

# Prevalence and tracing of persistent *Listeria monocytogenes* strains in meat processing facility production chain

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## Abstract

This work was aimed at determining the occurrence and diversity of *Listeria monocytogenes* in a traditional meat-processing facility to reveal persistent contamination. A total of 268 samples, including 196 environmental samples and 72 meat samples were collected during a four-year period, and 70 were found to be *L. monocytogenes* positive. Molecular serotyping of 77 isolates classified these strains into four serogroups, with the majority of 34 strains in serogroup IIa. To reveal the persistent contamination, subtyping by AscI/ApaI-PFGE was applied. Cluster analysis resulted in strain discrimination into 18 profiles. Strains with PFGE/MLST-ST classification as 1/ST9, 2/ST14 and 9/ST2 were predominant and considered persistent. Based on the results of PFGE, a spatial and temporal map of persistent *L. monocytogenes* contamination was drawn. Subtyping by PFGE was used as a reliable tool to reveal persistent contamination and, complemented with virulence characteristics, can contribute to the improvement of *L. monocytogenes* control programs in food processing.

## Practical application

*Listeria monocytogenes* is a food-borne pathogen with significant public health and economic impact. This study, focused on prevalence and tracing of *L. monocytogenes* strains in a traditional meat-processing facility, revealed persistent contamination by the pathogen. Data of PFGE profiling from repeated samplings over a four-year period facilitated to draw a spatial and temporal map of persistent *L. monocytogenes* strains spreading in a meat-processing facility and, subsequently, will allow optimisation of cleaning and disinfection measures to ensure hygiene. Based on the detection of virulence factors, persistent ECIII and *comK* prophage-positive strains were observed, indicating a higher risk of contamination by more virulent *L. monocytogenes* strains. The obtained results clearly indicate, that most attention should be paid to control strategy improvement in the meat processing chain focused preferably on prevention, not only to final products.

## KEYWORDS

*Listeria monocytogenes*, meat processing, persistence, subtyping, tracing

## 1 | INTRODUCTION

*Listeria monocytogenes* is an important foodborne pathogen responsible for listeriosis, representing a significant hazard to human health, mainly in high-risk groups of newborns, elderly, immuno-compromised individuals and pregnant women (Vazquez-Boland et al., 2001). Listeriosis is associated with the fatality rate as high as 20–30%, with 17.8% in 2012 (EFSA, 2014) and 15.6% in 2013 (EFSA, 2015) reported in EU, which is the highest among foodborne pathogens. An increasing inci-

dence of listeriosis has been reported in several European countries, including Slovakia (EFSA, 2014, 2015). Food contaminated with *L. monocytogenes* is considered the primary source of transmission to humans (Dussurget, Pizarro-Cerda, & Cossart, 2008). According to European regulations, a zero tolerance concept is in force for *L. monocytogenes* in ready-to-eat foods supporting the growth of *L. monocytogenes* before leaving the immediate producers control and food business operators manufacturing ready-to-eat foods shall sample the processing areas and equipment for *Listeria monocytogenes* as part of

their sampling scheme (Commission regulation No. 2073/2005, 2005). This is particularly important in food-processing environment with a character able to support the growth of *L. monocytogenes*. In this regard, type and source of raw material, production environment, as well as equipment complexity in meat processing facility provides the excellent conditions.

Environmental monitoring allowing the identification of potential contamination sources and transmission routes in meat production chain, particularly of persistent *L. monocytogenes* strains is important. The presence of persistent strains could be mostly due to cleaning and disinfection procedures ineffectiveness, allowing survival and spreading of *L. monocytogenes* strains. Another factor that may contribute to persistence is the nature of the strains, in view of the fact that some are better adaptable to food production environments (Thévenot et al., 2006). Despite the regular cleaning and disinfection procedures of food processing equipment and environments, presence of persistent *L. monocytogenes* PFGE types associated with specific production environments over long time periods has been documented (Bolocan et al., 2016; Di Ciccio et al., 2012).

In relation to food hygiene control, molecular subtyping methods can be used to characterize *L. monocytogenes* isolates from the food processing chain allowing the identification of persistent contamination. Pulsed-field gel electrophoresis (PFGE), the method with higher reproducibility and discriminatory power in comparison to other typing methods, is currently considered the "gold standard" to track *L. monocytogenes*, in the food production chain as well (Blatter, Giezendanner, Stephan, & Zweifel, 2010; Thévenot et al., 2006).

