MOLECULAR EPIDEMIOLOGY AND HEAT RESISTANCE OF
LISTERIA MONOCYTOGENES IN
MEAT PRODUCTS AND MEAT-PROCESSING PLANTS AND
LISTERIOSIS IN LATVIA

AIVARS BĒRZIŅŠ

ACADEMIC DISSERTATION

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University of Helsinki, for public examination in Auditorium Arppeanum, Snellmaninkatu 3,
Helsinki, on 12th November, 2010 at 12 noon.

Helsinki 2010
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>ALOA</td>
<td>agar <em>Listeria</em> according to Ottaviani and Agosti</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APHA</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>a&lt;sub&gt;w&lt;/sub&gt;</td>
<td>water activity</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>D-value</td>
<td>decimal reduction time (in minutes)</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELFA</td>
<td>enzyme-linked immunofluorescence assay</td>
</tr>
<tr>
<td>ES</td>
<td>EDTA-sodium lauroyl sarcosine buffer</td>
</tr>
<tr>
<td>ESP</td>
<td>EDTA-sodium lauroyl sarcosine proteinase K solution</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>fluoriscence in situ hybridization</td>
</tr>
<tr>
<td>FSIS</td>
<td>Food Safety and Inspection Service</td>
</tr>
<tr>
<td>IDF</td>
<td>International Dairy Federation</td>
</tr>
<tr>
<td>IMS</td>
<td>immunomagnetic separation</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization of Standardization</td>
</tr>
<tr>
<td>LMBA</td>
<td><em>Listeria monocytogenes</em> blood agar</td>
</tr>
<tr>
<td>MEE</td>
<td>multilocus enzyme electrophoresis</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
</tr>
<tr>
<td>MVLST</td>
<td>multi-virulence-locus sequence typing</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PALCAM</td>
<td>selective plating agar containing polymyxin B, acriflavin, lithium chloride, ceftazidime, aesculin and mannitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PIV</td>
<td>Tris-NaCl buffer, “Pett IV solution”</td>
</tr>
<tr>
<td>ppm</td>
<td>parts-per-million</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplification of polymorphic DNA</td>
</tr>
<tr>
<td>REA</td>
<td>restriction endonuclease analysis</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RTE</td>
<td>ready-to-eat</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SPHA</td>
<td>State Public Health Agency</td>
</tr>
<tr>
<td>TDT</td>
<td>thermal death time</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-HCl, EDTA buffer</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptic soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>TSBYE</td>
<td>tryptic soy broth with yeast extract</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair-group method using arithmetic averages</td>
</tr>
<tr>
<td>YE</td>
<td>yeast extract</td>
</tr>
<tr>
<td>z-value</td>
<td>temperature (in degrees Celsius) required for the thermal destruction curve to move one log cycle</td>
</tr>
</tbody>
</table>
ABSTRACT

The prevalence, contamination and heat resistance of *Listeria monocytogenes* were investigated in meat products and meat-processing plants. Moreover, trends of human listeriosis in Latvia were studied over a 10-year period from 1998 to 2007.

A high prevalence (40%) of *L. monocytogenes* was found in cold-smoked meat products compared with other heat-treated ready-to-eat meat products (0.7%) available in retail markets in Latvia. Pulsed-field gel electrophoresis (PFGE) and serotyping were applied to analyse the diversity of *L. monocytogenes* isolates present in ready-to-eat (RTE) meat products and meat-processing plants. A high genetic diversity was seen among *L. monocytogenes* isolates from cold-smoked meat products, suggesting the existence of various sources of contamination at different production stages in the meat-processing environment.

The manufacture of cold-smoked meat products involves no processing steps to eliminate *L. monocytogenes*, thus, contamination of the raw meat and contamination during processing can both contribute to *L. monocytogenes* in the finished product. Logistic multivariable regression model was successfully applied to identify the main factors associated with *L. monocytogenes* contamination during the manufacturing of cold-smoked pork products. Meat brining by injections was a significant factor (odds ratio 10.66; \( P < 0.05 \)) for contamination of products with *L. monocytogenes*. Of the cold-smoked meat-processing plant environmental samples, most contaminated sites were associated with brining machine and brining area. Long cold-smoking times (\( \geq 12 \) h) also had a significant predictive value (odds ratio 24.38; \( P < 0.014 \)) for a sample testing positive for *L. monocytogenes*.

A genetically diverse population of *L. monocytogenes* entered the meat-processing plant, where only some of the strains colonized and established a persistent microbial community within the plant over a 5-year period. *L. monocytogenes* PFGE types belonging to serotypes 1/2a and 4b were isolated from imported, defrosted, raw pork from Germany and Belgium in meat-processing plant B. In total, two *L. monocytogenes* PFGE types originating from raw meat were found also in finished RTE cold-smoked pork products, whereas one PFGE type was recovered later only from the meat-processing environment. One of the *L. monocytogenes* PFGE types, belonging to serotype 1/2c, was isolated from RTE cold-smoked meat products.
and from the feeding teeth of the brining machine, thus showing that improper cleaning, disinfection and poor hygiene design of the brining machine may cause *L. monocytogenes* contamination over time.

Post-package pasteurization of high- and low-fat content cooked sausages at temperatures higher than 55°C was found to be an effective method of post-process thermal treatment to reduce contamination of *L. monocytogenes*. However, heating to 55°C, 60°C and 62.5°C may not be practical in the meat industry because the process takes too long to reach a 3-log reduction. The formulation of high-fat content RTE cooked sausages may require modification to maintain product quality.

During the 10-year study period the overall incidence of listeriosis in Latvia was 0.4 per 100 000 population, with the highest incidences recorded in 2000 and 2002 (1.5 and 0.7 per 100 000 population, respectively). The highest incidence of listeriosis in Latvia was observed in 2000, which significantly exceeded incidence levels in all Baltic and Nordic countries, and was the highest among all EU member states during the same period. A marked clustering of human listeriosis cases was observed from September to December 2000, possibly indicating one large outbreak. The lack of serotyping and molecular typing methods for subtyping of *L. monocytogenes* isolates in the present surveillance system is one of the main reasons why there have been no officially documented listeriosis outbreaks in Latvia to date. Measures to allow the application of appropriate actions at the food industry level need to be implemented to prevent or significantly reduce the real burden of foodborne listeriosis in Latvia.
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by Roman numerals I to V:


The original articles have been reprinted with the kind permission from Journal of Food Protection (I, III), Elsevier (II, IV) and Schlütersche Verlagsgesellschaft mbH & Co. KG (V).
1. INTRODUCTION

*Listeria monocytogenes* is a foodborne pathogen that causes listeriosis in humans and animals. Although foodborne listeriosis is a relatively rare infectious disease, it is often associated with severe illness, mainly in unborn children, infants and the elderly, as well as in immunocompromised persons. Moreover, the disease is characterized by a high mortality (20-30%). *L. monocytogenes* is still among the most important causes of death from foodborne infections in developed countries. Only within the last 25 years has food been recognized as a most important mode of transmission for listeriosis in humans.

During the last two decades several notable outbreaks of listeriosis worldwide have been linked to different foods, including fish, meat, dairy and plant products. Overall, 98 listeriosis cases have been identified in Latvia from 1998 to 2009; however, no clear source of the infection has thus far been determined. Although listeriosis in Latvia is a notifiable infectious disease, the lack of continuous collection and subtyping of *L. monocytogenes* food and clinical isolates limits identification of listeriosis outbreaks, delaying the appropriate actions at the food industry level being taken to prevent or significantly reduce the real burden of listeriosis.

Delicatessen meat, fish and dairy products, ready-to-eat (RTE) foods and foods with extended shelf-lives are commonly consumed in modern society. Moreover, such foods with extended shelf-lives may support the survival and growth of *L. monocytogenes* in high numbers, thus leading to listeriosis. *L. monocytogenes* is commonly found in the food-processing environments, including different surfaces and equipment. Thus, special control measures must be taken by the food industry to minimize the prevalence in finished RTE products and in the food-processing environment. Moreover, it is important to trace the sources of *L. monocytogenes* in food-processing plants and the routes of transmission to prevent any persistent or non-persistent contamination.
2. REVIEW OF THE LITERATURE

2.1. Listeria monocytogenes and listeriosis

2.1.1. The genus Listeria and L. monocytogenes

A Gram-positive bacterium, *Listeria monocytogenes* was found for the first time in 1911 in rabbits in Sweden (Hülphers 1911). Murray *et al.* (1926) was the first to describe this bacterium after epidemics in rabbits and guinea pigs in research laboratories of Cambridge, UK. A year later, a similar disease in rabbits caused by the Gram-positive bacillus was described in South Africa by Pirie (1927), who later, in 1940, gave the genus the name *Listeria*. The disease was characterized by its ability to cause large mononuclear leucocytosis in laboratory animals; thus, Murray *et al.* (1926) suggested naming the bacterium *Bacterium monocytogenes*. Based on previous studies, the nomenclature *Listeria monocytogenes* was applied (Pirie 1940).

*Listeria monocytogenes* is a small, regular Gram-positive rod with rounded ends, 0.5 µm in a diameter and 1–2 µm in length, a facultative anaerobe that is catalase-positive and oxidase-negative. *Listeria* cells can be found as single units, in short chains or in Y and V forms. The forms of the cells may vary from short to coccoid rods. They are motile at 20-25°C due to flagellar activity and do not form any capsules or spores (Seeliger and Jones 1986, Rocourt and Buchrieser 2007).

The genus *Listeria* belongs to the family *Listeriaceae*, includes alongside the genus *Brochothrix*. Modern molecular biology methods and phylogenetic analysis have been widely used to study diversity of the genus *Listeria*, which currently contains six species: *L. monocytogenes*, *L. innocua*, *L. ivanovii* subsp. *londoniensis*, subsp. *ivanovii*, *L. seeligeri*, *L. grayi* and *L. welshimeri* (Feresu and Jones 1988, Boerlin *et al.* 1992, Rocourt *et al.* 1992, 2007, Graham *et al.* 1997). Moreover, two novel *Listeria* species: *Listeria marthii* sp. nov. and *Listeria rocourtiae* sp. nov., have recently been proposed to originate from the natural environment of lakes in New York, USA, and from Austrian pre-cut lettuce, respectively.
(Graves et al. 2009, Leclercq et al. 2009). Up to now L. monocytogenes and L. ivanovii were considered pathogenic species, causing disease in humans and animals (Slutsker et al. 1999, Vazquez-Boland et al. 2001, Rocourt and Buchrieser 2007, Wesley 2007). Recently, the genome sequences of L. monocytogenes (2,944,528 base pairs) and the non-pathogenic species L. innocua (3,011,209) have been compared. Large numbers of predicted genes encoding surface and secreted proteins, transporters and transcriptional regulators, consistent with the ability of both species to adapt to diverse environments, were found (Glaser et al. 2001).

2.1.2. Growth conditions and thermal resistance

L. monocytogenes is a psychrotrophic bacterium with the ability to grow at low temperatures. Slow growth of some L. monocytogenes strains was observed at a temperature of -1.5°C to -0.1°C (Junttila et al. 1988, Petran et al. 1989, Walker et al. 1990, Hudson et al. 1994). In other studies, the minimum growth temperature for L. monocytogenes under optimum pH and a_w varied between -1.6°C and 0.41°C and depended strongly on medium pH (Tienungoon et al. 2000). The ability to grow at low temperatures may vary among different L. monocytogenes strains (Begot et al. 1997, Lado et al. 2007, Byelashov et al. 2009, Chan et al. 2009).

The survival of L. monocytogenes cells during storage at frozen temperatures may vary with the temperature and freezing rate. Lower freezing temperature and rapid freezing rate were most favourable for L. monocytogenes survival, while a slow freezing at -18°C had a more lethal effect on cell survival. Freezing and storage at -18°C may inactivate 1 to 2 logs and injure >50% of the bacterial population (El-Kest et al. 1991, Lado et al. 2007). Palumbo et al. (1991) showed that low pH of foods (pH 4.7) may increase death and injury of L. monocytogenes during storage at frozen temperatures.

