food and Environmental Hygiene (*L. monocytogenes* AT3E, HT4E and HR5E).

The *L. monocytogenes* isolates used in study III were collected from the environment, equipment and food products of three meat-processing plants as part of their in-house sampling plans. Of 367 isolates, 40 isolates belonging to 34 RFLP types were used in the acid and heat experiments.

A mixture of three *L. monocytogenes* strains (*L. monocytogenes* B9, V1 and V3) isolated from meat and dairy plants were used in studies IV and V. The Department of Food and Environmental Hygiene (*L. monocytogenes* B9) and VTT Technical Research Centre of Finland (*L. monocytogenes* V1 and V3) provided the strains.

The strains used in studies I and III were categorised as persistent if they were found in a food plant on five occasions or more and for a period exceeding three months, and as non-persistent if they were found on less than five occasions (Lundén *et al.*, 2003a).

4.1.3 Starter cultures (II)

Dry sausages were prepared using two starter cultures. Starter A (Imperial Meat Products, Lovendegem, Belgium) included *Kocuria varians, Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus carnosus* and *Staphylococcus xylosus,* and starter B (Danisco, Niebüll, Germany) included *Lactobacillus curvatus, L. lactis, S. carnosus* and *S. xylosus.*

The protective strain was *L. plantarum* DDEN 2205 (previously designated as WHE 92 (Ennahar *et al.*, 1996)). Concentrations of starter cultures were 10^6 cfu/g, and the protective strain was used at two different concentrations.

4.2 Detection, enumeration and identification of *L. monocytogenes* (I–V)

The detection of *L. monocytogenes* in study I was performed according to the ISO method. The detection of *L. monocytogenes* in study II was also performed according to the ISO method, but modified using a *Listeria monocytogenes* blood agar (LMBA) medium (Lab M, Lancashire, UK) instead of the Oxford medium. Samples were enriched in half-Fraser broth (Oxoid, Basingstoke, UK) for 24 h at 30°C and then in Fraser broth (Oxoid) for 48 h at 37°C. Samples were streaked onto two selective agar plates (Palcam (Oxoid), and Oxford (Oxoid) (I) or LMBA (II)) after both enrichments. Five typical colonies from each selective agar plate were cultured on blood agar, and *L. monocytogenes* was identified by a catalase test, gram staining (I, II) and motility testing at 25 °C and confirmed with an API Listeria kit (bioMérieux SA, Marcy l'Etoile, France) (I).

The quantitative *L. monocytogenes* analysis in study II was performed using the 9-tube Most Probable Number (MPN) method. For MPN analyses, 10 g of dry sausage, taken from the middle of the sausage, was mixed with 90 ml of peptone saline and blended using a laboratory blender (Stomacher 400, Seward Medical, London, England) for 1 min. Further decimal dilutions were made to obtain sampling sizes of 1, 0.1, 0.01, 0.001, and 0.0001 g. Enrichment, plating and identification were performed as in *L. monocytogenes* detection.

Determination in study III was performed by transferring 1 ml of the acid-treated suspension to 9 ml of phosphate-buffered saline at pH 7.2 or 1 ml from the heat-treated glass tube to 9 ml of phosphate-buffered saline at room temperature. Appropriate dilutions were plated onto duplicate tryptic soy agar plates (Difco, Sparks, MD, USA), and colonies were counted after 48-h incubation at 37°C. Cell reduction was determined by calculating the difference in cell counts between the 0-sample and the 2-hour sample in the acid challenge and the 0-sample and the 40-min sample in the heat challenge.

In study IV, to determine the amount of *L. monocytogenes* attached to the surfaces prior to the cleaning treatments, one contaminated piece of each material was dismantled with tweezers and transferred to a stomacher bag with 400 ml of degassed sterile water. Degassing was performed in a small ultrasonic bath (Branson 2100-DTH, 70 W, 47 kHz, 2.8 litres, Branson Ultrasonics, Soest, The Netherlands)

for 5 min. The pieces in the individual bags were treated with ultrasound at 25 °C for 10 min in the same ultrasonic bath to remove *L. monocytogenes* cells from the pieces. *L. monocytogenes* cells detached into the liquid were determined by spread plating serial dilutions in duplicate onto tryptic soy agar plates. The colonies were counted after aerobic incubation at 37 °C for 24 h and 48 h. The cleaned pieces were treated similarly after the cleaning treatment. The lowest level of detection was 1 cfu/ml.

The conveyor test piece in study V was removed from the laminate bag prior to ultrasonic cleaning treatment and four parallel swab samples were collected with cotton swabs by wiping an area of 10cm by 6 cm. The swabs were vortexed in peptone-saline solution, and the solution was diluted and plated. Palcam (Difco) and LMBA (LabM) were used in the plating. After ultrasonic cleaning treatment, the test piece was swabbed at four locations. These locations were similar in area (i.e. each 10 cm by 6 cm) to those swabbed before the treatment. The locations of the swabbed areas were selected to be close to but not the same as the areas swabbed prior to ultrasonic treatment to exclude the possible effect of location on the conveyor belt. The colonies were counted after aerobic incubation at 37 °C for 24 h and 48 h.

4.3 Preparation of *L. monocytogenes* inoculum (II–V)

L. monocytogenes strains in study II were grown in a brain heart infusion broth (Difco) for 18 h at 37 °C. Concentration levels were measured by optical densities, and equal amounts of each strain were inoculated in a total amount of 10-ml peptone (0.1%) saline (0.85%). The final concentration of *L. monocytogenes* in the sausage mass was 10^3 cfu/g.