Serotyping represents another scheme of *L. monocytogenes* sub-species characterization, which is partially related to virulence potential. Serotypes 1/2a, 1/2b and 4b are responsible for more than 90% of human infections. Most outbreaks are caused by strains of serotype 4b whereas serotype 1/2a, frequently isolated from food, has been traditionally associated with sporadic cases (Swaminathan & Gerner-Smidt, 2007). However, the apparent increase in serotype 1/2a outbreaks in Europe and North America in last ten years has been reported (Lomonaco, Nucera, & Filipello, 2015). Traditional serotyping depending on expensive antisera is possible to be overcome by multiplex-PCR enabling to cluster *L. monocytogenes* strains into five molecular serogroups (Kérouanton et al., 2010).

Subtyping of *L. monocytogenes* isolates from listeriosis outbreaks revealed that many outbreaks were caused by a small number of *L. monocytogenes* epidemic clones (ECs) worldwide (Almeida et al., 2013; Knabel et al., 2012). ECs have been considered more virulent than other strains (Fugett, Schoonmaker-Bopp, Dumas, Corby, & Wiedman, 2007) and/or may have better ability to adapt in food production environments where they are able to persist for years (Lomonaco et al., 2015). In respect to higher virulence potential, it was recently suggested that prophages integrated in *comK* gene may represent a rapid adaptation island (RAI) that allows *L. monocytogenes* strains to quickly adapt to and produce biofilms in specific niches (Verghese et al., 2011). ECs linked to outbreaks resulting from contamination in meat processing plants were shown to carry the *comK* prophage and were able to form strong biofilms (Verghese et al., 2011).

Meat products are important food vehicles of *L. monocytogenes*. Along with increasing EU production, market globalization, increasing consumers' interest is focused on traditional farm products. However, the character of a small traditional farming production with less staff and/or less experienced staff in food safety management could represent a higher risk of contamination.

In this study, *L. monocytogenes* contamination of small-size meat-processing facility, mostly oriented to the production of traditional ready-to-eat soft sausages, was investigated during a four-year period. The facility was almost new, well equipped, with most stainless steel food-contact surfaces and equipment. Appropriate cleaning and sanitation procedures were performed regularly according to the good hygienic and sanitation practice on a systematic basis. From this facility, *L. monocytogenes* strains were isolated and characterized by molecular serogrouping, PFGE and MLST subtyping and the detection of selected virulence/persistence markers. The aim was to evaluate the prevalence and diversity of the pathogen, as well as to reveal the sources of contamination and the routes of transmission, focusing on persistence potentially associated with more virulent *L. monocytogenes* strains.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

The samples were collected from a meat-processing facility in Slovakia over a four-year period, from November 2010 to December 2014 with a total of 15 sampling visits and a frequency of two to three samplings per year in 2011 to 2013 and six samplings in 2014. The facility represented a production chain, in which raw pork and beef meat as initial raw materials, purchased from a couple of suppliers, were processed. The samplings of the production environment were performed two hours after the end of individual production runs. From a total of 268 samples, 196 environmental samples were collected from different sites along the production chain; 106 samples from food-contact; and 90 samples from non-food-contact areas and equipment (including floors, floor drains, walls, washbasins, protective overshoes). A number of 72 food samples of raw meat, semi-products and final products including 56 ready-to eat products were analysed (Table 1). Sample numbers analysed per individual sampling visits divided to environmental and food samples are presented in Table 2. The sampling programme was designed to include sites of difficult sanitation, thus most likely to harbour *L. monocytogenes*, and focused on those previously detected positive. Environmental swabs were collected using a sampling kit (3M, St. Paul, Minnesota, United States) containing a sterile biocide-free sponge pre-hydrated with neutralizing buffer solution in sample bag. The sponge was dragged back and forth in order to cover the area of  $30 \times 30 \text{ cm}^2$ , then the sponge was turned over and direction was changed  $90^\circ$  and the same sampled surface was swabbed again. To analyse raw materials and meat products, amounts of 25 g were collected. Disposable equipment, such as rags or protective overshoes were collected as whole pieces. Each sponge and sample were placed into sterile bags immediately after sampling, kept refrigerated during transportation and analysed within 24 h.