Growth of L. monocytogenes has been reported at pH values between 4.0 and 9.6 (Farber et al. 1989, 2007, Petran et al. 1989, Phan-Thanh et al. 1998, 2000). Although growth of L. monocytogenes below pH 4.0 has not been described, Parish and Higgins (1989) showed that some L. monocytogenes strains may survive up to four days in orange juices with pH 3.6 and more than 365 days in cheddar cheeses with pH 5.1, stored at 4°C and 6°C, respectively.
Moreover, the effect of pH on *L. monocytogenes* viability depends on other intrinsic or extrinsic factors and on physiological status of the cells. Refrigeration temperatures may inhibit the growth of *L. monocytogenes*, but favours its survival in acidic foods (Lado and Yousef 2007). Johnson *et al.* (1988) confirmed that *L. monocytogenes* may survive well, for example, in hard salami with a pH of 4.4 during storage at refrigeration temperatures. *L. monocytogenes* has the ability to adapt to an the environment with a low pH or to induce acid tolerance (O’Driscoll *et al.* 1996, Dykes *et al.* 2000, Caggia *et al.* 2009). Prolonged survival of acid-adapted *L. monocytogenes* cells compared with unadapted cells was confirmed in cottage cheese (pH 4.7) and salad dressings (pH 3.0) stored at refrigeration temperatures (Gahan *et al.* 1996, Lado and Yousef 2007). Skandamis *et al.* (2008) concluded that combinations of sublethal hurdles may affect *L. monocytogenes* acid and heat tolerance, especially in acidic environments with mild heating or in low moisture environments.

*L. monocytogenes* is able to multiply at *a_ω* values as low as 0.90 to 0.92 (Nolan *et al.* 1992). Although *L. monocytogenes* does not grow at *a_ω* < 0.90, it may survive in such environments for long periods. *L. monocytogenes* may tolerate high salt concentrations (23.8% NaCl) and survive in commercial cheese brines stored at 4°C for > 250 days (Larson *et al.* 1999). Survival of *L. monocytogenes* in the presence of > 12% NaCl decreases with increasing salt concentration or incubation temperature, for example, presence of 16% NaCl was listeriostatic for at least 33 days at ≤ 4°C and the presence of 26% NaCl decreased *Listeria* populations 2 and 3.5 logs at 0°C and 4°C for the same storage period (Sorrells *et al.* 1990, Lado and Yousef 2007).


Heat resistance of *L. monocytogenes* is notably dependant on strain variation, previous growth conditions and exposure to heat, acid and other stresses (Bunčić *et al.* 1991b, 2001, Juneja *et al.*
Heating *L. monocytogenes* at temperatures >56°C may cause ribosomal damage, protein unfolding and denaturation, and enzyme inactivation (Bunduki *et al.* 1995, Lado and Yousef 2007).

The time required to inactivate one log or 90% of the microbial population at a given temperature (D-value) and the temperature required for the thermal destruction curve to move one log cycle (z-value) have been used to describe the heat resistance of a certain strain (Heldman and Newsome 2003). Thermal inactivation rates of *L. monocytogenes* at any given temperature varied considerably among studies and when the pathogen was heated in different media (Heldman and Newsome 2003, Lado and Yousef 2007). Doyle *et al.* (2001) reviewed a large number of heat inactivation studies of *L. monocytogenes* in different foods and culture media, and concluded that heat resistance data for different strains demonstrates significant variability. Strains of the *L. monocytogenes* serovar 4b tend to be slightly more heat-resistant than those of serovar 1/2a (Bunčić *et al.* 2001). However, El-Shenawy *et al.* (1989) found that some of the most commonly studied *L. monocytogenes* strains, e.g. Scott A (serovar 4b), had lower heat resistance than V7 (serovar 1a). D- and z-values for *L. monocytogenes* strains in different meats and meat products may vary significantly (Table 1). *L. monocytogenes* can be eliminated by thermal processing, but the effectiveness of different heating regimens is affected by meat fat composition, heat source, rate of heating, and exposure of the bacteria to stresses such as acid, heat shock and preservatives (Doyle *et al.* 2001). Data from experiments with cooked versus raw beef and chicken suggested that *L. monocytogenes* can be more heat-resistant in cooked meat (Gaze *et al.* 1989). This may have implications for some post-packaging pasteurization processes (Doyle *et al.* 2001). D$_{62^\circ C}$ values for *L. monocytogenes* inoculated into fresh pork were about 1.5 times greater than those for cells inoculated into 3-month-old ground pork (Kim *et al.* 1994). Ground beef with high fat content (30.5%) was more protective of *L. monocytogenes* at 57.2°C and 62.8°C than low-fat (2%) beef (Fain *et al.* 1991). Kim *et al.* (1994) showed that slow heating (1.3°C/min) of inoculated ground pork samples allowed survival of more *L. monocytogenes* cells than rapid heating (8°C/min). Overall, heat shock may increase thermotolerance of *L. monocytogenes* in foods, which may be a concern for meat products that are heated slowly (Doyle *et al.* 2001).
<table>
<thead>
<tr>
<th>Product</th>
<th>Conditions</th>
<th>D-values (min) at temperature (°C) of</th>
<th>z-value</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>55</td>
<td>57.2</td>
</tr>
<tr>
<td>Beef</td>
<td>Raw</td>
<td>85</td>
<td>21</td>
<td>3.8</td>
</tr>
<tr>
<td>Beef, &lt;7% fat</td>
<td>Cooked, Scott A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, 20% fat</td>
<td>Inoculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, 3.1% fat</td>
<td>pH 7.2, 2.4% lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, 3.1% fat</td>
<td>pH 7.2, 4.8% lactate</td>
<td></td>
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<td></td>
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<tr>
<td>Beef, 1.5 M NaCl</td>
<td>Growth 0.09 M NaCl</td>
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<tr>
<td>Beef, 1.5 M NaCl</td>
<td>Growth 1.5M NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ground beef</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pork</td>
<td>Heating 2.2°C/ min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pork</td>
<td>Heating 8.0°C/ min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sausage</td>
<td>Pork only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sausage</td>
<td>23% beef, 77% pork</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ground pork</td>
<td></td>
<td>109</td>
<td>9.8</td>
<td>1.14</td>
</tr>
<tr>
<td>Chicken</td>
<td>Thigh/ leg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>Breast, raw</td>
<td>100</td>
<td>13</td>
<td>8.7</td>
</tr>
<tr>
<td>Cooked sausages</td>
<td>Sodium lactate 2.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>Cooked, Scott A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ham</td>
<td>No heat shock</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ham</td>
<td>Heat shocked</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sausage</td>
<td>Summer sausage</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.3. Isolation, identification and quantification

“The isolation of the infecting organism is not easy, and we found this to remain true even after we had established the cause of the disease”, stated Murray et al. (1926) when *L. monocytogenes* was firstly described. In the 1940s, the cold-enrichment procedure based on *L. monocytogenes* ability to grow in low temperatures was introduced as an alternative method to isolate *L. monocytogenes* from highly contaminated samples (Gray et al. 1948).

Until now, cold enrichment, selective enrichment, direct plating and several rapid methods have been used in various combinations to detect *L. monocytogenes* in foods and in clinical or environmental samples (Donnelly and Nyachuba 2007). The current *L. monocytogenes* isolation methods are based on less time-consuming procedures, involving selective enrichment and selective plating. Standard methods for detection and enumeration of *L. monocytogenes* issued by FDA, IDF and ISO are widely used by food and environmental laboratories worldwide (Anonymous 1995, 1996a, 1996b, Anonymous 2003a, 2004a, 2004b, Allerberger 2003). Since the 1950’s potassium tellurite, lithium chloride, nalidixic acid, acriflavine, polymyxin B, moxalactam and ceftazidime have been the most recognized selective agents in isolation of *Listeria* species (Donnelly and Nyachuba 2007). In addition, blood or chromogens can be used as selective agents or indicator substrates to differentiate *Listeria* spp. (Anonymous 1995, 1996a, 2003a, 2004a, Leclercq 2004, Greenwood et al. 2005).

Modern conventional isolation methods involve either a one- or two-step selective enrichment, followed by plating on selective plating media and incubating at 30°C or 37°C for 1-2 days (Anonymous 1995, 1996a). Quantitative and semi-quantitative methods are used for enumeration of *L. monocytogenes* in foods, involving a 1-h resuscitation in buffer media and surface plating on selective agar plates (Anonymous 1996b, 2004b).

The identification and confirmation of *L. monocytogenes* includes Gram-staining, catalase reaction, oxidase reaction, motility test at 25°C, β-haemolysis and fermentation of rhamnose and xylose (Anonymous 1996a, 2004a). In addition to the conventional *Listeria* identification tests, commercial biochemical kits and identification systems, such as API Listeria kits (bio
Mérieux, Marcy l’Etoile, France), BBL Crystal ID system (BBL™ Crystal™ Identification Systems, MD, USA) or phenotypic microarray system of Biolog (Hayward, CA, USA), are available. Moreover, the identification systems, including colorimetric DNA probe, latex bead-based lateral flow immunoassay, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunofluorescence assay (ELFA), immunomagnetic separation (IMS), fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR), may be used for rapid detection of *L. monocytogenes* (Karpíšková *et al.* 2000, Cocolin *et al.* 2002, Brehm-Stecher and Johnson 2007, O’Grady *et al.* 2009).

### 2.1.4. Subtyping

Serotyping has been widely used for subtyping of *L. monocytogenes*, based on their somatic (O) and flagellar (H) antigens. *L. monocytogenes* strains are currently subdivided into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (Seeliger and Höhne 1979). Classical analysis of *L. monocytogenes* by serotyping with traditional agglutination tests is limited by cost, availability and the need for technical expertise to perform the assay. Moreover, the reproducibility of serotyping is not always satisfactory (Doumith *et al.* 2004b, Graves *et al.* 2007). Multiplex PCR assay has been developed and is widely used to separate the four major *L. monocytogenes* serovars isolated from food and patients (1/2a, 1/2b, 1/2c, 4b) into distinct groups. The PCR test for differentiation of *L. monocytogenes* serovars is a more rapid and practical alternative to laborious classical serotyping (Uueda *et al.* 2002, Doumith *et al.* 2004b).

Overall, 95% of human listeriosis cases are caused by *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b and 4b, and therefore, serotyping alone is of limited value in epidemiological studies (Graves *et al.* 2007). Serotyping alone has poor discriminating power compared with other subtyping methods. Nevertheless, serotyping may provide valuable information for rapid screening of groups of strains isolated during suspected outbreaks (Lyytikäinen *et al.* 2000, Nadon *et al.* 2001, Lukinmaa *et al.* 2003, Doumith *et al.* 2004a, 2004b, Graves *et al.* 2007).

Bacteriophage typing is one of the conventional subtyping methods based on interaction of a particular bacteriophage with its host strain, resulting in host cell lysis. The method is suitable
for processing large numbers of cultures with a good discriminatory power; however, not all strains are typable, and results are not always reproducible (Rocourt et al. 1985, McLauchlin et al. 1996, Graves et al. 2007). Despite its high discriminating power and easy applicability to large numbers of strains, phage typing is available only at selected national and international reference laboratories due to the method needing maintenance of stocks of biologically active phages and control strains (Graves et al. 2007).

Molecular subtyping methods are widely applied to characterize *L. monocytogenes* isolates (Bille and Rocourt 1996, Graves et al. 2007). These methods are based on specific proteins or genes specific for certain species, which are passed from one generation to another. The application of these methods provides valuable information to confirm disease outbreaks, to trace sources of the contamination in the food chain or to monitor epidemic strains and their reservoirs (Gasanov et al. 2005, Graves et al. 2007).

Multilocus enzyme electrophoresis (MEE) is a method based on differences in electrophoretic mobility of metabolic enzymes in a gel electrophoresis, which is a result of charge differences caused by amino acid substitutions in the polypeptide sequence (Selander et al. 1986, Graves et al. 2007). The electrophoretic types obtained by MEE can further be used in different epidemiological studies. In the 1990s, MEE was used for epidemiological investigations of listeriosis outbreaks in the US and Europe. Caugnat et al. (1996) showed that the method has good typeability, but comparatively low discriminatory power. Moreover, Rørvik et al. (2000) suggested that further subtyping with other molecular methods is needed to obtain more discriminatory results.

Chromosomal DNA restriction endonuclease analysis (REA) using frequently cutting restriction endonuclease has been used in different studies. Gerner-Smidt et al. (1996) showed that REA subtyping of *L. monocytogenes* is sensitive, cost-effective and relatively easy to perform. In addition, the entire genome can be evaluated. The method has high discriminatory power, but it would not be suitable for large-scale comparisons or for building dynamic databases of DNA patterns for comparative evaluation (Gerner-Smidt et al. 1996, Graves et al. 2007).
The amplified fragment length polymorphism (AFLP) method is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al. 1995). The technique involves restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments and gel analysis of the amplified fragments (Vos et al. 1995, Keto-Timonen et al. 2003, Graves et al. 2007). AFLP has high reproducibility and a high PCR multiplex ratio; however, it requires an automated DNA sequencer and has complex protocols (Guerra et al. 2002, Graves et al. 2007). The major advantages of AFLP are high throughput, possibilities for automation and applicability not only for strain characterization but also for molecular evolution studies of L. monocytogenes (Mougel et al. 2002, Keto-Timonen et al. 2003, 2007). Autio et al. (2003) showed that AFLP method is also a valuable tool for strain characterization and analysis of L. monocytogenes contamination routes and ecology in food-processing plants.