L. monocytogenes isolates in study III were grown overnight on blood agar at 37°C. The culture was transferred to tryptic soy broth (Difco) and incubated overnight at 37°C. Further, 0.1 ml of culture was transferred to 10 ml of tryptic soy broth and incubated for 22 h at 37°C to receive a concentration of 10^8 to 10^9 cfu/ml.

In studies IV and V, the strains were grown in tryptic soy broth (Difco) at 37 °C for 24 h. After incubation, an inoculum of 100 µl of each strain was transferred to 8 ml of tryptic soy broth and the *L. monocytogenes* mixture was grown at 37 °C for 24 h. This mixture was used for inoculation in study IV. In study IV, an inoculum of 400 µl of each strain was mixed with organic soil after incubation. The concentration of each *L. monocytogenes* strain was 10^8 to 10^9 cfu/ml after incubation.

4.4 Preparation and sampling of dry sausage (II)

A dry sausage batch of 15 kg comprised of 5 kg of frozen lean pork, 5 kg of frozen pork back fat, 5 kg of fresh lean pork shoulder without rind, 2.8% salt, 170 ppm sodium nitrite, 1.1% milk protein, 0.7% dextrose, 0.35% spice mix and 0.04% sodium ascorbate. All ingredients were mixed in a bowl cutter, vacuum-packed and stuffed into fibrous collagen casings (Naturin, Weinheim, Germany). The sausages were

fermented and ripened according to the following processing parameters: the sausages were stored at 24 °C and 90% relative humidity for 72 h. On the first two days (at 24 h and 48 h), the sausages were smoked for 1 h. After 72 h, the sausages were dried at 14 °C and 75% relative humidity for 25 days to obtain the final product on day 28.

The sausages were weighed after stuffing (day 0) and on days 3, 7, 10, 14, 17 and 21 and pH was measured at 0, 24, 48 and 63 or 70 h after stuffing. For microbiological analyses, samples were taken after 4 h and on days 2, 7, 17 and 28 of ripening. Samples of control sausages without *L. plantarum* DDEN 2205 were taken immediately after stuffing. Raw materials were also sampled for the enumeration of *L. monocytogenes*. A total of 107 *L. monocytogenes* isolates were collected during or after ripening of the dry sausages.

4.5 AFLP (I)

4.5.1 DNA isolation of *Listeria* spp.

DNA was extracted according to the method of Pitcher *et al.* (1989), with slight modifications. The cells were lysed in Tris-HCl, EDTA buffer (TE) (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA)) containing lysozyme 25 mg/ml (Sigma, St. Louis, MO, USA), mutanolysin 245 U/ml (Sigma) and RNase 196 µg/ml (Sigma) at 37 °C for 2 h. DNA concentrations were measured using a BioPhotometer (Eppendorf, Hamburg, Germany). DNA samples were stored at -70 °C prior to AFLP analysis.

4.5.2 AFLP reaction and electrophoresis

The AFLP reactions were carried out as described earlier (Vos *et al.*, 1995, Thompson *et al.*, 2001), with a few modifications. Total genomic DNA (400 ng) was digested with 15 U *Hind*III (New England Biolabs, Beverly, MA, USA) and 15 U *Hpy*CH4IV (New England Biolabs), and ligated to restriction site-specific adapters. Samples were stored at -20 °C prior to polymerase chain reaction (PCR) amplification. DNA samples were diluted with sterile, distilled, deionised water and amplified by preselective PCR in a 20-µl reaction mixture containing 4 µl of diluted template DNA, 15 µl of Amplification Core Mix (Applied Biosystems, Foster City, CA, USA), 25 nM Hind-0 50 primer (Oligomer, Helsinki, Finland) and 125 nM Hpy-0 primer (Oligomer). After preselective amplification, the templates were diluted with sterile, distilled, deionised water and amplified by selective PCR in a 10-µl reaction mixture containing 1.5 µl of diluted template, 50 nM FAM-labelled Hind-A primer (Oligomer), 250 nM Hpy-A primer (Oligomer) and 7.5 µl of Amplification Core Mix. Preselective and selective PCR conditions were as described previously (Keto-Timonen *et al.*, 2006). The samples were prepared for capillary electrophoresis by adding 1 µl of the selective amplification product to 11.5 µl of Hi-Di Formamide (Applied Biosystems) and 0.5 µl of the internal size standard (GS-500 LIZ, Applied

Biosystems) to enable accurate band sizing. The mixture was denatured at 95 °C for 2 min. Denatured fragments were electrophoresed on POP-4 polymer (Applied Biosystems) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) in 1 x Genetic Analyzer Buffer with EDTA (Applied Biosystems). The electrophoresis conditions were 15 kV at 60 °C for 28 min. GeneScan 3.7 fragment analysis software (Applied Biosystems) was used for data pre-processing.

Reproducibility of the various different data sets was assessed by using *L. monocytogenes* strain ATCC 15313 as an internal reference, which underwent each step of the DNA extraction and AFLP analysis, thereby providing a standard for comparison between different data sets.

4.5.3 AFLP pattern analyses

The AFLP patterns were analysed using BioNumerics software version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between normalised AFLP patterns were calculated with the Pearson product-moment correlation coefficient. Clustering and construction of dendrograms were performed using the unweighted pair-group method with arithmetic averages.