**TABLE 1** Number of samples analysed and detected positive for *L. monocytogenes* in sampling period from 11-2010 to 12-2014 in meat-processing facility

	Number of samples Positive/analysed					
Sampling year	2010	2011	2012	2013	2014	Total
Environmental samples						
Food contact	1/9	1/20	1/22	5/12	8/43	16/106
Non-food contact	0/2	1/7	1/7	13/16	29/58	44/90
Subtotal	1/11	2/27	2/29	18/28	37/101	60/196
Food samples						
Ready-to-eat products	0/4	1/13	0/8	0/6	4/25	5/56
Raw meat products <sup>a</sup>	0/0	0/0	0/0	0/4	5/12	5/16
Subtotal	0/4	1/13	0/8	0/10	9/37	10/72
Total	1/15	3/40	2/37	18/38	46/138	70/268

<sup>a</sup>Final products intended to be treated by heat before consumption, including raw meat and raw meat semiprocessed.

## 2.2 | Detection and isolation

*L. monocytogenes* detection was performed using the standard culture method according to ISO 11290-1:1996. Briefly, after the 24-h pre-enrichment in half-Fraser broth, and then after the 24-h and 48-h selective subculture in Fraser broth (both Merck, Darmstadt, Germany), samples were streaked on the chromogenic Agar Listeria acc. to Ottaviani and Agosti (ALOA, Merck) in order to obtain well-isolated colonies. Up to five typical colonies from the positive samples, were streaked on Tryptose Soy Yeast Extract Agar (TSYEA, Merck) plates.

Isolated strains were maintained in 20% glycerol or freeze-dried for long-term storage at  $-18^{\circ}\text{C}$ .

## 2.3 | Real-time PCR

To confirm *L. monocytogenes* identity, all isolates were analysed by the specific TaqMan real-time PCR targeted to *actA* gene (Oravcová et al., 2006). DNA samples were prepared from overnight cultures in Tryptose Soy Yeast Extract Broth (TSYEB, Merck) using InstaGene Matrix (BioRad, Hercules, California) and the amplification was performed in

**TABLE 2** Prevalence of *L. monocytogenes* PFGE profiles isolated from individual samplings during the period from 11-2010 to 12-2014

Sampling	Sampling date	Number of samples Positive/analysed				Number <sup>a</sup> of strains	Ascl/Apal-PFGE profile																			
		FC	NFC	F	1		2	3	4	5	6	9	11	15	17	18	19	20	21	22	23	24	25			
30	11-10	1/9	0/2	0/4	1	1																				
34	03-11	0/6	0/2	0/5	0																					
37	06-11	0/6	1/2	1/5	2		1	1																		
39	10-11	1/8	0/3	0/3	1				1																	
46	02-12	0/7	0/2	0/2	0																					
54	05-12	1/7	0/2	0/2	1		1																			
62	11-12	0/8	1/3	0/4	1					1																
69	07-13	3/7	4/7	0/3	7	3	2					2														
74	10-13	2/5	9/9	0/7	11	1	3				1	5				1										
75	02-14	3/6	6/8	0/5	9	1	3				1	2		2												
76	04-14	3/12	4/11	0/0	9 <sup>b</sup>	2	1				1	1	1	3												
80	06-14	0/12	5/7	0/0	6 <sup>b</sup>	1	3					2														
82	08-14	0/7	7/10	2/2	9	3					1	5														
86	11-14	1/2	5/10	2/17	8		4								1		1			1		1		1		
87/88	12-14	1/4	2/12	5/13	12 <sup>b</sup>	2										3	1	2	1	1	1			1		
Total		16/106	44/90	10/72	77	14	18	1	1	1	4	17	1	5	1	4	2	2	1	2	1	1	1	1		

Abbreviations: FC, food-contact samples; NFC, non-food-contact samples; F, food samples.

<sup>a</sup>After the elimination of identical isolates from individual sample.

<sup>b</sup>Several isolates with different PFGE-profile obtained from individual samples.

Opticon 2 (MJ Research, Waltham, Massachusetts). All identified *L. monocytogenes* isolates were subjected to molecular characterization.

## 2.4 | PCR serogrouping

Multiplex gel-based PCR, (Kérouanton et al., 2010) targeted to serotype-specific gene markers was applied to cluster *L. monocytogenes* strains into five molecular serogroups IIa, IIb, IIc, IVa and IVb. The modified protocol using *prfA* gene marker (Rossmanith, Krassnig, Wagner, & Hein, 2006) specific for *L. monocytogenes* was described previously (Véghová et al., 2015). Amplification was performed in Veriti thermal cycler (Applied Biosystems Foster City, California, USA) and PCR products were analysed by 1.5% agarose gel electrophoresis.

