Listeria monocytogenes in ready-to-eat vacuum and modified atmosphere packaged meat and fish products of Estonian origin at retail level

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1. Introduction

Listeria monocytogenes is the causative agent of a severe foodborne infection, listeriosis which shows increasing trend during recent years in Europe (EFSA, 2015). The notification rate for human listeriosis in 2013 was 0.44 cases per 100,000 population which indicates an 8.6% increase compared with 2012 (EFSA, 2015). According to the data of the Estonian Health Board (Terviseamet, 2015) the average notification rate of human listeriosis cases was 0.15 per 100,000 inhabitants from 2011 to 2014 in Estonia.

The main route of transmission of L. monocytogenes to humans is through the consumption of contaminated food (Allerberger & Wagner, 2010). The majority of the listeriosis outbreaks have been linked to certain RTE foods with extended storage time, and is related with the fact that L. monocytogenes can grow to high counts at refrigeration temperatures (Ericsson et al., 1997; Gottlieb et al., 2006; Lyttikainen et al., 2000; McLauchlin, Hall, Velani, & Gilbert, 1991; Miettinen et al., 1999; Sim et al., 2002; Stephan et al., 2015).

RTE meat and fish products with a long shelf-life are associated with the high risk of transmission of L. monocytogenes. In RTE fish and fishery products the prevalence of L. monocytogenes has been 10.8%, and the criterion of 100 CFU/g during the shelf-life of a product was exceeded in 1.6% of the samples in EU year 2013 (EFSA, 2015). Furthermore, L. monocytogenes was detected in 3.4% and 2.3% of pig and bovine RTE meat products, and L. monocytogenes was found at the levels exceeding the 100 CFU/g in 1.0%, 0.0% and 0.4% of the RTE poultry, bovine and pig meat samples, respectively (EFSA, 2015).
Present study aimed to determine the prevalence, counts and genetic similarity of *L. monocytogenes* in Estonian origin RTE fish and meat products. Data will further be used for quantitative risk assessment of *L. monocytogenes*.

2. Materials and methods

2.1. Sampling

A total of 185 RTE meat and 185 RTE fish product samples were collected monthly during two years period at Estonian retail level from the most consumed RTE fish and meat products in the biggest supermarkets. Samples originated from 13 fish and 13 meat enterprises. RTE fish and meat VP and MAP products of Estonian origin having a long shelf-life, more than four weeks, were collected. Among the RTE fish products cold-smoked, hot-smoked, salted (including gravad fish) and matured fish products were sampled. Most of the salted fish products were lightly salted with final sodium chloride content of 1.3–1.5%. Among the RTE meat products cold-smoked, hot-smoked, cooked and fermented meat products were sampled.

At retail outlets before sampling the storage temperature was recorded and all the products were kept at proper refrigerated temperature conditions not exceeding 4 °C, and transported to the laboratory immediately. The samples were stored under the refrigerated temperatures in accordance with product labeling information at maximum allowed storage temperature. All analyses of the products were performed at use-by-date.

2.2. Isolation and enumeration of *L. monocytogenes*

The isolation of *L. monocytogenes* was carried out in Estonian Veterinary and Food Laboratory and all the analyses and confirmation tests were performed in accordance with instructions of the detection methods described by EVS-EN ISO 11290-1:2000/A1:2004. Examination for *L. monocytogenes* included a primary and secondary enrichment. All samples were incubated in half Fraser broth (Oxoid, Basingstoke, Hampshire, England) at 30 °C for 24 h. After the incubation, 0.1 ml was transferred to a tube containing 10 ml of Fraser broth and incubated at 37 °C for 48 h. After the incubation, the half- and full-strength Fraser broths were plated-out on ALOA agar (Lab M Ltd., Bury, Lancashire, UK) and PALCAM agar (Oxoid). Selective agar plates were incubated at 37 °C for 24–48 h. Typical colonies (n = 5) presumed to be *Listeria* spp. were streaked from both PALCAM and ALOA agars onto the tryptone soya yeast extract agar (TSYEA) made in house from single components (Oxoid) and plates were incubated at 37 °C for 24 h. The confirmation tests were performed using the pure culture obtained from TSYEA. Isolates that were catalase and Gram positive and with characteristic tumbling motility were inoculated on 5% sheep blood agar (Oxoid) plates to determine the haemolytic reaction, β-haemolysin for *L. monocytogenes*. For further confirmation, carbohydrate utilization and CAMP tests were performed. All confirmed *L. monocytogenes* isolates were stored at −80 °C in bacterial protect tubes (TSC Ltd, Heywood, Lancashire, UK).