4.6 Isolation of DNA and PFGE (I, II, III)

Typing of selected strains (n=48) in study I and isolates from meat-processing plants (n=367) in study III, and identification of inoculated *L. monocytogenes* strains in study II was performed using PFGE. DNA isolation and PFGE were performed as described by Autio *et al.* (1999) and Lundén *et al.* (2003a) with proteinase K (I, III) or with Pronase (Roche Diagnostics GmbH, Mannheim, Germany) instead of proteinase K (II). A single colony from blood agar was inoculated into brain heart infusion broth (Difco) and cells were harvested from 2 ml of the broth after overnight incubation at 37 °C. Cells were embedded into 2% low-melting-point agarose (InCert agarose; FMC Bioproducts, Rockland, ME, USA). The plugs were lysed for 3 h at 37°C and washed with ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 µg/ml proteinase K [Finnzymes]) at 50 °C for 1 h. After proteolysis, the plugs were washed in TE for 1 h, and proteinase K was inactivated using 1 mM Pefablock SC (Roche Diagnostics) at 37 °C overnight (I).

Restriction endonuclease digestion of agar-embedded DNA was performed using restriction enzymes *Apa*I (I, III) and *Asc*I (I, II, III) (New England Biolabs) as described by the manufacturer. DNA fragments were electrophoresed through 1.0% (wt/vol) agarose gel (SeaKem gold; FMC Bioproducts) in 0.5 Tris-borate-EDTA (45 mM Tris, 4.5 mM boric acid [pH 8.3] and 1 mM sodium EDTA) at 200 V at 14 °C in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden). The pulse time ramped from 1 s to 35 s for *Apa*I and *Asc*I for 18 h. A low-range PFG marker (New England Biolabs) was used for fragment size determination. The gels were stained with ethidium bromide and digitally photographed under ultraviolet

transillumination with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA).

PFGE patterns were analysed by BioNumerics software (Applied Maths) (I, II). The similarity analysis of the PFGE patterns was performed using the Dice coefficient (position tolerance 1%) (I).

4.7 Acid and heat treatments (III)

4.7.1 Acid treatment

Following incubation, 1 ml of the culture was transferred to 9 ml of tryptic soy broth (Difco), which was centrifuged at 10 000 g for two minutes (Sigma 3K30, Osterode, Germany). The pellet was resuspended in 10 ml of tryptic soy broth at pH 2.4. Hydrochloric acid (1 mol/l) was used to acidify the suspension. The cell concentration was determined immediately after resuspension (0-sample) and after two h. The test was performed at least twice.

4.7.2 Heat treatment

Two glass tubes with 4.95 ml of tryptic soy broth were placed in a water bath at 55°C (Haake V26, Karlsruhe, Germany) and one glass tube with 4.95 ml of tryptic soy broth at room temperature. One of the glass tubes in the water bath was used for the measuring suspension temperature (control tube). When the temperature of the tryptic soy broth measured within the control tube reached 55°C, 0.05 ml of the suspension containing *L. monocytogenes* cells was inoculated into the glass tube in the water bath and into the glass tube at room temperature. The suspension was pipetted below the surface of the tryptic soy broth to avoid splashes against the walls of the tube. The upper level of the suspension in the glass tube was below the water level in the bath. Cell concentration was determined immediately after pipetting from the suspension at room temperature (0-sample) and after 40 min from the glass tube in the water bath. The test was performed at least twice.

4.8 Ultrasonic cleaning (IV, V)

4.8.1 Conveyor belts

Conveyor belt materials of stainless steel mesh (cold-rolled, AISI 304, ESFO BV Transportbanden, Enschede, The Netherlands), polypropylene (Intralox 1100, Amsterdam, The Netherlands) and acetal (Ammeraal Beltec, Heerhugowaard, The Netherlands) were used in study IV. The belts were cut into pieces measuring 15cm by 10 cm.

The conveyor belt in study V was made of stainless steel (cold-rolled AISI 304; ESFO B.V.) with a loop to the ultrasound bath. The conveyor belt was 0.75 m wide

and 4.5 m long. Its velocity was adjustable, from 0.04 m/s to 0.11 m/s. For the study, pieces of conveyor belt were removed. Each piece was 0.75 m wide and 0.15 m long.

4.8.2 Organic soil

Organic soil was made according to Wirtanen *et al.* (1997) from cream, fat-free milk powder, modified starch and whey powder. The ingredients were mixed, boiled and distributed to bottles (IV). The organic soil used in the inoculation was 2% minced pork meat in a 0.1% peptone-saline solution (V). Both organic soils were sterilised (121.C, 15 min) and stored at 4 °C.

4.8.3 Inoculation of conveyor belt pieces

Conveyor belt pieces (IV) or one piece of conveyor belt (V), were washed and dried. Dry pieces were autoclaved to reach sterilisation (121.C, 1 bar, 15 min). The pieces were transferred aseptically to individual stomacher bags (Stomacher 400, Seward Medical) and 50 ml of organic dirt was added to the bags. The bags were left to stand for 30 min, after which 4 ml of *L. monocytogenes* suspension (10^8 cfu/ml) and 350 ml of tryptic soy broth were added (IV). The autoclaved piece was placed in a laminate bag (Amilen Polyamide/Polyethylen, Oy Suomen Union Verpackungs Ab, Östersundom, Finland), and 200 ml of organic dirt with *L. monocytogenes* and 1000 ml of 0.1% peptone-saline solution were inserted into the laminate bag (V). The conveyor belt pieces were incubated at 37 °C for 72 h to attach *L. monocytogenes* to the conveyor belt. After incubation, the conveyor belt pieces were rinsed with sterile water.