## 2.5 | Pulsed-field gel electrophoresis

All *L. monocytogenes* isolates were analysed using last updated Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes* (PNL04, last update April 2014). After DNA extraction, the prepared agarose plug slices were digested by the restriction enzymes *Ascl* and *Apal* (New England BioLabs, Ipswich, Massachusetts) and followed by electrophoresis performed in 1.5% SeaKem Gold agarose (Lonza, Rockland, Maine) using CHEF Mapper III (Bio-Rad). *Salmonella* ser. Braenderup H9812 digested by *XbaI* was used as a size reference standard. Running parameters were previously described (Véghová et al., 2015). In order to check the identity of the isolates, up to five *L. monocytogenes* colonies from each individual sample were analysed. In the case, when more than one PFGE profile was obtained from the same sample, one representative isolate of each individual PFGE profile was involved in further investigation. The *Ascl*/*Apal*-PFGE patterns of individual *L. monocytogenes* isolates were analysed by BioNumerics software (Applied Maths, Kortrijk, Belgium). Similarity levels were calculated using Dics coefficient with 1.0% band tolerance, cluster analysis was performed by UPGMA method.

## 2.6 | Multilocus sequence typing

The MLST scheme of amplification and sequencing of seven gene fragments based on the procedure described by Salcedo, Arreaza, Alcalá, de la Fuente, and Vazquez, (2003) and modified by Ragon et al. (2008), according to the Protocol PF8 Genotyping of Pathogens and Public Health Platform, Institut Pasteur ([http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers\\_Lmono.html](http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Lmono.html)) was used to subtype representative *L. monocytogenes* strains. The modifications were as follows: DNA were isolated using DNeasy Blood and Tissue kit (Qiagen), amplification of *ldh* locus was optimized using  $MgCl_2$  concentration increased to 1.8 mmol/L and primers concentration of 10 pmol per reaction. DNA sequencing was performed BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3130xL Genetic Analyzer (both Applied Biosystems). Multilocus sequence types (STs) were assigned using BioNumerics software (Applied Maths) and the *Listeria* MLST database (<http://www.pasteur.fr/mlst>).

## 2.7 | Detection of virulence genes

Virulence genes *inlA*, *inlB*, *inlC* and *inlJ* were detected by two duplex PCR analyses using the *inlA*, *inlC* and *inlJ* primers (Liu, Lawrence, Austin, & Ainsworth, 2007) as well as *inlB* primers (Kacálková, Pangallo, Drahovská, Oravcová, & Kuchta, 2003). For *inlA/inlB* duplex PCR (product size 800 bp/343 bp), optimized concentrations of primers were used: 1  $\mu$ mol/L for *inlA*, and 250 nmol/L for *inlB*. The thermal programme consisted of the initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and polymerization at 72 °C for 2 min, and final polymerization at 72 °C for 2 min. Duplex PCR *inlC/inlJ* (product size 517 bp/238 bp), with concentration of *inlC* primers of 1.2  $\mu$ mol/L and *inlJ* primers of 0.8  $\mu$ mol/L, were performed using the thermal programme according to Liu, Lawrence, Austin, and Ainsworth, (2007). Detection of the *prfA* gene (Rossmanith et al, 2006) was included in the modified protocol for multiplex PCR molecular serogrouping (as described earlier).

## 2.8 | Detection of epidemic clones and *comK* prophage

Detection of genetic markers for epidemic clones ECI, ECII and ECIII was performed by previously described multiplex PCR (Chen & Knabel, 2007) with some modifications. Three primer sets were used at the modified final concentrations 80 nmol/L of each. PCR was performed in thermocycler Veriti Thermo cycler (Applied Biosystems) with a shortened PCR programme consisting of initial denaturation for 3 min at 95 °C, 15 cycles (denaturation 30 s at 94 °C, annealing 30 s at 55 °C and polymerisation 30 s at 72 °C, with annealing temperature lowered by 1 °C every third cycle), 15 cycles (denaturation 30 s at 94 °C, annealing 30 s at 50 °C and polymerisation 30 s at 72 °C) and final polymerisation 8 min at 72 °C. Detection of *comK* prophage junction fragments was performed according to Verghese et al. (2011). DNA was prepared by simple lysis in 1% Triton X-100 (Merck) at 95 °C and amplification was performed in four independent PCR with primer pairs ComKattBF/ComKattBR; ComKattBF/ComKattPR; ComKattPF/ComKattBR and ComKattPF/ComKattPR.