Ribotyping involves strain characterization for restriction fragment length polymorphisms (RFLP) associated with ribosomal operons. The reproducibility and typeability of this method are good; however, it is less discriminating than MEE or REA (Nørrung and Gerner-Smidt 1993, Graves et al. 2007). Baloga and Harlander (1991) revealed that in RFLP the most discriminating enzyme for L. monocytogenes is the restriction endonuclease EcoRI. This method has been used in several epidemiological studies and also to trace the sources of L. monocytogenes contamination in food-processing plants (Swaminathan et al. 1996, Norton et al. 2001, Thimothe et al. 2004, Graves et al. 2007, Meloni et al. 2009).

Random amplification of polymorphic DNA (RAPD) is a PCR-based method in which a single arbitrarily selected primer anneals to nearly complementary sequences on the target DNA at a low temperature (37°C). The primer anneals to several locations on the target and amplifies an array of DNA fragments of different sizes, yielding a DNA pattern suitable for typing (Björkroth et al. 1996, Martinez et al. 2003, Graves et al. 2007). The RAPD method has high discriminatory power and the ability to screen a large number of samples. Until now, it has been used in epidemiological investigations, listeriosis outbreak studies and surveys in the food industry (Destro et al. 1996, Fonnesbech Vogel et al. 2001, Graves et al. 2007).

Pulsed-field gel electrophoresis (PFGE) is a method based on separation of chromosomal DNA fragments in a pulsed-field electrophoresis chamber. The chromosomal DNA is

Multilocus sequence typing (MLST) involves automated DNA sequencing to characterize the alleles present at different housekeeping genes. The method is highly discriminatory and provides excellent assessment of genetic relatedness between strains; it is therefore highly useful for the investigation of genetic structure, evolution and population biology of pathogenic microorganisms (Maiden et al. 1998, Graves et al. 2007). Salcedo et al. (2003) described an MLST-based subtyping method for L. monocytogenes that uses the sequence diversity data of seven housekeeping genes (abcZ, bglA, cat, dapE, dat, ldh, lhkA). The MLST-based subtyping method has previously been compared with PFGE. Although obvious congruence was found among groupings obtained using sequence analysis of housekeeping genes and those obtained using PFGE, PFGE was much more discriminating than MLST (Salcedo et al. 2003).

Multi-virulence-locus sequence typing (MVLST) is another novel DNA-sequence-based subtyping method that targets virulence and virulence-associated genes. Comparison of MVLST with housekeeping gene-based MLST analysis showed that MVLST provided higher discriminatory power for serotype 1/2a and 4b strains than MLST (Zhang et al. 2004).
MVLST had improved discriminatory power for subtyping genetically diverse *L. monocytogenes* isolates and identified isolates associated with large multistate listeriosis outbreaks in the US (Chen et al. 2007). Thus, MVLST subtyping has been successfully applied to characterize and differentiate epidemic clones and outbreak strains of *L. monocytogenes* (Chen et al. 2005, 2007).

### 2.1.5. Foodborne listeriosis

*L. monocytogenes* was already recognized as a human pathogen in the 1920s (Nyfeldt 1929); however, the route of transmission was unclear until the 1980s, when a large number of outbreaks indicated that *L. monocytogenes* had been transmitted from foods to humans (Schelch et al. 1983, Farber and Peterkin 1991, Gellin et al. 1991, Hof 2003, Swaminathan and Gerner-Smidt 2007). Seeliger (1961) observed that most domestic animals are susceptible to *Listeria* infection, but listeriosis occurs mainly in ruminants. Only *L. monocytogenes* has been recognized as a pathogen that may cause foodborne listeriosis in humans, but a few rare human infections have been caused by *L. seeligeri* (Rocourt et al. 1986), *L. innocua* (Perrin et al. 2003), *L. ivanovii* (Cummings et al. 1994, Lessing et al. 1994, Guillet et al. 2010) and *L. grayi* (Rapose et al. 2008), mostly in heavily immunocompromised persons. Although the infectious dose for *L. monocytogenes* may be relatively high (>10^3 CFU/g), the infection can also be caused by a prolonged daily consumption of *L. monocytogenes*-contaminated foods containing 10^1 to 10^5 CFU/g (McLauchlin 1996, Maijala et al. 2001).

Listeriosis is a foodborne infection that does not result in a significant disease in healthy adults, but severe illness may occur in the unborn child, infants, the elderly and patients with compromised immune systems. Listeriosis in humans is a rare infection, but its mortality rate may vary between 20% and 30% (Gellin and Broom 1989, Farber and Peterkin 1991, Norton and Braden 2007). Although approximately 20% of listeriosis cases may occur in healthy adults, most cases have been found among pregnant women, neonates, the elderly or immunocompromised adults (Schuchat et al. 1991, Ramaswamy et al. 2007).

Listeriosis in pregnant women may result in foetal loss, stillbirth, premature delivery or neonatal infection. The disease may occur at any time during pregnancy, but it is most frequently documented during the third trimester (Goddijn 1997, Mylonakis et al. 2002, Hof
2003, Al-Tawfiq 2008). Most listeriosis cases during pregnancy occur in otherwise healthy women. Epidemiological studies on a series of listeriosis cases during pregnancy revealed that only 4% had a possible predisposing condition (Mylonakis et al. 2002). Although most cases of neonatal listeriosis present with severe illness, symptoms in women with listeriosis during pregnancy may be non-specific, and they often manifest as a mild illness (Painter and Slutsker 2007). *L. monocytogenes* infection of the foetus is thought to result from transplacental transmission following maternal bacteraemia, although some infections could also occur through an ascending spread from vaginal colonization (Mylonakis et al. 2002, Painter and Slutsker 2007).

Neonatal listeriosis has two clinical forms: early onset and late onset (Painter and Slutsker 2007). Early-onset neonatal listeriosis occurs in infants infected *in utero* and results in illness at birth or shortly thereafter. Frederiksen (1992) showed that 45-70% of neonatal listeriosis is of early-onset. The late-onset form may occur from one to several weeks after birth (Gellin et al. 1991). Although transplacental transmission of *L. monocytogenes* is the presumed source of infection in early onset of neonatal listeriosis, the route of infection in late onset is not well understood (Goddijn et al. 1997, Painter and Slutsker 2007). Mortality rates for both forms of the neonatal disease are usually 20-30% (McLauchlin 1967, Lorber 1997, Painter and Slutsker 2007).

Invasive listeriosis in non-pregnant adults is characterized by bacteraemia, CNS infections and sepsis, with a case fatality rate of approximately 30% (Mylonakis et al. 2002). Hussein et al. (2000) showed that *L. monocytogenes* was the second most frequently isolated bacteria causing acute bacterial meningitis in adults. Non-pregnant adults with listeriosis most frequently present with sepsis, meningitis or meningoencephalitis, with such symptoms as fever, malaise, ataxia, seizures and altered mental status (Farber and Peterkin 1991, Gellin et al. 1991, Mylonakis et al. 2002, Painter and Slutsker 2007).

A non-invasive form of listeriosis may occur as a gastrointestinal illness in healthy persons, with an incubation period of one day and fever as the most frequently reported symptom (Schlech 1997, Ooi and Lorber 2005). Susceptible persons are at higher risk for invasive listeriosis, but no host risk factors have thus far been associated with non-invasive listeriosis.
The factors that lead to febrile gastroenteritis versus invasive illness remain unclear (Painter and Slutsker 2007).

The proportion of listeriosis cases due to foodborne transmission has been estimated to be 99% (Mead et al. 1999). During the last two decades large numbers of sporadic cases and outbreaks of listeriosis have been described; however, limited information is available on the suspected vehicles of the disease (Norton and Braden 2007). Several foodborne listeriosis outbreaks have been associated with raw milk and dairy products (Allerberger and Gaggenbichler 1989, Goulet et al. 1995, Lyytikäinen et al. 2000, Anonymous 2003b), fish and fishery products (Riedo et al. 1994, Ericsson et al. 1997, Miettinen et al. 1999b, Tham et al. 2000) and vegetables and different RTE salads (Schlech et al. 1983, Salamina et al. 1996, Aureli et al. 2000). Although listeriosis has been associated with different foods of animal and non-animal origin, meat and processed meat products have been involved in most of the major foodborne listeriosis outbreaks worldwide (Table 2). The first laboratory-confirmed association of pork and poultry products with invasive listeriosis occurred in 1988, when a case was linked to consumption of contaminated turkey franks. Thereafter, patients were hospitalized with sepsis caused by L. monocytogenes serotype 1/2a (Schwartz et al. 1988). The vast majority of meat products involved in listeriosis outbreaks or sporadic cases were processed, vacuum-packaged or stored for long periods in home refrigerators (Jacquet et al. 1995, Loncarevic et al. 1997, Goulet et al. 1998, de Valk et al. 2001). One of the largest documented outbreaks of listeriosis occurred in France in 1992. It was associated with consumption of pork tongue in aspic jelly, and in most of the cases serotype 4b was found. Among the 279 outbreak-associated cases, 182 (65%) occurred in nonpregnant adults, 92 (33%) in pregnant women and 5 (2%) in children. The outbreak resulted in 63 deaths in non-pregnancy-associated cases, 22 foetal deaths and 7 neonate deaths, for an overall case fatality rate of 33% (Goulet et al. 1993, Jacquet et al. 1995). Successful identification of the food vehicle in an outbreak and subtyping of L. monocytogenes isolates from patients and food were critical for linking foods to human infections (Miettinen et al. 1999b, Lyytikäinen et al. 2000, Norton and Braden 2007).
Table 2. Major foodborne listeriosis outbreaks due to meat products in the European Union, Australia, New Zealand, Canada and USA.

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>No. of cases (deaths)</th>
<th>Implicated vehicle</th>
<th>Serotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>Australia</td>
<td>9 (6)</td>
<td>Processed meats, paté</td>
<td>ND</td>
<td>Watson et al. 1990</td>
</tr>
<tr>
<td>1992</td>
<td>France</td>
<td>279 (85)</td>
<td>Pork tongue in jelly</td>
<td>4b</td>
<td>Jacquet et al. 1993</td>
</tr>
<tr>
<td>1993</td>
<td>France</td>
<td>38 (10)</td>
<td>Rillettes</td>
<td>4b</td>
<td>Goulet et al. 1993</td>
</tr>
<tr>
<td>1996</td>
<td>Australia</td>
<td>5 (1)</td>
<td>Diced, cooked chicken</td>
<td>ND</td>
<td>Hall et al. 1996</td>
</tr>
<tr>
<td>1999</td>
<td>USA</td>
<td>11 (ND)</td>
<td>Paté</td>
<td>ND</td>
<td>Anonymous 1999</td>
</tr>
<tr>
<td>1999-2000</td>
<td>France</td>
<td>10 (3)</td>
<td>Rillettes</td>
<td>4b</td>
<td>de Valk et al. 2001</td>
</tr>
<tr>
<td>2000</td>
<td>USA</td>
<td>30 (7)</td>
<td>RTE deli turkey meat</td>
<td>1/2a</td>
<td>Anonymous 2000</td>
</tr>
<tr>
<td>2000</td>
<td>New Zealand</td>
<td>30 (ND)</td>
<td>RTE deli meats</td>
<td>1/2</td>
<td>Sim et al. 2002</td>
</tr>
<tr>
<td>2001</td>
<td>USA</td>
<td>16 (ND)</td>
<td>Deli meats</td>
<td>1/2a</td>
<td>Frye et al. 2002</td>
</tr>
<tr>
<td>2002</td>
<td>USA</td>
<td>54 (8)</td>
<td>RTE deli turkey meat</td>
<td>4b</td>
<td>Gottlieb et al. 2006</td>
</tr>
<tr>
<td>2006-2007</td>
<td>Germany</td>
<td>16 (ND)</td>
<td>RTE scalded sausage</td>
<td>4b</td>
<td>Winter et al. 2009</td>
</tr>
<tr>
<td>2008</td>
<td>Austria</td>
<td>16 (ND)</td>
<td>Jellied pork</td>
<td>4b</td>
<td>Pichler et al. 2009</td>
</tr>
<tr>
<td>2008</td>
<td>Canada</td>
<td>57 (22)</td>
<td>RTE meat products</td>
<td>ND</td>
<td>Anonymous 2009</td>
</tr>
</tbody>
</table>

*multiple state outbreak in USA; ND, no data available.