4.8.4 Ultrasonic cleaning

The stomacher bags in study IV were filled with 400 ml of 2% washing detergent, either potassium hydroxide (Solo VC27, Diversey Johnson, Sturtevant, WI, USA) or potassium hydroxide–sodium hydroxide (MP3-Mip SP, Ecolab, St. Paul, MN, USA). The concentration of potassium hydroxide in the first-mentioned washing detergent according to the manufacturer was 0.3% to 0.6%, and 0.1% to 0.3% in the latter washing detergent. The concentration of sodium hydroxide was 0.3% to 0.6%. The stomacher bags were degassed for 5 min in an ultrasonic bath (38 litres, FinnSonic M40, 600 W, 30 kHz, FinnSonic, Lahti, Finland). The conveyor belt pieces were then placed in the degassed bags, which were transferred back to the ultrasonic bath and attached to a frame. Cleaning was carried out at 30 °C and 45 °C for 30 s and 60 s with two detergents, resulting in eight different cleaning treatments. After the cleaning treatment, sterile water was used to rinse the detergent from the pieces, and the *L. monocytogenes* count was determined. Every treatment was performed with two replicate pieces, and each treatment was performed twice.

The ultrasonic bath (300 litres) in study V was fitted with four heating resistors and an ultrasound rod (2000 W, 25 kHz; Weber Ultrasonics, Clarkston, MI, USA).

The temperature of the liquid in the bath could be set from 10 °C to 90 °C. The ultrasonic bath was filled with 240 litres of water and a cleaning detergent (15% to 20% potassium hydroxide; Solo VC27, Johnson Diversey) with a concentration of 1.47% (working solution according to the manufacturer). The inoculated conveyor belt test piece was reattached to the conveyer belt. Cleaning with ultrasound was performed at 30 °C for 30 s and at 45 °C and 50 °C for 30, 20, 15 and 10 s with an ultrasonic frequency of 25 kHz. Cleaning without ultrasound was performed at 45 °C and 50 °C for 30 s. Each cleaning procedure was repeated three times.

4.9 Statistical analysis (I, III–V)

Associations between AFLP types and compartments or lines were determined by categorical analysis using the Chi-square test or Fisher's exact test if the expected values were less than five (I).

Statistical analysis of the differences in persistent and non-persistent strains to acid and heat stress was performed using the Mann-Whitney test, which is suitable for data that are not normally distributed. The acid resistance data were also analysed with the Student's t-test after exclusion of the exceptionally acid-susceptible strains (strains 12 and 18), which distorted the normal distribution (III).

The difference between *L. monocytogenes* cell counts before and after ultrasonic cleaning treatment was calculated, and the effect of the treatment was expressed as the logarithmic reduction. The differences in logarithmic reduction between different ultrasonic cleaning temperatures, times and detergents were analysed statistically with the paired t-test. Analysis of variance (ANOVA) was used with multiple comparisons Tukey test to analyse differences between conveyor belt materials (IV). An independent samples t-test assuming unequal variance was conducted to analyse the effect of ultrasound at different treatment temperatures. ANOVA was used to determine whether differences in reduction of *L. monocytogenes* counts under various temperature and time conditions were significant at the 0.05 level. When significant differences were obtained, Tamhane's T2 posthoc test was performed to determine which values differed from all other values (V). The statistical analyses were performed with the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc., Chicago, IL, USA) (III, IV, V) and Epi Info 6 (CDC, Atlanta, GA, USA) (I).

5 RESULTS

5.1 Contamination in a food-processing plant (I)

Overall, 319 *L. monocytogenes* isolates were collected during the eight-year surveillance period. The isolates were divided into four serotypes and 18 different AFLP types, five of which were persistent.

In compartment I, 177 isolates were recovered from the environment and from equipment in the compartment. The most contaminated lines were A and B, with 99 and 59 isolates recovered, respectively. Frequently contaminated sites were the conveyors located after the coolers and packaging machines. Isolates of compartment I were divided into three different AFLP types, which were all persistent. AFLP type A1 was repeatedly isolated and persisted throughout the eight-year surveillance, comprising 93% of the isolates in the compartment. Type A1 was also significantly more common ($P < 0.05$, Fisher exact test) in lines A and B than in lines C, D and E. AFLP type A11 was specific to compartment I, whereas AFLP type A1 was recovered from all three compartments in the plant, and A14 was isolated from compartments I and II. During the surveillance period, a new packaging machine was purchased for both lines A (in 2005) and B (in 2003). Soon after the new packaging machines were used, they were found to be contaminated with the predominant AFLP type, A1. The original packaging machine of line A was dismantled, cleaned and disinfected and then relocated to line E at the beginning of 2006. *L. monocytogenes* was not recovered from this packaging machine during the final seven months of the surveillance.

Overall 92 *L. monocytogenes*-positive samples were detected in compartment II. The isolates were divided into nine different AFLP types. All five non-persistent AFLP types and two out of four persistent AFLP types, A7 and A10, were specific to this compartment. AFLP type A7 was dominant and repeatedly isolated. The most contaminated sites were the conveyor before cooler, the cooler and the packaging machine. Only one *L. monocytogenes* isolate was recovered from compartment III during the 8-year surveillance. The organism was isolated from a movable container and belonged to the persistent AFLP type A1.