# 3 | RESULTS AND DISCUSSION

## 3.1 | Prevalence of *L. monocytogenes*

The total of 268 collected samples from a small-scale meat processing facility was represented by 196 (73%) environmental samples and 72 (27%) food samples. From the environmental samples, 106 (54%) were collected from food-contact surfaces and 90 (46%) from non-food-contact surfaces. From the food samples, 56 (80%) represented final ready-to-eat products and 16 (20%) were samples of raw meat, semi-products or final products intended for consumption after heat treatment.

*L. monocytogenes* prevalence in the samples collected from 15 samplings during the four-year period is summarized in Table 1. Out of all analysed samples, 70 (26.1%) were found to be positive for the

presence of *L. monocytogenes*, in most samples being in a mixture with other, predominantly non-pathogenic *Listeria* species.

Among the 70 positive samples, 60 (i.e., 31% from 196 analysed) were associated with the production environment including 16 and 44 food-contact and non-food-contact, respectively. From 10 positive food samples (i.e., 13.9% from 72 analysed), only five were ready-to-eat final products, but none from *L. monocytogenes* positive final products exceeded the limit of 100 CFU/g.

According to the presented results, *L. monocytogenes* contamination ranged from low levels of 7.5% in 2011 and 5.3% in 2012 to extremely higher levels of 47.3% in 2013 and 33.3% in 2014, with a massive increase from June 2013. However, the majority of positive samples (62.9%) was associated with non-food-contact surfaces and equipment.

From 70 positive samples, 77 individual *L. monocytogenes* isolates were obtained after the elimination of replicates by comparing PFGE profiles obtained from one sample. Based on experience from this study, multiple typical colonies should be always analysed, as more than one genetic subtype may be present in individual samples.

### 3.2 | Molecular analysis of serogroups and lineages

Molecular serotyping by multiplex PCR classified the 77 *L. monocytogenes* isolates into four different serogroups, with the majority of 34 (44.1%) strains in serogroup IIa, followed by 22 (28.6%) strains in serogroup IVb, 15 (19.5%) strains in serogroup IIc and 6 (7.8%) strains in serogroup IIb. Regarding the classification of lineages, 49 (63.6%) of the analysed *L. monocytogenes* strains represented lineage II (serogroups IIa and IIc) and 28 (36.4%) lineage I (serogroup IVb and IIb).

The results obtained in the study are in agreement with the observations in other European countries. *L. monocytogenes* strains of lineage II and serotype 1/2a (presenting up to 95% of serogroup IIa strains) followed by 1/2b or 1/2c, have been the most frequently isolated from food production facilities, including meat-processing facilities (Martín et al., 2014; Nucera et al., 2010). Serogroup IIb followed by IIa was reported as predominant in meat products in China (Wang et al., 2015). The prevalence of *L. monocytogenes* strains of serogroup IVb (lineage I) was higher in comparison to other authors. This might have been caused by the repeated isolation of individual strains (PFGE profile 9, ST 2) from different samplings or sampling sites grouped in one absolutely predominant profile in the serogroup.

### 3.3 | Molecular detection of virulence genes, epidemic clones and comK prophage

All the *L. monocytogenes* isolates analysed harboured virulence potential represented by the presence of *actA*, *prfA*, *inlA*, *inlB*, *inlC* and *inlJ* genes. Molecular markers specific for three epidemic clones (ECI–ECIII) of the respective *L. monocytogenes* serogroups were analysed. Positive results for ECIII were observed in 18 strains of IIa serogroup specifically associated with PFGE profile 2 corresponding to ST 14. These strains were isolated from different environmental swabs and food samples in six samplings during a period longer than one year. Moreover, these

strains were positive in PCR with ComKattBF/ComKattPR and ComKattPF/ComKattBR primers indicating integration of the A118-related prophage into *comK* gene.

Based on these results it should be assumed that these strains were able to persist in the food processing environment. Such contamination may cause intermittent spread of contaminants to food products and probably cause disease outbreaks, as suggested previously (Gilmour et al., 2010; Gottlieb et al., 2006; Graves et al., 2005). A sporadic case of listeriosis in 1988 and outbreak in 2000 were attributed to mutually similar strains originated from the same food-production facility and all were positive to ECIII marker (Olsen et al., 2005).