The incidence of foodborne listeriosis reported varies between countries, from 0.2 to 8.3 cases per million population (Gellin et al. 1991, Farber and Peterkin 1991, EFSA 2007, 2009, Goulet et al. 2008). The overall notification rate of listeriosis in humans in the EU was 0.3 cases per population of 100 000 in 2005-2007 (EFSA 2007, 2009). The total reported number of confirmed listeriosis cases in the EU has increased from 2000 to 2006; however, a slight decrease was observed in 2007 (EFSA 2007, 2009). Within Europe, 55.6% of all human listeriosis cases were reported in patients aged over 65 years. Although listeriosis continues to occur in association with pregnancy, it is now predominantly an infection of immunocompromised individuals in the older population segment (EFSA 2007). Moreover, most listeriosis cases are due to consumption of RTE foods able to support growth and containing markedly higher numbers of *L. monocytogenes* than 100 CFU/g (EFSA 2007).
2.2. **Listeria monocytogenes in foods**


2.2.1. **L. monocytogenes in meat and meat products**

The prevalence of *L. monocytogenes* in different meats and meat products is shown in Table 3. *L. monocytogenes* may occur frequently in raw pork (Autio *et al.* 1999, Nørrung *et al.* 1999, Kanuganti *et al.* 2002), raw beef (Bunčic *et al.* 1991a, MacGowan *et al.* 1994) and raw poultry products (Miettinen *et al.* 2001, Berrang *et al.* 2002, Rørvik *et al.* 2003, Praakle-Amin *et al.* 2006), although the origin of the contamination is not fully understood. The prevalence of *L. monocytogenes* on carcasses at slaughter is usually low, varying between 0 and 12% (Autio *et al.* 2000, Kanuganti *et al.* 2002, Farber *et al.* 2007). However, Gill *et al.* (1995) showed that due to poor sanitation at the slaughter plant high prevalence of listeriae (96%)
was found on pork carcasses. The prevalence of *L. monocytogenes* in pig tongues and tonsils was 14% and 12%, respectively, whereas contamination of hearts, kidneys, and livers was 6% (Autio *et al.* 2000).

*L. monocytogenes* is commonly found in comminuted meats and raw meat products (ground pork, beef and raw meat sausages). The prevalence of *L. monocytogenes* in ground meats and raw meat products may vary greatly, between 4.5% and 100% (Bunčić 1991a, Jay 1996, Fantelli and Stephan 2001, De Cesare *et al.* 2007, Farber *et al.* 2007, Miyasaki *et al.* 2009). Kanuganti *et al.* (2002) showed that *L. monocytogenes* was detected less often in tissues of freshly slaughtered pigs (0.87-2.4% of positive samples) than in ground pork (45-50.2% of positive samples). Moreover, Skovgaard and Nørrung (1989) reported that although only 1.7% of pig faecal samples contained *L. monocytogenes*, the pathogen was detected in 12% of ground pork samples. Breer and Schöpfer (1988) reported that of the 209 samples of beef, pork and pork products collected, listeriae were recovered from 11% to 45% with the prevalence for *L. monocytogenes* ranging from 0 to 15%. In comminuted meat products, the corresponding prevalences were 40-65% and 8-15%.

The prevalence of *L. monocytogenes* in RTE meat products may vary markedly, often depending on the type of product or its manufacturing processes (Table 3). Overall, *L. monocytogenes* is rarely isolated in RTE meat products or RTE foods containing meat. The results of comprehensive nationwide *L. monocytogenes* monitoring programme revealed that the proportions of *L. monocytogenes*-positive samples in RTE meat products have generally been low, ranging from 0 to 9.6% and from 0 to 8.1% in the EU member states and USA, respectively (Farber *et al.* 2007, EFSA 2009). No major differences emerged between detection rates of *L. monocytogenes* in RTE products made from pork, beef or poultry (EFSA 2007, 2009).
Table 3. Prevalence of *Listeria monocytogenes* in different raw and ready-to-eat (RTE) foods of animal origin.

<table>
<thead>
<tr>
<th>Food type</th>
<th>No. of samples tested/ No. of positive samples</th>
<th>Prevalence %</th>
<th>Levels in CFU/g</th>
<th>Country of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw meat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minced meat (pork, beef)</td>
<td>26/ 18</td>
<td>69</td>
<td>ND</td>
<td>Yugoslavia</td>
<td>Bunčić 1991a</td>
</tr>
<tr>
<td>Ground beef</td>
<td>39/ 3</td>
<td>8</td>
<td>4-560</td>
<td>USA</td>
<td>Yu <em>et al.</em> 1995</td>
</tr>
<tr>
<td>Ground pork</td>
<td>20/ 5</td>
<td>25</td>
<td>4-240</td>
<td>USA</td>
<td>Yu <em>et al.</em> 1995</td>
</tr>
<tr>
<td>Pork sausage</td>
<td>17/ 1</td>
<td>6</td>
<td>240-21000</td>
<td>USA</td>
<td>Yu <em>et al.</em> 1995</td>
</tr>
<tr>
<td>Beef</td>
<td>658/ 39</td>
<td>6</td>
<td>ND</td>
<td>UK</td>
<td>Jay 1996</td>
</tr>
<tr>
<td>Poultry (broiler legs)</td>
<td>240/ 169</td>
<td>70</td>
<td>ND</td>
<td>Estonia</td>
<td>Praakle-Amin <em>et al.</em> 2006</td>
</tr>
<tr>
<td>Poultry (broiler pieces)</td>
<td>61/ 38</td>
<td>62</td>
<td>ND</td>
<td>Finland</td>
<td>Miettinen <em>et al.</em> 2001</td>
</tr>
<tr>
<td>Poultry (marinated broiler)</td>
<td>186/ 67</td>
<td>36</td>
<td>0.3-300</td>
<td>Finland</td>
<td>Aarnisalo <em>et al.</em> 2008</td>
</tr>
<tr>
<td>Ground pork sausage</td>
<td>96/ 22</td>
<td>23</td>
<td>ND</td>
<td>USA</td>
<td>Duffy <em>et al.</em> 2001</td>
</tr>
<tr>
<td>Heat-treated meat products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paté</td>
<td>31/ 0</td>
<td>0</td>
<td>-</td>
<td>UK</td>
<td>MacGowan 1994</td>
</tr>
</tbody>
</table>
Table 3 continues.

<table>
<thead>
<tr>
<th>Food type</th>
<th>No. of samples tested/ No. of positive samples</th>
<th>Prevalence %</th>
<th>Levels in CFU/g</th>
<th>Country of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paté</td>
<td>7/ 0</td>
<td>0</td>
<td>-</td>
<td>Australia</td>
<td>Seneviratna 1990</td>
</tr>
<tr>
<td></td>
<td>36/ 5</td>
<td>14</td>
<td>1100</td>
<td>Spain</td>
<td>Lafarge et al. 1994</td>
</tr>
<tr>
<td></td>
<td>2676/ 287</td>
<td>11</td>
<td>$10^3 - 10^6$</td>
<td>UK</td>
<td>McLauchlin 1991</td>
</tr>
<tr>
<td>Raw fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>510/ 44</td>
<td>8</td>
<td>ND</td>
<td>Finland</td>
<td>Miettinen et al. 2005</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>60/ 1</td>
<td>2</td>
<td>ND</td>
<td>Finland</td>
<td>Autio et al. 1999</td>
</tr>
<tr>
<td>RTE fish products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Preserved fish</td>
<td>282/ 62</td>
<td>22</td>
<td>ND</td>
<td>Denmark</td>
<td>Nørrgaard et al. 1999</td>
</tr>
<tr>
<td>Different RTE fish</td>
<td>232/ 32</td>
<td>14</td>
<td>ND</td>
<td>Denmark</td>
<td>Nørrgaard et al. 1999</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>33/ 3</td>
<td>9</td>
<td>ND</td>
<td>Norway</td>
<td>Rørvik et al. 1991</td>
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<tr>
<td>Smoked, cold-salted fish</td>
<td>110/ 22</td>
<td>20</td>
<td>10-9900</td>
<td>Finland</td>
<td>Johansson et al. 1999</td>
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<td>Raw cow’s milk</td>
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<td>3</td>
<td>ND</td>
<td>France</td>
<td>Meyer-Broseta et al. 2003</td>
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<td></td>
<td>361/ 13</td>
<td>4</td>
<td>ND</td>
<td>UK</td>
<td>Greenwood et al. 1991</td>
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<td>RTE dairy products</td>
<td></td>
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<tr>
<td>Cream</td>
<td>52/ 0</td>
<td>0</td>
<td>-</td>
<td>USA</td>
<td>Ryser 2007</td>
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<td>Chocolate milk</td>
<td>415/ 5</td>
<td>1</td>
<td>ND</td>
<td>USA</td>
<td>Ryser 2007</td>
</tr>
<tr>
<td>Butter</td>
<td>30/ 0</td>
<td>0</td>
<td>-</td>
<td>USA</td>
<td>Ryser 2007</td>
</tr>
<tr>
<td>Ice cream</td>
<td>659/ 23</td>
<td>3</td>
<td>ND</td>
<td>USA</td>
<td>Ryser 2007</td>
</tr>
<tr>
<td>Soft cheese</td>
<td>85/ 12</td>
<td>14</td>
<td>$&lt;10^5 - 10^6$</td>
<td>England</td>
<td>Pini et al. 1988</td>
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<tr>
<td></td>
<td>45/ 2</td>
<td>4</td>
<td>$&lt;10^5$</td>
<td>France</td>
<td>Pini et al. 1988</td>
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<td></td>
<td>44/ 7</td>
<td>16</td>
<td>$&lt;10^5 - 10^4$</td>
<td>Italy</td>
<td>Pini et al. 1988</td>
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<tr>
<td></td>
<td>6/ 0</td>
<td>0</td>
<td>-</td>
<td>Denmark</td>
<td>Pini et al. 1988</td>
</tr>
<tr>
<td>White mould cheese</td>
<td>119/ 15</td>
<td>13</td>
<td>ND</td>
<td>France</td>
<td>Loncarevic et al. 1995</td>
</tr>
<tr>
<td></td>
<td>19/ 0</td>
<td>0</td>
<td>-</td>
<td>Denmark</td>
<td>Loncarevic et al. 1995</td>
</tr>
</tbody>
</table>

ND, no data available.
2.2.2. *L. monocytogenes* in other foods of animal origin

*L. monocytogenes* has been recovered from aquatic environments, fish and fishery products. Although *L. monocytogenes* were not recovered from a freshwater stream, the prevalence of *L. monocytogenes* increased with the degree of human activity: 2% in seawater fish, 10% in freshwater fish farms, 16% in fish slaughterhouses and 68% in fish smokehouses (Hansen *et al.* 2006). Miettinen *et al.* (2005) showed that prevalence of *L. monocytogenes* may vary greatly between different fish farms, from 0 to 100%. The gills of farmed fish were found to be the most contaminated parts, with a prevalence of up to 95.6%, and only 4.4% of the *L. monocytogenes*-positive samples were obtained from skin or viscera (Miettinen and Wirtanen 2005). The prevalence of *L. monocytogenes* in RTE fish products that are not heat-treated, such as cold-smoked or otherwise lightly processed products, is usually higher than in heat-treated products (Table 3). High prevalences of *L. monocytogenes* (11-50%) have been found in cold-smoked and Nordic gravad (raw-salted) fish products (Rørvik *et al.* 1995, Jørgensen and Huss 1998, Keto and Rahkio 1998, Lyhs *et al.* 1998, Johansson *et al.* 1999, Dauphin *et al.* 2001), whereas in heat-treated or hot-smoked fish products the prevalence of *L. monocytogenes* varied between 0 and 12% (Keto and Rahkio 1998, Lyhs *et al.* 1998, Johansson *et al.* 1999, Jemmi *et al.* 2002).

Raw milk, products made from raw milk and unfermented and fermented dairy products may contain variable numbers of *L. monocytogenes* (Miettinen *et al.* 1999a, Nørrung *et al.* 1999, Ryser 2007). Latorre *et al.* (2009) showed that high prevalence of *L. monocytogenes* in raw bulk tank milk can be associated with contamination from *L. monocytogenes* biofilms in the milking system or farm environment. Thus, raw milk contaminated with *L. monocytogenes* could be a source of contamination for a milk-processing plant (Waak *et al.* 2002). Although higher prevalence of *L. monocytogenes* is usually associated with soft cheeses and blue mould cheeses, at 30% and 11%, respectively (Jacquet *et al.* 1993), Rudolf and Scherer (2001) showed that prevalence can vary up to 17% also in other types of hard cheeses, but confirmed previous observations that *L. monocytogenes* is most often found in soft and semi-soft cheeses. Surprisingly, this study revealed that a higher prevalence of *L. monocytogenes* was observed in cheeses made from pasteurized milk (8%) than in cheeses manufactured from raw
milk (4.8%), suggesting that cross-contamination within the dairy plant was the most important factor associated with contamination.