L. monocytogenes was isolated from raw materials on 18 occasions. These materials were grated cheese, sweet pepper and a cooked chicken product. The isolates belonged to eight different AFLP types, i.e. seven non-persistent and one persistent type. *L. monocytogenes* was isolated 31 times from non-heat-treated finished products produced in compartment II, and four non-persistent and three persistent AFLP types were observed. Predominant AFLP type A1 was not recovered from the finished products.

AFLP type A18 was isolated from both the raw materials (grated cheese) and the finished products produced in compartment II over a three-month period. The supplier of the cheese was replaced, after which AFLP type A18 was detected only once, from a sample batch of grated cheese purchased from a different supplier.

During reproducibility testing, the internal reference *L. monocytogenes* ATCC 15313 exhibited identical AFLP banding patterns during each run, based on fragment sizes. However, small variation in peak heights was observed. An 89% cut-off value was therefore selected to define the AFLP type of *L. monocytogenes strains* on the basis of a visual examination of the banding patterns of all strains studied.

5.2 Survival of *L. monocytogenes* strains in a dry sausage model (II)

L. monocytogenes was detected at the end of the ripening period in sausages without *L. plantarum* DDEN 2205, at 12–14 MPN/g, and in sausages with starter A and a low level of *L. plantarum* DDEN 2205, < 0.1–0.1 MPN/g. *L. monocytogenes* was not detected in the other two sausage treatments after 17 and 28 days of ripening. All sausages with *L. plantarum* DDEN 2205 contained less than 100 MPN/g at all sampling times.

Strain AT3E was recovered most frequently in sausages and was detected in all sausages at some point during ripening. Strain HR5E was detected only in sausages without *L. plantarum* DDEN 2205. HT4E was not detected in any of the samples, and the other inoculated strains (DCS 31, DCS 184) were detected from several sausages. The raw material was contaminated with an *L. monocytogenes* strain, and the strain was also detected in sausages during and at the end of ripening. PFGE revealed that the natural contaminant was the same genotype in all raw materials and sausages.

Weight losses over 21 days of ripening were 200–240 g (27–32%). The pH values of the sausages decreased from an initial 5.6–5.7 to 4.7–5.0 after 48 h of fermentation and to 4.5–4.7 after 63–70 h of fermentation.

5.3 Survival of *L. monocytogenes* in acid and heat stress (III)

The acid stress resulted in mean reductions of log 1.3 cfu/ml and log 1.8 cfu/ml in all persistent and in non-persistent strains, respectively. A significant difference was not detected between the acid tolerance of persistent and non-persistent strains by the non-parametric test (Mann-Whitney U-test, P = 0.078). However, the persistent strains exhibited significantly higher acid tolerance than the non-persistent strains after the exclusion of the exceptionally acid-susceptible strains (strains 12 and 18; Student's t-test, P = 0.02).

No significant differences in heat tolerance between persistent and non-persistent strains were observed. However, individual *L. monocytogenes* strains exhibited large differences in both acid and heat tolerance. The difference between strains in acid tolerance was over 6 log units. The highest difference observed between strains in heat resistance was 3 log units. The most acid-tolerant strains and the most heat-resistant strains were persistent, and the most acid-susceptible strains and the most heat-susceptible strains were non-persistent. Isolates representing identical RFLP

types exhibited different acid tolerance and partly different heat tolerance. The RFLP type of the *L. monocytogenes* isolates was obtained by combining both PFGE restriction enzyme profiles into one unique profile, resulting in 12 persistent RFLP types and 23 non-persistent RFLP types. Four types were represented by two or more strains in the study.

5.4 Survival of *L. monocytogenes* after ultrasonic treatment (IV, V)

L. monocytogenes attached to the tested materials in varying degrees in study IV. In most cases, the highest *L. monocytogenes* counts after inoculation were attained from stainless steel and acetal, and the lowest from polypropylene. On the other hand, *L. monocytogenes* was easily detached from stainless steel, and after the cleaning treatment no *L. monocytogenes* were detected in most treatments. A logarithmic reduction of less than 3 log units was only detected on polypropylene in three treatments. The logarithmic reductions on polypropylene were smaller than on stainless steel and on acetal in all treatments, and the difference between these materials and polypropylene was statistically significant (Tukey, $P < 0.001$). The logarithmic reductions in stainless steel were statistically greater than in acetal as well (Tukey, $P < 0.05$).

A higher temperature enhanced the efficiency of the ultrasonic cleaning treatments (IV, V). The difference in the logarithmic reductions between the 45-°C and 30-°C treatments was statistically significant (paired t-test, $P < 0.001$) (IV). The enhanced effect of a higher temperature was significant at all treatment temperatures (ANOVA, Tamhane's T2 posthoc test, $P < 0.01$). *L. monocytogenes* was effectively removed from the conveyor belt by ultrasonic cleaning treatment at 45 °C and 50 °C. At the lowest temperature (30 °C), the reduction with the longest time tested (30 s) was only 2.86 log units. In treatments at 45 °C and 50 °C without ultrasound the enhanced effect of the higher temperature was not observed (V).

The difference in the logarithmic reductions between 30- and 60-second treatments was not statistically significant (paired t-test, $P > 0.05$) (IV). The difference in logarithmic reduction was not significant between 10- to 30-second treatment times at 45 °C and 50 °C (ANOVA, Tamhane's T2 posthoc test, $P > 0.05$) (V).