Enumeration of *L. monocytogenes* was carried out according to the method described by EVS-EN ISO 11290-2:2000/A1:2004. In all cases enumeration was performed at the use-by-date. For making initial suspensions and dilutions (10⁻¹ to 10⁻³) buffered peptone water (ISO, Oxoid) was used. The procedure included 1-h resuscitation in buffered peptone water at room temperature, and surface plating on ALOA agar of 1.0 ml of 10⁻¹, and 0.1 ml of each of the 10⁻² and 10⁻³ dilutions to duplicates of the ALOA plates. 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.

2.3. Serological typing

In house method of EU Reference Laboratory for *L. monocytogenes* (AFSSA, 2009) was followed for conventional serotyping. Somatic (O) and flagellar (H) antigenes were identified by using specific antisera produced by Denka Seiken (Tokyo, Japan).

2.4. In situ DNA isolation and pulsed-field gel electrophoresis (PFGE)

In situ DNA isolation and PFGE were performed as described earlier by Autio et al. (2002), Agarose-embedded DNA was digested with restriction endonuclease Ascl (New England Biolabs, Ipswich, USA). PFGE patterns were analyzed using BioNumerics software, version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium), and similarity between PFGE patterns was expressed as a Dice coefficient (position tolerance 1.0%). Clustering and construction of dendrograms were performed by using the unweighted pair-group method with arithmetic averages.

2.5. Statistical analyses

All individual results were recorded using MS Excel 2010 software (Microsoft Corporation, Redmond, Wash.), and statistical analysis was performed with the Statistical Package R in order to determine if there were statistically significant differences at 95% confidence interval.

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**Table 1**

*L. monocytogenes* in RTE meat and fish products of Estonian origin.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of positive samples/positive %</th>
<th>CI95% of positive %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTE meat</td>
<td>185</td>
<td>11/5.9</td>
<td>3.4–10.3</td>
</tr>
<tr>
<td>RTE fish</td>
<td>185</td>
<td>31/16.8</td>
<td>12.1–22.8</td>
</tr>
<tr>
<td>Total</td>
<td>370</td>
<td>42/11.4</td>
<td>8.5–15.0</td>
</tr>
</tbody>
</table>

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**Table 2**

*L. monocytogenes* presence and counts in different RTE fish and meat products.

<table>
<thead>
<tr>
<th>Product category</th>
<th>No. of samples</th>
<th>Negativea</th>
<th>Positiveb</th>
<th>10 ≤ 100</th>
<th>&gt;100</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTE meat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold-smoked</td>
<td>66</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hot-smoked</td>
<td>70</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cooked</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fermented</td>
<td>20</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>All RTE meat</td>
<td>174</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RTE fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold-smoked</td>
<td>62</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hot-smoked</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Matured</td>
<td>38</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Salted</td>
<td>52</td>
<td>17</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>All RTE fish</td>
<td>154</td>
<td>31</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total (%)</td>
<td>328 (88.6%)</td>
<td>42 (11.4%)</td>
<td>6 (1.6%)</td>
<td>1 (0.3%)</td>
<td></td>
</tr>
</tbody>
</table>

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*a* Absence in 25 g.  
*b* Presence in 25 g.
and 99% confidence levels in the prevalence and counts of *L. monocytogenes* in RTE fish and meat products of different categories, producers and retail outlets using the Kruskal–Wallis rank sum test and Chi-square test.

### 3. Results and discussion

The overall prevalence of *L. monocytogenes* in RTE meat and fish products is shown in Table 1. The contamination of RTE fish products (16.8%) with *L. monocytogenes* was significantly higher than of RTE meat products (5.9%). Similarly, the European baseline survey carried out in 2010 and 2011 concluded, that the EU wide prevalence of *L. monocytogenes* was highest in fish products (10%) and lower in meat products 2.1% at the end of the products shelf-life (EFSA, 2013). Similar prevalence of RTE fish has been reported in different studies performed in Bulgaria (Gyurova, Krumova-Vulcheva, Daskalov, & Gogov, 2015), Finland (Lyhs, Hatakka, Maki-Petays, Hyytia, & Korkeala, 1998), Iran (Fallah, Saeid-Dehkordi, & Mahzounieh, 2013) and Northern Spain (Garrido, Vitas, & Garcia-Jalon, 2009). Another Spanish study by González, Vitas, Díez Leturia, and García-Jalón (2013) found only 4.8% of smoked salmon products to be *L. monocytogenes* positive with low levels. In a previous Estonian study by Kramarenko et al. (2013) performed in 2008–2010 the prevalence of *L. monocytogenes* in RTE fish was 5.7% and in RTE meat products 2.0%. According to the EFSA (2015) the overall *L. monocytogenes* prevalence in RTE fish and fishery products in EU was higher than in RTE meat products. *L. monocytogenes* prevalence found in current study showed higher contamination percentages compared to the EU average. However, in the present study the analyses focused only to the VP and MAP.
ready-to-eat products with long shelf-life. Additionally, 39% and 76% of the RTE meat and fish products sampled in present study were not heat-treated e.g., were cold-smoked or lightly salted, therefore belonging to the known high risk food categories. Also, Lado and Youssef (2007) reported that L. monocytogenes can grow in vacuum packages (VP) as well as in modified atmosphere packages (MAP) which extend the shelf-life of foods, giving the opportunity for L. monocytogenes to multiply to high numbers towards the end of shelf-life, especially if the recommended temperature during the storage is not maintained.