While consumption of eggs and egg products has not yet been linked to a single case of listeriosis, the presence of *L. monocytogenes* on egg shells that contain minute cracks, along with the ability of this pathogen to survive 90 days and >14 days on eggs stored at 5°C and 10°C, respectively, to persist on inoculated eggs treated with sodium hypochlorate containing 100 ppm available chlorine, and to grow in artificially contaminated eggs stored at refrigerated as well as ambient temperatures suggests that eggs cannot be completely ignored as a possible source of listerial infection (Bartlett 1993, Ryser 2007).

2.3. *Listeria monocytogenes* in the meat-processing environment

2.3.1. Contamination of meat and meat products with *L. monocytogenes*


*L. monocytogenes* has been recovered from different slaughterhouse environments, carcasses, by-products and faeces of animals (Bunčić 1991a, Giovannacci et al. 1999, Autio et al. 2000, Kanuganti et al. 2002, Thévenot et al. 2006). Skovgaard and Nørrung (1989) showed that *L. monocytogenes* has been occasionally isolated on farms from faeces and skin of healthy pigs,
whereas Felon et al. (1996) found that the prevalence of *L. monocytogenes* in pig faecal samples may range from 0 to 47%. Although it is agreed that carcasses may become contaminated from faecal content, Autio et al. (2000) showed that *L. monocytogenes* originating from tongues and tonsils may also contaminate slaughtering equipment, which may further cause the spread of the pathogen to carcasses. Previous studies confirmed that pigs were more likely to harbour *L. monocytogenes* in their tonsils than to excrete bacteria in faeces (Bunčić 1991a). Carcasses are thought to be contaminated also when the large intestines are ruptured during evisceration (Skovgaard and Nørrung 1989). The presence of *L. monocytogenes* on carcasses has been strongly associated with contamination of the slaughterhouse equipment and environment (Autio et al. 2000, Miettinen et al. 2001, Kanuganti et al. 2002, Hellström et al. 2010).

An extensive study of meat contamination in the meat-processing industry indicated that contamination of raw pork may significantly increase after chilling and cutting (Nesbakken et al. 1996). Moreover, Van der Elzen and Snijders (1993) found the environmental prevalence of *L. monocytogenes* in chilling-cutting areas can be as high as 71-100%, suggesting that post-slaughter processing is a significant cause of meat contamination. Processed meat products may become contaminated with *L. monocytogenes* from raw meat (Samelis and Metaxopoulos 1999, Chasseignaux et al. 2001, Berrang et al. 2002), from contaminated surfaces during the manufacturing process (Lundén et al. 2002, Heir et al. 2004) or from personnel (Holah et al. 2003, Gudbjörnsdóttir et al. 2004). Meat-processing equipment, such as slicing, dicing or packaging machinery, has been associated with *L. monocytogenes* contamination in several studies (Lundén et al. 2002, 2003, Suihko et al. 2002). Moreover, Suihko et al. (2002) found that skinning machines, slicing machines and conveyor belts were important sources of *L. monocytogenes* contamination in meat-processing plants. Contamination may also occur via tools or hands, footwear and gloves and aprons of the personnel involved in manufacturing (Gudbjörnsdóttir et al. 2004). Rørvik et al. (1997) concluded that job rotation between departments in a food-processing plant can be a significant risk factor associated with *L. monocytogenes* contamination.

Chasseignaux et al. (2001) observed that contamination level in meat-processing plants increased along the processing line, likely the result of ineffective cleaning and disinfection procedures. Moreover, complex and poorly cleanable and disinfectable machinery in the
meat-processing plants may facilitate spread of *L. monocytogenes* contamination (Chasseignaux *et al.* 2001, Lundén *et al.* 2002).

2.3.2. Meat-processing plant contamination with persistent and non-persistent *L. monocytogenes* strains

A particular feature of *L. monocytogenes* contamination in food-processing environments is the persistence of the contamination (Rørvik *et al.* 1995, Miettinen *et al.* 1999a, Autio *et al.* 2003, Lundén *et al.* 2004, 2005), often lasting for months or years (Lundén *et al.* 2005, Cornu *et al.* 2006).


Lundén *et al.* (2003) reported the existence of persistent and non-persistent *L. monocytogenes* strains in several meat-processing plants. Chasseignaux *et al.* (2001) showed that some *L. monocytogenes* clones have been recovered in meat-processing plants over several months, while others were eliminated during the cleaning and disinfection, suggesting that contamination was mainly associated with meat processing. Processing machines, such as slicing machines, packaging machines and conveyor belts, have been found to frequently be contaminated with *L. monocytogenes* (Giovannacci *et al.* 1999, Lundén *et al.* 2002, Suihko *et al.* 2002). Lundén *et al.* (2003) observed that persistent *L. monocytogenes* strains were widely
spread in the meat-processing plant, contaminating two or more processing lines, whereas none of the persistent strains were present in raw meat (Nesbakken et al. 1996, Lundén et al. 2003). Genetic diversity among *L. monocytogenes* isolates from meat-processing industry plants suggests the involvement of a number of sources and factors in persistent product contamination (Heir et al. 2004, Lundén et al. 2005, Folsom et al. 2006, Vorst et al. 2006).
3. AIMS OF THE STUDY

The objectives of this thesis were to investigate the prevalence, contamination and heat resistance of *Listeria monocytogenes* in meat products and meat-processing plants and to analyse the trends of listeriosis in Latvia. Specific aims were as follows:

1. to investigate the prevalence and genetic diversity of *L. monocytogenes* in different ready-to-eat meat products at retail markets in Latvia (I).

2. to evaluate the factors associated with *L. monocytogenes* contamination of cold-smoked pork products and to determine sources and routes of *L. monocytogenes* contamination in cold-smoked meat-processing plants (II-III).

3. to investigate heat resistance of *L. monocytogenes* in high- and low-fat cooked-sausages during post-package pasteurization (IV).

4. to analyse and describe the trends of listeriosis in Latvia over a 10-year period, from 1998 to 2007 (V).
4. MATERIALS AND METHODS

4.1. Sampling

4.1.1. Listeria monocytogenes prevalence studies (I, II)

Ready-to-eat vacuum-packaged, sliced meat products, sampled from 2003 to 2005, comprised several production lots for each of nine product groups from two supermarket chains. A total 523 packages of products from 20 meat-processing plants were collected. The temperatures of the air in the display counters were determined during sampling. All available RTE vacuum-packaged meat products of Baltic origin presented in meat counters were represented in the sampling. The packages were transported to the laboratory on ice and then stored at 6°C, before being analysed within 5 days of the end of shelf-life stated on each package. Each vacuum package with 70-150 g of sliced product was aseptically opened and a 25-g sample was taken and pummelled with 225 ml of half-strength Fraser broth (Oxoid, Basingstoke, UK) in a stomacher. After the sampling, all product packages were aseptically vacuum-packed again and kept at 6°C to await enumeration analyses.

4.1.2. Contamination study in a cold-smoked meat-processing plant (III)

A total of 183 samples were collected in meat-processing plant B for contamination analyses, including samples of the product at different manufacturing stages (n = 136) and environmental samples (n = 47) covering all manufacturing surfaces over a 3-month period in 2009 (III, Table 1). Additionally, 73 RTE cold-smoked pork samples from the same meat-processing plant had been analysed in 2004, and their L. monocytogenes isolates were compared with isolates obtained in 2009. Pork samples were taken aseptically after defrosting of raw pork, after dry salting and/or brining injections, after primary ripening and after secondary ripening as a sliced, vacuum-packaged product. The samples were transported to the laboratory on ice packs and were analysed within 3 h of arrival, except RTE cold-smoked, sliced, vacuum-packaged pork samples, which were stored at 6°C, before being analysed at the end of the stated shelf-life. RTE cold-smoked pork vacuum packages were aseptically opened, and a 25-g sample was taken and homogenized with 225 ml of half-Fraser broth (Oxoid, Basingstoke, UK) in a stomacher.
Environmental samples of the processing plant were taken before and during production. The samples from the surfaces of the processing environment and equipment were taken by sterile cellulose Whirl-Pak® sponges pre-moistened in a Speci-Sponge® bag containing 10 ml of neutralizing buffer (monopotassium phosphate, sodium thiosulfate, and aryl sulfonate complex), which neutralizes surfaces. The processing environment and equipment were intensively swabbed with 11.5 cm × 23 cm single-use sponges for 2-3 min. A total volume of 200 ml of brining solution was taken during the meat brining and mixed, 1 ml of which was then transferred to the 9 ml of half-Fraser broth (Oxoid) and further incubated at 30°C for 24 h. Finally, a set of samples of plastic boot-covers from six employees was taken after 30 min of use in different working areas.

4.2. *Listeria monocytogenes* strains, culture preparation and maintenance (I-IV)

All *L. monocytogenes* experimental strains and isolates obtained from meat products and the meat-processing environment were stored at -70°C. *L. monocytogenes* strains Scott A, F5069 and V7 were used in heat resistance studies. All strains were maintained on Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA) slants with 0.6% Yeast Extract (YE, Difco, Sparks, MD, USA). Each *L. monocytogenes* strain was transferred to 150 ml of Tryptic Soy Broth (TSB, Difco, Sparks, MD, USA) with 0.6% YE and incubated at 37°C for 18 h. After achieving a stationary growth phase, 2 ml of each strain suspension was transferred to a centrifuge tube and vortexed to ensure a homogeneous *L. monocytogenes* cocktail. *L. monocytogenes* three-strain cocktail (500 μl) of was placed into a centrifuge tube containing 0.5 ml of TSB and 0.6% YE. The *L. monocytogenes* cocktail suspension was centrifuged at 9300g for 10 min and resuspended in 0.5 ml of phosphate-buffered saline at pH 7.5 to a final concentration of 1 × 10⁸ CFU ml⁻¹ of the inoculum.

4.3. Isolation, identification and enumeration of *Listeria monocytogenes* (I-IV)

Isolation of *L. monocytogenes* was performed according to the International Organization for Standardization method (Anonymous 1996a, 2004a), with the modifications suggested by Johansson (1998). Examination for *L. monocytogenes* included a two-step enrichment. All samples were incubated in half-strength Fraser broth at 30°C for 24 h. After incubation, 0.1
ml was transferred to full-strength Fraser broth and incubated at 37°C for 48 h. After incubation, the half- and full-strength Fraser broths were plated on PALCAM listeria selective agar (Oxoid) and *Listeria monocytogenes* blood agar (LMBA) containing TSA base (Difco, Becton Dickinson, Sparks, MD, USA), 5% sterile sheep blood and, per litre, 10 g of lithium chloride (Merck KgaA, Darmstadt, Germany) and 10 mg of ceftazidime (Abtek Biologicals Ltd., Liverpool, UK) or chromogenic agar *Listeria* according to Ottaviani and Agosti (ALOA, AES Chemunex, Combourg, France). Selective agar plates were incubated at 37°C for 24-48 h. Five typical colonies were streaked from both PALCAM and LMBA or ALOA onto 5% sheep blood Columbia agar (Difco) and then incubated at 37°C for 24 h. Isolates that were β-haemolytic, catalase-positive and Gram-positive were presumed to be *L. monocytogenes*. Presumptive *L. monocytogenes* were confirmed using the API Listeria kit (bio Mérieux, Marcy l’Etoile, France).

Enumeration of *L. monocytogenes* was carried out according to the method of the International Organization for Standardization (Anonymous 1996b) for 53 of 158 positive samples. Enumeration was performed immediately upon expiry of each product’s shelf-life. The lower limit of enumeration was 10 CFU/g. The procedure included a 1-h resuscitation in buffered peptone water at 20°C and surface plating on PALCAM and LMBA of 1.0 ml of 10⁻¹, and 0.1 ml of each of the 10⁻² and 10⁻³ dilutions. The plates were incubated at 37°C for 24-48 h. Typical colonies were selected and plated on 5% sheep blood agar. *L. monocytogenes* was confirmed as described above.