The logarithmic reduction was higher in the cleaning treatments utilising potassium hydroxide detergent than those utilising combined potassium and sodium hydroxide detergent, and the difference was statistically significant (paired t-test, $P < 0.05$) (IV).

Ultrasonic cleaning was more effective at removing *L. monocytogenes* than high temperature and cleaning detergent without ultrasound. The difference between treatments with and without ultrasound was significant: 3.15 log units at 45 °C (independent samples t-test, $P < 0.001$) and 1.31 log units at 50 °C (independent samples t-test, $P < 0.05$).

6 DISCUSSION

6.1 Contamination in a food-processing plant (I)

Ready-to-eat foods have frequently been related to listeriosis (Pichler *et al.*, 2009; Smith *et al.*, 2011; de Castro *et al.*, 2012), and thus the whole food process from raw materials to the finished product requires thorough consideration of food safety risks. The contamination levels of three processing compartments in the ready-to-eat and ready-to-reheat food plant were considerably different. The equipment and environment of the post-heat-treatment area of compartment I were heavily contaminated with persistent *L. monocytogenes* strains. The flow of sporadic *L. monocytogenes* strains from raw materials seemed to be limited by heat treatment, because these strains were not recovered in the post-heat-treatment area. Product type, deficient cleaning routines and lack of compartmentalisation in compartment I may have predisposed the most contaminated lines, A and B, to persistent contamination. The conveyors were frequently contaminated with food residues, whereas the conveyors in lines C, D and E were not contaminated with product fillings. During high season, lines A and B were cleaned only every other day, impeding the efficient removal of the attached food residues, which may have contributed to the persistence of *L. monocytogenes*. Equipment with complex structures are known to favour the persistence of *L. monocytogenes* (Autio *et al.*, 1999; Miettinen *et al.*, 1999b*;* Lundén *et al.*, 2002: Lundén *et al.*, 2003a), and the proper cleaning of conveyors is often difficult, predisposing them to contamination (Lyytikäinen *et al.*, 2000; Rørvik, 2000). The AFLP type A1 was recovered as early as 1998. Although raw- and post-heat-treatment areas were later separated, the existing *L. monocytogenes* contamination could not be eliminated. The new packaging machines purchased for lines A and B were soon contaminated with *L. monocytogenes*, while the new packaging machine on line E remained free of contamination throughout the surveillance. Special attention should therefore be paid to sufficient compartmentalisation in compartments that produce cooked meals.

On the other hand, the largest variation in different AFLP types was detected in compartment II, with several persistent and non-persistent strains. Compartment II produced uncooked chilled food, and the high diversity of strains may result from the range of different raw materials, such as vegetables, meat products and dairy products, introducing the organism to the processing environment. AFLP type A18 was repeatedly discovered from grated cheese and finished products. After replacing the cheese supplier, type A18 was no longer detected in the products. AFLP type A7 was first recovered from the uncooked finished products and the cooler in compartment II, and later in grated cheese. In addition to cheese, sweet pepper was contaminated with *L. monocytogenes* on several occasions during the same year. The raw materials were not regularly tested for *L. monocytogenes*, and the initial contamination may have been introduced to the establishment through the raw materials. To avoid the contamination of finished products, the plant began to cook

the sweet pepper before its use in production. Special attention should be paid to the continuous quality control of raw ingredients in compartments that produce uncooked ready meals, and high-risk raw materials should be heat-treated before use in production.

The processing line in compartment II was reconstructed by straightening and shortening the line by removing the conveyors. The cooler, which had repeatedly been contaminated with *L. monocytogenes*, was removed from the line. These adjustments resulted in reduced prevalence rates of *L. monocytogenes* and persistent AFLP types A7 and A10 were not recovered in either the environmental or equipment samples after the implementation of structural changes. The contamination status can thus be improved by structural adjustments to the production line.

Only one environmental sample tested positive for *L. monocytogenes* in compartment III, producing heat-treated ready-to-eat products, and no contamination of equipment or products was observed. The raw materials used were flour and egg, which are not typically contaminated with *L. monocytogenes*. Furthermore, the raw and post-heat-treatment areas were clearly separated, and uncooked products had never been produced in the area. The only positive sample contained a persistent AFLP type A1 and was detected on a movable container that had been transferred to the compartment without cleaning. The transfer of persistent *L. monocytogenes* contamination has been reported to occur between food-processing plants with a dicing machine (Lundén *et al.*, 2002). Thorough cleaning and disinfection of all machines and equipment before relocation to another compartment or processing plant are therefore essential.

Each compartment of the chilled food processing plant had a unique contamination pattern, which was demonstrated by the sustained surveillance and utilisation of the genotyping method. AFLP analysis proved to be an efficient tool for contamination route studies.

6.2 Survival of *L. monocytogenes* strains in a dry sausage model (II)

The decrease in pH to 4.5–4.7 during ripening was not solely sufficient to eliminate *L. monocytogenes,* because of the ability of *L. monocytogenes* to grow in pH as low as 4.0 (George *et al.*, 1988; Lado & Yousef, 2007). The other hurdles in dry sausages, such as low water activity and pediocin produced by *L. plantarum* DDEN 2205, eliminated the bacterium. The conditions during fermentation and ripening can adversely affect the production of bacteriocin. *L. plantarum* WHE 92 consistently produces pediocin in culture broths between pH 4.0 and 6.0 (Ennahar *et al.*, 1996), and thus the pH in dry sausages did not prevent the production of pediocin.