The prevalence of L. monocytogenes in RTE meat products has been generally low but controversially in a recent Greek study by Manios et al. (2014) overall 28% of tested RTE meat products were contaminated. Also in a Belgium study L. monocytogenes was detected in 27.8% (25/90) smoked fish samples (Uyttendaele et al., 2009).

Enumeration of L. monocytogenes in RTE meat and fish products of Estonian origin (Table 2) resulted with only one RTE fish product exceeding the limit of 100 CFU/g at the end of the shelf-life. All the RTE meat products of Estonian origin were in compliance with the EU food safety criterion. In comparison, 1.6% of RTE fish and 0.7% of RTE meat products was found to exceed the criterion of 100 CFU/g in the EU wide survey in average (EFSA, 2015).

L. monocytogenes was not detected in 328 samples (89%) and only 1.6% of the RTE fish and meat products contained L. monocytogenes in range of 10–100 CFU/g and 0.3% more than 100 CFU/g at the end of shelf-life (Table 2). For a healthy human population, foods where the levels do not exceed the 100 CFU/g limit are considered to pose a very low or negligible risk (EFSA, 2015).

L. monocytogenes positive samples were found from the products of three out of thirteen meat plants. One meat plant (coded as M2) had proportion of L. monocytogenes positive RTE meat products 19% while for others it was ranging from 0%–6.7%. Among different RTE meat product categories the highest L. monocytogenes prevalence (3.8%) was found for cold-smoked meat products (Table 2). Also in previous Baltic studies cold-smoked pork and beef products have been found to have high L. monocytogenes prevalence (Berižiņš, Horman, Lundén, & Korkeala, 2007; Berižiņš, Terentjeva, & Korkeala, 2009). In other RTE meat categories the L. monocytogenes prevalence was low.

PFGE analysis revealed that one pulsotype (P11) predominated in the products of company M2 (Fig. 1; Table 3). In addition, all strains were isolated from cold-smoked meat products of different batches, which suggests a possible persistent L. monocytogenes contamination in the food processing plant. L. monocytogenes can persist in food processing plants even for several years and cause postprocessing contamination of the food products (Keto-Timonen et al., 2008; Lundén, Autio, Sjöberg, & Korkeala, 2003).

L. monocytogenes positive samples were found from the products of seven out of thirteen fish plants (Table 2). Among different RTE fish product categories the highest L. monocytogenes prevalence (12%) was found for salted fish products (Table 2). The prevalence was higher than in other fish product categories (p-value <0.05). In the present study only one RTE product, salted salmon fillet slices, was found to exceed the criterion of 100 CFU/g at the use-by-date.

We found one predominant pulsotype (P3) for fish plant F3 (Fig. 1) which was associated with various salmon products at different sampling times. It may indicate the existence of persistent L. monocytogenes in the fish plant. At the same time latter pulsotype was also found in products of other plants (F1, F5, M1, M3) which means that dominating strains in certain food enterprises are not always plant-specific. Similar findings were described by Autio et al. (2002).

Serotyping revealed that all L. monocytogenes isolates belonged to the 1/2a serotype which is similar to our previous findings (Kramarenko et al., 2013).

4. Conclusion

Study showed that in vacuum and modified atmosphere packaged RTE fish products the prevalence of L. monocytogenes was higher than in similarly packaged RTE meat products. However, for both RTE fish and meat products the counts of L. monocytogenes were in compliance with the food safety criterion of 100 CFU/g during the products self-life, except for one product. Prevalence differences in RTE fish and meat products between food plants were found. Only serotype 1/2a was determined among L. monocytogenes isolates. PFGE genotyping revealed that the few predominant pulsotypes may be associated with particular food companies.

Acknowledgements

This study was supported by the Estonian Scientific Council (Sihtasutus Eesti Teadusagentuur, ETAg) Grant No. 9315, and by Ministry of Agriculture of Estonia, applied science project T13057/VTTH.

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