### 4.4. Serotyping of *Listeria monocytogenes* isolates (I-III)

At least two *L. monocytogenes* isolates of each PFGE type were serotyped with *Listeria* antisera (Denka Seiken, Tokyo, Japan). Determination of the O-antigen was carried out with heat-inactivated bacteria using the slide agglutination method. A dense bacterial antigen suspension was prepared by suspending *L. monocytogenes* cells cultured on a TSA agar plate with 0.2% sodium chloride. Suspension was heated at 121°C for 30 min, followed by centrifugation at 3000 rpm for 20 min, and the precipitate was resuspended with a small amount of 0.2% sodium chloride. The precipitate suspension was then tested with *Listeria* antisera following the manufacturer’s instructions. Determination of the H-antigen was carried out using a test tube method with the bacteria cultured in liquid media. *L.
monocytogenes cells were passed through the semi-liquid BHI media (0.2% agar) with a Craigie’s tube three times. Ready-to-use cell suspension was prepared by incubating cells in TSB broth at 30°C overnight. After incubation, an equal amount of physiological saline was added. Thereafter, two drops of each H-antisera was placed into separate test tubes and then 0.5 ml of the cell suspension was added to each. One tube not containing the antisera was used as a control. Results were recorded following the manufacturer’s instructions.

4.5. DNA isolation and pulsed-field gel electrophoresis (PFGE) typing (I-III)

DNA isolation was performed with modifications as described by Björkroth et al. (1996) and Autio et al. (2002). After overnight incubation, 2 ml of the culture was diluted in 5 ml of PIV buffer (10 mM Tris, 1 M NaCl) and concentrated by centrifugation at 1100 × g for 15 min at 4°C. Plugs for PFGE were prepared with concentrated cell suspension in PIV and 2% (w/v) low melting point agarose (InCert; FMC Bioproducts, Rockland, ME, USA). Cells in prepared plugs were lysed in a solution containing per ml 20 μg of RNase, 1 mg of lysozyme and 10 U of mutanolysin in lysis buffer containing 6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate and 0.5% sodium lauryl sarcosine, at 37°C for 3 h with slow shaking. Lysis was continued with a 1-h wash with ESP solution containing proteinase K (Sigma) 100 μg/ml, 0.5 M EDTA and 10% sodium lauryl sarcosine at 50°C. ESP wash was repeated twice under the same conditions. Afterwards, the plugs were washed in buffer containing 10 mM Tris and 0.1 mM EDTA at 50°C for 1 h. A cutting enzyme, AscI 20 U/μl (New England BioLabs, Beverly, MA, USA), was used for digestion at 37°C for 16 h.

PFGE was performed with 1.0% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts, Rockland, ME, USA) in buffer containing 45 mM Tris, 4.5 mM boric acid and 1 mM sodium EDTA at 8°C in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden) operated at 200 V. Pulse times ranged from 1 to 35 s for 18 h. The size of the fragments was determined with a low-range PFG marker (New England BioLabs, Beverly, MA, USA), Gels were stained with ethidium bromide. Photo images were obtained with the Alpha Imager 2000 photo documentation system (Alpha Innotech, San Leandro, CA, USA). The images were saved as TIFF files for further analysis.
4.6. Analysis of PFGE typing results (I-III)

Macrorestriction patterns (MRP) of AscI were analysed with BioNumerics software version 4.01 (Applied Maths, Kortrijk, Belgium) and applied Dice coefficient correlation to identify similarities among PFGE types. A dendrogram was constructed with the unweighted pair-group method using arithmetic averages (UPGMA). The position tolerance was set to 1.2%, with the optimization value at 1.0%.

4.7. Manufacturing process of cold-smoked, sliced, vacuum packaged pork (II, III)

A questionnaire was distributed to obtain detailed information on manufacturing practices at seven meat-processing plants. Cold-smoked pork production involves several operations (II, Fig. 1). After cutting, the raw meat is dry-salted or dry-salted and injected with brine. The salt and/or brine may contain nitrite, nitrate, spices and/or starter cultures. The ripening time before cold-smoking varies among manufacturers, from 7 to 21 days. The meat is maintained at ≤ 6°C and is turned regularly. More salt can be added and manually rubbed in during this primary ripening. Cold-smoking temperatures vary among producers, from 20°C to 30°C. The time of exposure to natural smoke also varies among companies, from 3 to 48 h. Secondary ripening takes place at some plants for 7-20 days at a temperature <14°C in ripening chambers with humidity controlled at 75%. Products are sliced and vacuum-packaged before they are distributed to the market. The shelf-lives of the vacuum-packaged products are set by the manufacturers, ranging from 20 to 120 days. The manufacturing process includes much handling of the product by personnel, especially during salting, ripening, slicing and vacuum-packaging. This type of product is consumed without any heat treatment.

4.8. Heat resistance studies of *Listeria monocytogenes* (IV)

Commercially produced, sliced, cooked sausages of a diameter of 100 mm with a high-fat (26%) or a low-fat (3.2%) content were used in heat resistance studies. Slices of cooked sausages were aseptically transferred to sterile vacuum packages. Before sealing, vacuum packages and samples were weighed separately and spot-inoculated with 100 μl of the strain cocktail suspension in ten different areas of the slices, vacuum-packaged and incubated at 4°C for 1 h to allow attachment of *L. monocytogenes* cells to the product surface.
Vacuum-packaged samples were placed into a hot water pasteurization system at temperatures 55°C, 60°C, 62.5°C and 65°C. After heat treatment, at selected times, packages were placed into an ice bath for 10 min. Vacuum packages were then opened and the product was diluted with 0.1% peptone in a 1:10 dilution and placed into stomacher for 1 min. A volume of 11 ml was transferred to 99 ml of 0.1% peptone. A duplicate 0.1-ml amount of the dilution was plated onto TSA and 0.6% YE plates and incubated at 37°C for 4 h to resuscitate heat-injured cells. Thereafter, plates were overlaid with modified Oxford agar (MOX, Difco, Sparks, MD, USA). MOX agar plates were incubated at 37°C for an additional 24-26 h. Enumeration of viable \textit{L. monocytogenes} cells was performed according standard APHA methods (Anonymous 1992). All thermal inactivation experiments were replicated three times. Log-survived cells were plotted against sampling time to determine D-values. Log D-values were plotted against heating temperatures to obtain z-values.

4.9. Surveillance of human listeriosis in Latvia (V)

Registration of human listeriosis cases has been carried out in Latvia since 1998. Since then, general hospitals, maternity hospitals and clinical laboratories have reported listeriosis cases to the SPHA as a part of an obligatory notification system. Available data were analysed for the period 1998-2007. A listeriosis case was defined by positive culture of \textit{Listeria monocytogenes} isolated from a sterile site or by identification of anti-\textit{Listeria} antibodies from a suspected \textit{L. monocytogenes} infection case. Since 2002, all listeriosis cases have been confirmed by bacteriological analysis as stated by the official case definition. All mother-child cases were registered as two separate cases. Until now, serotyping and molecular subtyping of human listeria isolates have not been carried out.

Detailed clinical information was available for 15 of the 90 patients. Seasonal distribution of listeriosis cases was analysed over the same study period. Population data over the study period were obtained from the Central Statistical Bureau of Latvia. In 1998, the population of Latvia was 2.42 million, with 0.78 million living in Riga, the capital. Since 1998, the total population of the country has gradually decreased to 2.28 million in 2007.
The population aged 60 years or more increased from 19.8% in 1998 to 21.9% in 2007, whereas the proportion of females in this population segment decreased from 72.6% in 1998 to 65.3% in 2007. Clinical information about listeriosis cases was obtained either from the SPHA and its branches or from the hospitals where the disease was identified. Gender of the patient was recorded for 36% of all cases reported. All listeriosis patients were categorized by cohort. Detailed case reports, including the outcome of the disease, were described and recorded in case record forms for listeriosis cases registered in Riga and the Riga district.

4.10. Statistical analysis (I-V)

Chi-square test or Fisher’s exact test was performed to compare the numbers of *L. monocytogenes*-positive products produced by different meat-processing plants and the prevalence data in different groups of meat products (I, II).

To identify factors in the manufacturing process of cold-smoked, sliced, vacuum-packaged pork products associated with *L. monocytogenes* contamination, all manufacturing steps were defined as variables, and analysis of variance (ANOVA) was used to determine significant differences (*P* < 0.05) for the prevalence of *L. monocytogenes* (Statistical Package for Social Sciences 12.0 for Windows, SPSS Inc., Chicago, IL, USA). A non-parametric Spearman rank-order correlation coefficient with a two-tailed *P*-value was calculated for cross-correlations between manufacturing steps and the presence of *L. monocytogenes*. The odds ratio (OR) with a 95% confidence interval was calculated for each manufacturing step, and *L. monocytogenes* detected in cold-smoked, sliced, vacuum-packaged pork was set as a dependent variable in the logistic multivariable regression model. The manufacturing steps identified as most significant in the bivariate analysis were set as independent variables in the model. Predictive values of independent variables were analysed for the dependent variable by computing the coefficient estimates (B-values), *P*-values for the B-values and ORs with 95% confidence intervals from the B-values (II). General linear model procedure was applied for ANOVA using the least-squares difference test on thermal inactivation kinetics in heat resistance studies (SAS Inc., Cary, NC, USA) (IV).
5. RESULTS

5.1. Prevalence and genetic characterization of *Listeria monocytogenes* in ready-to-eat meat products (I, II)

Overall, 30% (158/523) of the RTE, sliced, vacuum-packaged meat product samples were positive for *L. monocytogenes* (Table 4). The prevalence of *L. monocytogenes* in cold-smoked meat products (40%) was significantly higher than in cooked and hot-smoked meat products (0.8%) \( (P < 0.001) \). *L. monocytogenes* was found in 13 (65%) of the 20 investigated meat-processing plants. In 11 (73%) of the 15 cold-smoked meat-processing plants, the prevalence of *L. monocytogenes* in cold-smoked meat products was higher than 10%. Moreover, 70% (109/158) of *L. monocytogenes* isolates were found in RTE cold-smoked meat products originating from meat-processing plants A, B and C. The overall prevalence of *L. monocytogenes* in different RTE, cold-smoked meat products in plants A, B and C was 51%, 22% and 48%, respectively. A total of 44 (83%) of the 53 *L. monocytogenes*-positive samples tested contained <100 CFU/g at the end of the product’s shelf-life. None of the *L. monocytogenes*-positive samples contained more than 1000 CFU/g; however, 9 samples (17%) contained 100-1000 CFU/g.

Altogether 158 *L. monocytogenes* isolates from RTE, sliced, vacuum-packaged meat products were subtyped using serotyping and PFGE typing. Overall, 96% of all *L. monocytogenes* isolates belonged to serotypes 1/2a and 1/2c, while the remaining 4% belonged to serotypes 1/2b, 3b and 4b. Based on genetic similarity, 30 different PFGE types were determined (I, Fig. 1; II, Fig. 2). *L. monocytogenes* isolates obtained from plants A \( (n=43) \), B \( (n=18) \) and C \( (n=49) \) were grouped into 7, 7 and 11 PFGE types, respectively. Overall, three PFGE types were recovered over a 9-month period from different cold-smoked pork production lots from plant C. Identical PFGE type was found in six different production lots of cold-smoked pork and beef products from meat-processing plant A over a 2-month period. Moreover, *L. monocytogenes* isolates belonging to one PFGE type (serotype 1/2c) were found repeatedly over a 1-month period in two different production lots of dried beef “Basturma” and cold-smoked beef in plant A.
Table 4. Prevalence of *Listeria monocytogenes* in different, sliced, vacuum-packaged meat products originating from 20 meat-processing plants at retail markets in Latvia.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Meat product</th>
<th>No. of lots tested</th>
<th>No. of packages tested</th>
<th>No. (%) of positive packages</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cold-smoked beef</td>
<td>6</td>
<td>54</td>
<td>31 (57)</td>
</tr>
<tr>
<td></td>
<td>Dried beef “Basturma”</td>
<td>2</td>
<td>10</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td>Cold-smoked pork</td>
<td>3</td>
<td>20</td>
<td>11 (55)</td>
</tr>
<tr>
<td></td>
<td>Salami-type sausages</td>
<td>3</td>
<td>13</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cooked smoked ham</td>
<td>2</td>
<td>7</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cooked smoked beef</td>
<td>1</td>
<td>11</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>17</td>
<td>115</td>
<td>43 (37)</td>
</tr>
<tr>
<td>B</td>
<td>Cold-smoked pork</td>
<td>13</td>
<td>83</td>
<td>18 (22)</td>
</tr>
<tr>
<td>C</td>
<td>Cold-smoked pork</td>
<td>9</td>
<td>100</td>
<td>48 (48)</td>
</tr>
<tr>
<td></td>
<td>Liver paté</td>
<td>4</td>
<td>14</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>13</td>
<td>114</td>
<td>49 (43)</td>
</tr>
<tr>
<td>D</td>
<td>Cold-smoked pork</td>
<td>4</td>
<td>22</td>
<td>1 (5)</td>
</tr>
<tr>
<td>E</td>
<td>Salami-type sausages</td>
<td>2</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cooked smoked ham</td>
<td>3</td>
<td>12</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cooked sausages</td>
<td>1</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cooked smoked turkey</td>
<td>1</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>7</td>
<td>27</td>
<td>0 (0)</td>
</tr>
<tr>
<td>F</td>
<td>Salami-type sausages</td>
<td>1</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Cold-smoked pork</td>
<td>4</td>
<td>13</td>
<td>9 (69)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>5</td>
<td>18</td>
<td>9 (50)</td>
</tr>
<tr>
<td>G</td>
<td>Salami-type sausages</td>
<td>3</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>H</td>
<td>Cooked smoked ham</td>
<td>3</td>
<td>25</td>
<td>0 (0)</td>
</tr>
<tr>
<td>I</td>
<td>Cooked sausages</td>
<td>1</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>J</td>
<td>Liver paté</td>
<td>2</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td>K</td>
<td>Cold-smoked pork</td>
<td>2</td>
<td>10</td>
<td>1 (10)</td>
</tr>
<tr>
<td>L</td>
<td>Cold-smoked pork</td>
<td>1</td>
<td>11</td>
<td>8 (73)</td>
</tr>
<tr>
<td>M</td>
<td>Cold-smoked pork</td>
<td>2</td>
<td>25</td>
<td>10 (40)</td>
</tr>
<tr>
<td>N</td>
<td>Cold-smoked pork</td>
<td>3</td>
<td>14</td>
<td>0 (0)</td>
</tr>
<tr>
<td>O</td>
<td>Cold-smoked pork</td>
<td>3</td>
<td>24</td>
<td>16 (67)</td>
</tr>
<tr>
<td>P</td>
<td>Cold-smoked pork</td>
<td>ND</td>
<td>4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other (Q-T)</td>
<td>Cold-smoked pork</td>
<td>ND</td>
<td>11</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>523</td>
<td>158</td>
<td>158 (30)</td>
</tr>
</tbody>
</table>
5.2. *Listeria monocytogenes* contamination in cold-smoked pork products (II, III)