L. monocytogenes was detected in lower levels in sausages with the pediocin-producing strain *L. plantarum* DDEN 2205 than in sausages without the strain. *L. monocytogenes* decreased more rapidly in sausages with starter A when the concentration of the pediocin-producing culture was high. Starter A with low

concentrations of a pediocin-producing strain did not result in sausages without *L. monocytogenes* after 28 days of ripening, but starter B with the same concentration of the pediocin-producing strain resulted in sausages without *L. monocytogenes* already after two days of ripening. Starter cultures contained different lactic acid bacteria, which probably explain the difference in survival of *L. monocytogenes*. Starter A, with the high concentration of pediocin-producing *L. plantarum* DDEN 2205, and starter B, with the low concentration of *L. plantarum* DDEN 2205, seem to be effective combinations for eliminating *L. monocytogenes* from dry sausages during ripening.

The survival of *L. monocytogenes* during sausage ripening differed among the strains. Strain HT4E was not detected in any of the sausages, and HR5E was inhibited in all sausages with the pediocin-producing *L. plantarum* strain. Strain AT3E was detected in all sausages at some point during ripening, and strains DCS148 and DCS31 were detected in all sausages, except those with the highest concentration of protective culture. Altogether, strains DCS31 and AT3E were detected in sausages more often than other strains. Manifold differences occur in sensitivity to pediocin between *L. monocytogenes* strains (Ennahar *et al.*, 2000b; Katla *et al.*, 2003), thus emphasising the necessity to use several strains in inoculation studies. The natural *L. monocytogenes* contaminant was isolated in all sausages except those without a pediocin-producing strain. This *L. monocytogenes* strain was probably more resistant to pediocin, and a better competitor than the inoculated strains in sausages with *L. plantarum* DDEN 2205. The hurdle concept for dry sausage making should be effective against the most resistant strains of *L. monocytogenes*.

During ripening, all sausages reached the EU-regulated limit (EU, 2005) of *L. monocytogenes* for ready-to-eat foods such as dry sausages. The *L. monocytogenes* amount decreased throughout ripening and reached <100 MPN/g at 17 days in all sausages.

Bacteriocin-producing starter cultures such as *L. plantarum* provide an appealing hurdle in dry sausage processing assisting in the elimination of *L. monocytogenes* contamination. Possible variations in susceptibility and development of resistance in *L. monocytogenes* strains can create problems when other hurdles are not used. Starter cultures with different bacteriocin-producing strains or competitive strains might prevent the development of resistant *L. monocytogenes* strains.

6.3 Survival of *L. monocytogenes* in acid and heat stress (III)

Of the 40 *L. monocytogenes* strains originating from meat establishments and challenged with acidic conditions, the persistent strains exhibited higher tolerance than non-persistent strains. A large variation in tolerance to acid stress (6 log units) was found between all strains. Such a difference in acid tolerance may affect the survival of the strains in food establishment environments. Persistent strains did not exhibit higher tolerance to heat stress than non-persistent strains. Regardless of the persistence, heat tolerance was also highly variable between strains, by much as 3 log

units. Although persistence did not correlate with heat tolerance, the most acid- and heat-sensitive strains were non-persistent.

The persistent *L. monocytogenes* contamination of a food establishment is caused by several issues. The properties of a strain, such as adherence to surfaces (Lundén *et al.*, 2000; Beresford *et al.*, 2001), biofilm formation (Norwood & Gilmour, 2001) and acid and heat tolerance have an effect on the survival and persistent contamination of *L. monocytogenes* in food establishments. Cleaning and disinfection will probably eliminate the highly sensitive *L. monocytogenes* strains more efficiently. The conditions in food establishments, such as hard-to-clean places in the environment, the structure of the processing lines and the equipment where *L. monocytogenes* can persist, influence the persistence of *L. monocytogenes* strains in food establishments (Lundén *et al.*, 2003a; Carpentier & Cerf, 2011).

The *L. monocytogenes* strains belonging to the same RFLP type were observed to differ in acid and heat tolerance, demonstrating that strains within an RFLP type can exhibit different phenotypes. These strains may have differences in the genome not revealed in the RFLP typing, therefore expressing different stress tolerance. The possible earlier adaptation to acid or heat also results in increased tolerance through adaptive responses (O'Driscoll *et al.*, 1996). Persistent contamination is therefore the sum of several interacting factors, and different acid and heat tolerance levels should be taken into account in food establishments when designing sanitation procedures to also eliminate those *L. monocytogenes* strains with the highest tolerance to acid and heat.

6.4 Ultrasonic treatment in the prevention of *L. monocytogenes* (IV, V)

Despite the high amount of *L. monocytogenes* in stainless steel prior to the washing treatment, the amount was either small or undetectable after washing. All tested ultrasonic cleanings were efficient in cleaning the conveyor made of stainless steel. The cleaning treatment was considered effective if *L. monocytogenes* reduction after treatment was at least 3 log units (Mosteller & Bishop, 1993). *L. monocytogenes* contamination was present on polypropylene after each cleaning treatment, and not all the tested ultrasonic cleanings were effective. The cleaning efficacy of *L. monocytogenes* was significantly greater in stainless steel than in plastic materials, which can be explained by the hardness of steel. Ultrasonic cleaning is more efficient on hard surfaces than on soft materials. The surface hardness of stainless steel is greater than that of plastic materials, and also the attachment of *L. monocytogenes* to stainless steel is weaker than it is to polymeric materials (Midelet & Carpentier, 2002; Veluz *et al.*, 2012). Acetal has a greater surface hardness compared with polypropylene, which can explain the greater logarithmic reduction obtained in acetal than in polypropylene.