5.2.1. Manufacturing of cold-smoked pork products and factors associated with *L. monocytogenes* contamination (II)

Manufacturing of cold-smoked meat products was studied in seven meat-processing plants producing similar products; the prevalence of *L. monocytogenes* varied from 0 to 67%. All production processes were divided into manufacturing steps and analysed as separate variables. The prevalence of *L. monocytogenes* was significantly higher (*P*<0.05) in the cold-smoked meat products of plants A (designated IV in study II), C (I), M (III) and O (VII), than in products from plants B (II), D (VI) and N (V). The presence of *L. monocytogenes* correlated with the use of brining injections and cold-smoking time. Based on bivariate correlations, the four most significant variables: brining procedures, cold-smoking time, cold-smoking temperature, and total time at a temperature between 10°C and 30°C, were selected for use in a multivariate logistic regression model. Brining injections were a significant (*P*<0.05) factor in product contamination with *L. monocytogenes*. A long cold-smoking time (≥12 h) had a significant (*P*<0.014) predictive value for testing positive for *L. monocytogenes*, whereas cold-smoking at 24-30°C for 8-48 h carried a significant (*P*<0.002) predictive value for testing negative for *L. monocytogenes*.

5.2.2. Sources and routes of *L. monocytogenes* contamination in cold-smoked meat processing (III)

Meat plant B was chosen to study contamination patterns of *L. monocytogenes* in a cold-smoked pork-processing environment. Overall, 12 raw, defrosted pork samples imported from Germany and Belgium were positive for *L. monocytogenes* (18%). All environmental samples of the meat cutting area before production were negative for *L. monocytogenes*; however, the number of *L. monocytogenes*-positive environmental samples increased after the cutting area. A total of 10 environmental samples (55%) were positive for *L. monocytogenes* in the brining area. Most of the contaminated environmental samples were associated with the brining machine and the brining area. Overall, four (80%) out of the five samples taken from personnel of the brining area, including coverall, apron, rubber glove and plastic boot-cover
samples, were positive for *L. monocytogenes*. One (25%) of four samples taken from the brining machine was positive already before the beginning of production. *L. monocytogenes* was recovered from smooth surfaces, spaces, feeding teeth and the plastic curtain of the brining machine. The number of *L. monocytogenes*-positive pork samples clearly increased after brining injections or more specifically after contact with the brining machine. Overall, six (60%) of the 10 raw pork samples were positive for *L. monocytogenes* after brining injections.

The one PFGE type belonging to serotype 4b was found in all processing stages from the raw, defrosted pork, pork-processing area to finished RTE cold-smoked pork, whereas other PFGE types (serotypes 1/2a and 1/2c) were found either in raw pork or in the meat-processing environment. Raw pork contained eight different *L. monocytogenes* PFGE types, three of which were found also in the finished cold-smoked pork products. Moreover, two of the *L. monocytogenes* PFGE types belonging to serotype 1/2a that were isolated from raw pork were found later on the feeding teeth of the brining machine. All meat-processing environment-associated PFGE types isolated from the finished RTE cold-smoked pork products were also associated with the brining area. Overall, four *L. monocytogenes* PFGE types belonging to serotypes 1/2a and 4d were found only in the meat-processing environment and not in the incoming raw pork or in the finished RTE cold-smoked pork products.

5.3 Heat resistance of *Listeria monocytogenes* after post-package pasteurization (IV)

D-values at all heating temperatures for high-fat content (26%) and low-fat content (3%) cooked sausages decreased with increasing temperatures. No differences were found in D-values between high- and low-fat content cooked sausages at any of the heating temperatures. D-values at 55°C, 60°C, 62.5°C and 65°C for high-fat cooked sausages were 112.3, 7.4, 5.1 and 1.7 min and for low-fat content sausages 122.0, 14.5, 7.7 and 1.5 min, respectively. No significant differences in z-values were found for either sausage type.

Low-fat cooked sausages had significantly lower (*P*<0.05) cook loss than the high-fat formulation at all temperatures and times evaluated. Moreover, within a sausage type, the amount of cook loss was greater (*P*<0.05) as heating temperature increased. The high-fat formulation had excessive excessive lipid and moisture loss in the vacuum package, whereas
packages of low-fat sausages showed no obvious lipid and moisture loss. Thus, high-fat cooked sausages released more moisture during pasteurization, especially at higher temperatures (IV, Figs 3 and 4).

5.4 Human listeriosis cases in Latvia (V)

A total of 90 listeriosis cases were identified in Latvia for the period 1998-2007 (V, Table 1). The highest number of cases, 36, was registered in 2000. Most cases of listeriosis were registered in Riga and Riga district: 65 (72%) and 13 (15%), respectively. Only 12 cases (13%) were found elsewhere in Latvia (Daugavpils, Liepāja and Ventspils). During the 10-year study period the overall incidence of listeriosis was 0.4 per 100 000 population. The two highest incidences were recorded in 2000 and 2002 (1.5 and 0.7 per 100 000 population, respectively).

A marked clustering of listeriosis cases was observed over the September to December 2000 period, indicating a large outbreak. Since 2002, a significant decrease in listeriosis cases was observed in Latvia. Overall, 71% of the reported listeriosis cases were observed in individuals of the working population aged between 18 and 49 years (V, Table 1). Listeriosis in individuals aged over 60 years accounted for 10% of the cases. Children aged less than six years accounted for 4% of cases. Listeriosis occurred most often (69%) in female patients aged between 18 and 39 years in Riga and Riga district. However, only three perinatal cases were officially registered during 2001 and 2005.

Complete clinical information was collected for 13 *L. monocytogenes* infections in humans in Riga and Riga district. Four of these cases, two of which were infants, had a fatal outcome. Overall, 77% of all described clinical cases were characterized by meningoencephalitis and/or sepsis. One case in 2002 was described as a gastrointestinal manifestation of listeriosis. No information was found to indicate the presence of any underlying conditions in the listeriosis patients. The overall mortality, including spontaneous abortions and perinatal infant deaths, was 6.7% or six of the 90 cases.
Seasonal distribution of human listeriosis cases in Latvia from 1998 to 2007 is shown in the original publication (V, Fig. 2). No significant seasonal variations were observed, except for a clustering of listeriosis cases during the September-December period in 2000.
6. DISCUSSION


The prevalence of \textit{L. monocytogenes} in sliced, vacuum-packaged, cooked or hot-smoked meat products and sliced, vacuum packaged, cold-smoked meat products varied from 0 to 10\% and from 0 to 73\%, respectively (Table 4). Cold-smoked, sliced, vacuum-packaged beef and pork products showed the highest levels of contamination with \textit{L. monocytogenes}. Both cold-smoked meat products have similar manufacturing methods involving no processing steps to eliminate \textit{L. monocytogenes} from the finished products, which may explain the high prevalence of \textit{L. monocytogenes}. Regardless of the variable shelf-life of products (20-120 days) set by the meat-processing plants, levels of \textit{L. monocytogenes} at the end of shelf-lives were mostly <100 CFU/g. This may suggest that cold-smoked meat products have some \textit{L. monocytogenes}-inhibiting factors. The wide use of starter cultures in the manufacture of cold-smoked meat products may explain the low levels of \textit{L. monocytogenes}. The use of starter cultures in the production of meat products has been observed to be an important step to control \textit{L. monocytogenes} growth (Farber and Peterkin 1991, Ingham \textit{et al.} 2004, Thévenot \textit{et al.} 2005, Pal \textit{et al.} 2008). The naturally occurring microbial population of cold-smoked meat products is also, to some extent, able to control growth of \textit{L. monocytogenes}. However, contamination of \textit{L. monocytogenes} in cold-smoked meat products is still of some concern to manufacturers and retailers.

\textit{L. monocytogenes} serotypes 1/2a, 1/2b, 1/2c, 3b and 4b were found in RTE meat products. Several studies have shown that \textit{L. monocytogenes} strains isolated from meat-processing plants are predominantly of serotypes 1/2a, 1/2b and 1/2c (Lundén \textit{et al.} 2000, Thévenot \textit{et al.} 2006). Moreover, especially serotype 1/2c has been demonstrated to be able to adhere to and persist on food processing surfaces (Norwood and Gilmour 1999, Lundén \textit{et al.} 2002). Lundén \textit{et al.} (2002, 2003) subsequently showed that serotype 1/2c strains also possess the highest adherence properties on stainless steel surfaces, thus facilitating development of persistent microbial population in meat-processing plants.
PFGE typing results revealed a high genetic diversity of *L. monocytogenes* isolates obtained from meat products of several meat-processing plants. This may indicate the existence of various sources of contamination at different production stages in meat plants. In addition to the number of non-persistent strains, persistent PFGE types were detected over several months in meat-processing plants A, B and C. Moreover, identical PFGE types were recovered from different production lots of vacuum-packaged, cold-smoked pork and beef produced by the same meat-processing plant over a 2-month period. In meat processing plant A, two of the seven PFGE types were found repeatedly in cold-smoked pork, dried beef “Basturma” and cold-smoked beef. This may indicate that continuous *L. monocytogenes* contamination existed in the production line of both pork and beef products. One *L. monocytogenes* PFGE type was recovered from six production lots of cold-smoked beef and pork products, revealing *L. monocytogenes* contamination as a continuous and recurrent contamination problem in meat-processing plant A.

6.2. *Listeria monocytogenes* contamination in cold-smoked pork products (II, III)

6.2.1. Manufacturing of cold-smoked pork products and factors associated with *L. monocytogenes* contamination (II)

The manufacture of cold-smoked pork involves no processing steps to eliminate *L. monocytogenes*, thus contamination of the raw meat and contamination during processing can both contribute to *L. monocytogenes* in the finished product. Several investigators have shown that the occurrence of *L. monocytogenes* in the plant environment and on processing surfaces has a significant positive relationship with the presence of this organism in finished products (Eklund *et al.* 1995, Autio *et al.* 1999, Chasseignaux *et al.* 2001; Aarnisalo *et al.* 2003, Heir *et al.* 2004, Lin *et al.* 2006).

The multivariate logistic regression model indicated that brining, cold-smoking time, and cold-smoking temperature were factors affecting product contamination with *L. monocytogenes*. Autio *et al.* (1999) previously showed that injected brines were an important source of the *L. monocytogenes* found in cold-smoked rainbow trout. Recirculation of contaminated brine through injection machines may also increase dissemination of *L. monocytogenes* during production (Autio *et al.* 1999, Gailey *et al.* 2003, Greer *et al.* 2004).
Eradication programmes, including disassembly of machines, thorough cleaning and disinfection of all equipment components, have been successful in eliminating *L. monocytogenes* from brining equipment (Autio et al. 1999). 

A long cold-smoking time (≥12 h) was also predictive of a sample testing positive for *L. monocytogenes*, but use of a cold-smoking temperature between 24°C and 30°C may reduce *L. monocytogenes* contamination. Eklund et al. (1995) observed that cold-smoking temperatures of 22-30°C decreased surface populations of *L. monocytogenes* in cold-smoked fish products. However, *L. monocytogenes* injected into the flesh increased during cold-smoking at these temperatures, and Rørvik et al. (2000) found that brine-injected *L. monocytogenes* can grow during cold-smoking. Thus, prolonged cold-smoking may allow the growth of *L. monocytogenes* in cold-smoked pork if contaminated brine has been injected prior to cold-smoking.

**6.2.2. *Listeria monocytogenes* contamination patterns in cold-smoked meat processing (III)**

The number of *L. monocytogenes*-positive environmental samples significantly increased in the brining area, representing brining machine and personnel working with brining procedures as the most contaminated sites in meat-processing plant B. After thorough surface sampling of the brining machine before and during use, three different contamination sites were identified. Feeding teeth of the brining machine harboured *L. monocytogenes* already before production and after cleaning and disinfection, whereas an additional two *L. monocytogenes* contamination sites were found on the plastic curtain, smooth surfaces and spaces of the brining machine during production. Moreover, all samples taken from coveralls, rubber gloves and plastic boot-covers of personnel from the brining area were positive for *L. monocytogenes*. Thus, the overall prevalence of *L. monocytogenes* in raw pork (18%) increased to 60% after brining injections. The brining area of the meat-processing plant was located next to the cutting area, where intensive movement of personnel between different production areas was observed, thus possibly increasing the area and sites of *L. monocytogenes* contamination. Rørvik (2000) also showed that rotation of assigned duties among personnel from different departments may spread *L. monocytogenes* in the fish-processing facilities. Autio et al. (1999) demonstrated that brining and post-brining areas were the most-contaminated sites in cold-
smoked fish-processing facilities. Moreover, brining equipment may increase contamination and facilitate spread of *L. monocytogenes* to the fish or meat products during processing (Autio et al. 1999, Greer et al. 2004).

Only one *L. monocytogenes*-positive sample was detected in the slicing and packaging area, and none of the positive samples was found in the smoking area of meat-processing plant B. Both areas of the meat-processing plant were well separated from the other facilities of the raw and processed meats, thus maintaining good manufacturing and hygienic procedures in an effort to avoid *L. monocytogenes* contamination during production. The degree of compartmentalization had an impact on *L. monocytogenes* contamination status in the meat-processing plants. Thus, less compartmentalized areas of the processing line have been more frequently contaminated and for longer periods of time than the processing lines with well-separated areas (Tompkin 2002, Lundén et al. 2003).

The prevalence of *L. monocytogenes* was significantly higher (*P < 0.001*) in the finished cold-smoked pork products where brining injections were applied than in RTE cold-smoked pork products where dry salting and ripening alone were used in meat-processing plant B. The brining area and the staff of these facilities were the most contaminated sites.

In total, two *L. monocytogenes* PFGE types were found in raw pork and finished cold-smoked pork products, whereas one PFGE type originating from raw pork was recovered later only from the meat-processing environment. Hoffman et al. (2003) concluded that environmental contamination is largely separate from that of incoming raw materials and includes strains persisting, possibly for years, within the fish-processing plant. Our findings revealed that a genetically diverse population of *L. monocytogenes* entered the meat-processing plant with raw material, but only some of the strains colonized and established a persistent microbial community within the plant over a 5-year period. Lundén et al. (2005) emphasized that *L. monocytogenes* is an exceptionally well-adapted pathogen to food-processing environments and may persist within a plant from a few months to several years. Raw materials are probably the most important source of contamination (Berrang et al. 2002).

The feeding teeth of the brining machine harboured three different PFGE types belonging to serotypes 1/2c, 1/2a and 4b. One PFGE type was found on the feeding teeth of the brining
machine and in finished RTE cold-smoked pork products 5 years earlier, thus showing persistent \textit{L. monocytogenes} contamination in the brining machine. The contaminated feeding teeth of the brining machine have an uneven surface that is in direct and continuous contact with meat, thus facilitating spread of \textit{L. monocytogenes}. Two \textit{L. monocytogenes} PFGE types were found in raw meat and in brining area during production. The complexity and poor hygienic design of the brining machines could facilitate further spread of the \textit{L. monocytogenes} in the meat-processing environment, especially when improper cleaning and disinfection procedures are applied (Autio et al. 1999, Lundén et al. 2003, Greer et al. 2004).

Our study showed that two different \textit{L. monocytogenes} PFGE types belonging to serotypes 1/2a and 1/2c were recovered from the different sites of the brining machine and caused persistent contamination of RTE cold-smoked pork products over a 5-year period. Moreover, the brining machine also harboured three PFGE types belonging to serotypes 1/2a and 4d, which were found on the feeding teeth, smooth surfaces and spaces and plastic curtain of the machine.

\textbf{6.3. Impact of post-package heat treatment in controlling \textit{Listeria monocytogenes} in vacuum-packaged meat products (IV)}

Post-package pasteurization of high- and low-fat cooked sausages at temperatures higher than 55°C was an effective post-processing thermal treatment method to reduce contamination of \textit{L. monocytogenes}. However, heating at 55°C, 60°C and 62.5°C may not be practical in the meat industry because the process takes too long to reach a 3-log reduction.

D-values for high-fat content (26%) and low-fat content (3.2%) cooked sausages were similar to those reported for RTE smoked turkey and roast beef. Differences in D-value and z-value for smoked ham may be attributed to different inherent properties of the food and product formulation (Doyle et al. 2001). A product with higher fat content is expected to have higher thermal tolerance (Mackey et al. 1990, Doyle et al. 2001); however, there are many other ingredients and factors in cooked sausages that can affect thermal resistance. In addition, a decrease in water activity also increases microbial heat resistance.
Thermal inactivation kinetics differed when comparing our results for cooked sausages with other studies with other RTE meat products because of inherently different properties in each food matrix. Kinetic differences among meat products may be attributed to determination of “come-up” times, strain-to-strain variations, methods used for heat treatment and variations in plating media used to resuscitate and enumerate injured cells.

High-fat cooked sausages released more moisture during pasteurization, especially at higher temperatures. Greater cook loss (fat and moisture) in high-fat products is usually related to fat translocation to the surface of the product. Although post-package pasteurization of sliced, cooked, vacuum-packaged sausages is an effective post-processing thermal treatment to reduce contamination of *L. monocytogenes*, moisture loss may cause undesirable features from both aesthetic and economic viewpoints.

### 6.4 Human listeriosis in Latvia in the past, present and future (V)

Continuous registration of listeriosis cases has been carried out in Latvia by State Public Health Agency since the end of the 1990s. However, only limited epidemiological information has been recorded to date. The incidence of listeriosis varied from 0.04 to 1.5 per 100,000 of the population in 1999 and 2000, respectively. The highest incidence of listeriosis in Latvia was observed in 2000, which significantly exceeded incidence levels in all Baltic and Nordic countries, and was the highest among all EU member states for the period. The majority of the listeriosis cases (87%) were found in Riga and Riga district, which represents 40% of the population of the whole country during the study period.

A tremendous increase in listeriosis cases was observed from September to December 2000 in Riga and Riga district. This manifested as a marked clustering of listeriosis cases, possibly indicating a large listeriosis outbreak during the four-month period. Unfortunately, no detailed information about these cases was collected from the hospitals, and no subtyping methods were used for *L. monocytogenes* isolates to get more detailed information about the outbreak. Such a lack of coordinated response suggests that some of the other listeriosis outbreaks may have also been left unidentified in the past.
A total of 9 (10%) of 90 listeriosis cases occurred in patients older than 60 years. In contrast to other studies (Lyytikäinen et al. 2000, de Valk et al. 2001, Antal et al. 2007, EFSA 2007, 2009, Painter et al. 2007), we found that the age group over 60 years was not predominant. This may be explained by the rare occurrence of listeriosis resulting in some cases going unidentified (Daņileviča et al. 2004). The discrepancy might also arise from masking by underlying clinical conditions that are often found in older people. On the other hand, delicatessen products, which contain various numbers of *L. monocytogenes*, are often too expensive for pensioners to afford.

Since 2004, a national control programme for *Listeria monocytogenes* has been implemented. This measure was based on Regulation EC No. 2160/2003 of the European Parliament and of the Council on the control of *Salmonella* and other specified foodborne zoonotic pathogens. Moreover, specific control measures have also been implemented by the food industry since Regulation EC 2073/2005 on microbiological criteria for foodstuffs was enforced (Anonymous 2005, Smulders et al. 2008). Concrete control measures were also implemented to minimize the occurrence of *L. monocytogenes* in meat products. The implementation of these measures by the competent authorities and food industry could be one of the reasons why the overall incidence of listeriosis has decreased in Latvia from 0.7 per 100 000 population in 2003 to 0.2 per 100 000 population in 2007.

Our study also revealed that the present listeriosis surveillance system requires improvements. More thorough listeriosis case reports should be disseminated to all hospitals to provide more detailed information and promote proper epidemiological investigations. Moreover, routine-based serotyping and molecular subtyping of *L. monocytogenes* are needed to detect case clusters and common-source foodborne outbreaks. Previous studies have shown that serotyping and molecular subtyping by PFGE are useful in identifying specific clusters of listeriosis cases or outbreaks in addition to merely identifying the source of the outbreak (Gellin et al. 1989, 1991, Sim et al. 2002, Lukinmaa et al. 2003, 2004, Antal et al. 2007). The lack of serotyping and molecular typing methods used for subtyping of *L. monocytogenes* isolates in the present laboratory-based surveillance system is one of the main reasons why particular food items were not implicated or traced to particular listeriosis case in Latvia. Serotyping and molecular typing should be introduced for all clinical isolates of *L. monocytogenes* to identify listeriosis outbreaks as early as possible so that the appropriate
actions at the food industry level can be undertaken. This should prevent or significantly reduce the real burden of listeriosis in Latvia.
7. CONCLUSIONS

1. The prevalence of *L. monocytogenes* in cold-smoked meat products (40%) was significantly higher than in heat-treated RTE meat products (0.7%) (*P* < 0.001). The majority of *L. monocytogenes*-positive samples (83%) contained <100 CFU/g at the end of the product’s shelf-life. Moreover, high genetic diversity was found among *L. monocytogenes* isolates, suggesting the existence of various sources of contamination at different production stages in the cold-smoked meat-processing environment.

2. The manufacture of cold-smoked meat products involves no processing steps to eliminate *L. monocytogenes*, thus contamination of the raw meat and contamination during processing can both contribute to *L. monocytogenes* in the finished product. The logistic multivariable regression model was a successful tool for identifying the main factors associated with *L. monocytogenes* contamination during the manufacturing of cold-smoked pork products. Brining by injections was a significant factor (odds ratio 10.66; *P*<0.05) for contamination of products with *L. monocytogenes*. Moreover, the most contaminated sites were associated with the brining machine and brining area. One of *L. monocytogenes* PFGE types, belonging to serotype 1/2c, was isolated from RTE cold-smoked meat products and from the feeding teeth of the brining machine. Thus, improper cleaning, disinfection and poor hygiene design of the brining machine may have caused *L. monocytogenes* over a 5-year period. Long cold-smoking times (≥ 12 h) also had a significant predictive value (odds ratio 24.38; *P*<0.014) for a sample testing positive for *L. monocytogenes*.

3. Post-package pasteurization of high- and low-fat cooked sausages, at temperatures higher than 55°C, was an effective means of reducing of *L. monocytogenes* contamination. Fat content did not affect *L. monocytogenes* heat resistance in cooked sausages at 55°C, 60°C or 65°C. No difference in D-value was found between high- and low-fat cooked sausages at any of the heating temperatures studied; however, the formulation of high-fat RTE cooked sausages may require some modifications to maintain product sensory quality.
4. During the 10-year study period the overall incidence of listeriosis was 0.4 per 100 000 population, with the highest incidences recorded in 2000 and 2002 (1.5 and 0.7 per 100 000 population, respectively). A marked clustering of human listeriosis cases was observed from September to December 2000, possibly indicating one large outbreak. In addition, the lack of serotyping and molecular typing methods for subtyping of \textit{L. monocytogenes} isolates in the present surveillance system is one of the main reasons why there are no officially documented listeriosis outbreaks in Latvia to date. Such measures would allow appropriate actions to be taken at the food industry level, thus either preventing or significantly reducing the real burden of foodborne listeriosis in Latvia.
REFERENCES