The higher cleaning temperature (45 °C) resulted in statistically significantly greater cell count reductions in the plastic conveyor materials than did the lower cleaning temperature (30 °C). Low temperature (30 °C) combined with the

ultrasound and detergent failed to have the desired effect. At low temperatures, the fat from organic dirt remained adhered to the conveyor belt, and such organic matter can protect *L. monocytogenes* cells. At low temperatures it is difficult to remove fat from surfaces, even when combined with ultrasound. Higher temperatures significantly enhanced the cleaning result. Treatments at 45 °C and 50 °C resulted in a reduction of more than 3 log units at all tested treatment times, indicating the importance of thermal energy.

The cleaning results were similar with 30- to 45-second cleaning times. Ultrasonic cleaning at 45 °C was more effective when treatment time was increased from 10 to 30 s, but the reduction of *L. monocytogenes* at 50 °C was more than 5 log units after only 10 seconds. An improvement in the effectiveness of ultrasonic treatments thus appears to be achieved by increasing treatment temperature rather than by increasing treatment time. Ultrasonic treatment has been found to dislodge 83% of a biofilm on stainless steel in 10 s (Oulahal-Lagsir *et al.*, 2000). In a later study, a two-second treatment of conveyor belts with steam at 95 °C and ultrasound resulted in a significant reduction of the aerobic counts by 2.5 to 3.16 cfu/50 cm^2, and *Enterobacteriaceae* spp. counts were reduced to a level below the detection limit (Musavian *et al.*, 2015). Ultrasonic cleaning is effective even with short treatment times, which facilitates the application of ultrasonic cleaning of conveyors in the food-processing environment. It is not always possible to routinely dismantle the conveyors during everyday cleaning, thus reducing the efficacy of the cleaning. The difference between detergents used in ultrasonic cleaning was statistically significant, but small, making both detergents usable for ultrasonic cleaning.

The elimination of *L. monocytogenes* from the conveyor belt was significantly greater with ultrasound than without it. Thermal energy and the detergent alone did not result in a satisfactory result, but when these conditions were combined with ultrasound the elimination of *L. monocytogenes* was efficient. In later study, Axelsson *et al.* (2013), also found that the amount of removed bacterial cells increased with combined ultrasound treatments by a 1- to 2-log reduction compared with washing with detergent alone. Ultrasound reaches areas that are otherwise difficult to clean, and the stainless steel conveyor belt with multiple joints and hard-to-reach places can therefore be adequately cleaned. The evidence of erosion on stainless steel due to the use of ultrasound has been reported, and these effects should therefore be considered in the ultrasonic cleaning of food-processing environments (Chiu *et al.,* 2005).

Ultrasonic cleaning detached *L. monocytogenes* and organic dirt quickly and efficiently from the conveyor belts. Short ultrasonic cleaning treatment may provide a new possibility for cleaning conveyor belts that are difficult to clean using conventional methods.

7 CONCLUSIONS

1. The contamination status of the processing compartments of the ready-to-eat and ready-to-reheat food plant was considerably different. The product type, deficient cleaning routines and lack of compartmentalisation predisposed the ready-to-eat food-processing lines to persistent contamination. The largest variation in different AFLP types was detected in the uncooked chilled food compartment, as a result from the wide range of different raw materials. Continuous quality control of raw ingredients should be in place in uncooked chilled food establishments. The reconstruction of a processing line resulted in reduced prevalence rates of *L. monocytogenes* and the eradication of two persistent AFLP types. Contamination status can thus be improved by structural adjustments to the processing line. AFLP analysis proved to be an efficient tool for contamination route studies.

2. Low water activity and pediocin produced by *L. plantarum* DDEN 2205 eliminated *L. monocytogenes* in dry sausages during ripening. Starter A with a high concentration and starter B with a low concentration of *L. plantarum* DDEN 2205 seem to be effective combinations for eliminating *L. monocytogenes*. The survival of *L. monocytogenes* differed between the strains. Bacteriocin-producing starter cultures such as *L. plantarum,* provide an appealing hurdle in dry sausage processing for the elimination of *L. monocytogenes* contamination, when used in combination with other hurdles such as low water activity and low pH.

3. The persistent *L. monocytogenes* strains exhibited higher acid tolerance than non-persistent strains, although this difference remained small. Persistent strains did not exhibit higher tolerance to heat stress than non-persistent strains. Acid and heat tolerance were highly variable between all *L. monocytogenes* strains, and the most acid- and heat-sensitive strains were non-persistent. These differences affect the survival of bacterial strains in food establishments. Variable acid and heat tolerances should be taken into account during sanitation procedures to also eliminate those *L. monocytogenes* strains with the highest acid and heat tolerance.

4. The ultrasonic cleaning detached *L. monocytogenes* and organic dirt quickly and efficiently from the conveyor belts. Cleaning efficacy was greater in stainless steel than in plastic materials, due to the surface hardness of the steel. The effectiveness of ultrasonic cleaning treatments improved with increasing temperature rather than time. Ultrasonic cleaning was not effective with the lowest temperature used (30 °C). The elimination of *L. monocytogenes* from the conveyor belt using ultrasound was significantly greater than without ultrasound. Ultrasonic cleaning is effective even with

short treatment times, which provides possibilities for the application of ultrasonic cleaning of conveyors in the food-processing environment.

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