### 3.4 | Typing by PFGE and MLST

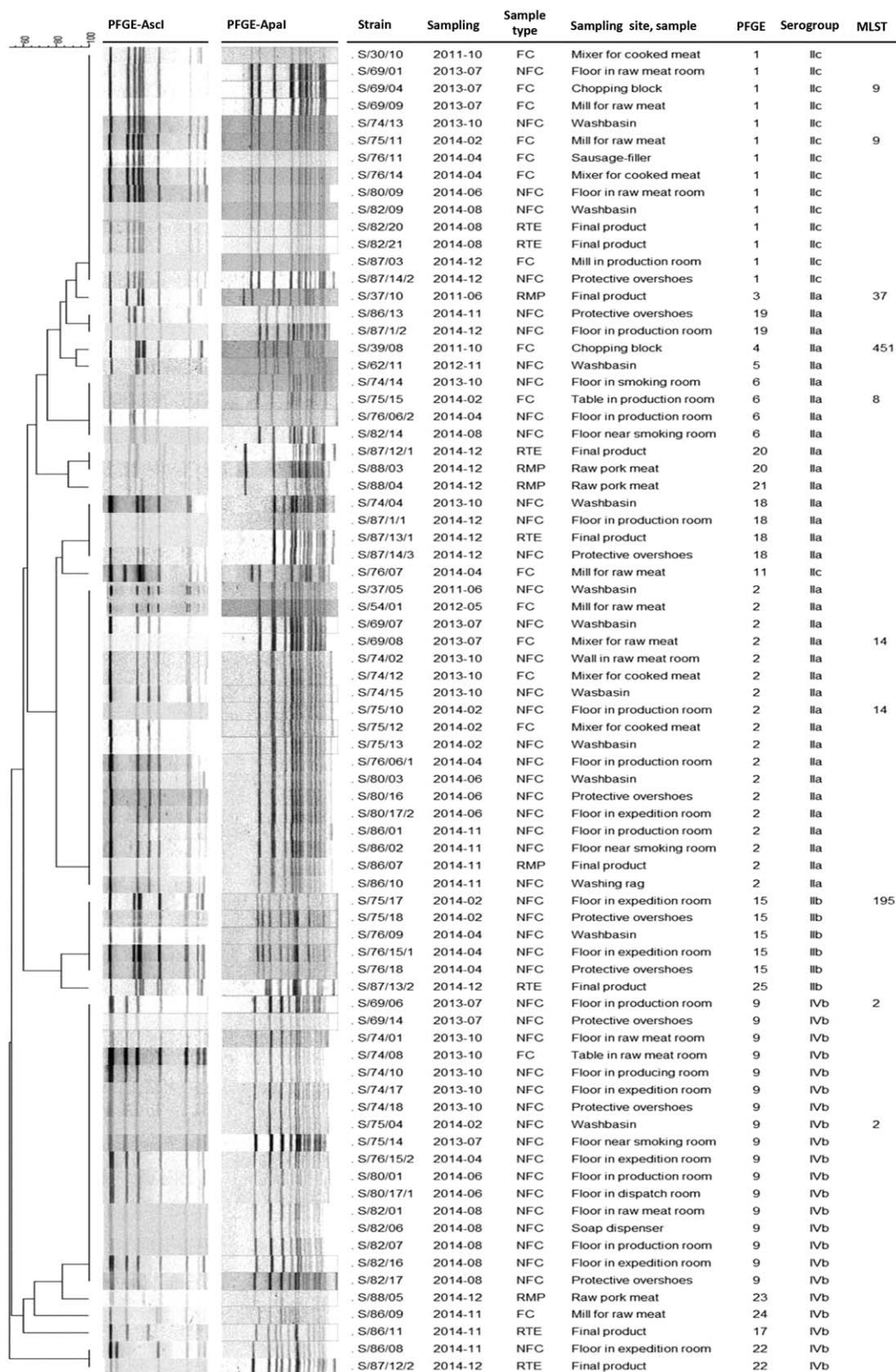
Analysis of AscI/ApaI-PFGE patterns of 77 individual isolates *L. monocytogenes* at the similarity level of 100% resulted in strain discrimination into 18 clusters. Isolates that showed 100% similarity of PFGE patterns were considered indistinguishable, assigned to the same PFGE profile. A number of 34 strains of serogroup IIa were classified into 9 PFGE profiles, with the largest group of 18 (53%) strains in PFGE profile 2, corresponding to ST 14. Rest of 16 isolates of IIa strains were classified to 8 different PFGE profiles. From 15 strains of serogroup IIc, 14 (93%) were grouped to absolutely dominant PFGE profile 1, corresponding to ST 9. All 49 strains of lineage II (serogroups IIa and IIc) formed one cluster at the level of 60% similarity.

Twenty-two strains of serogroup IVb were classified to 5 PFGE profiles, with 17 (77%) strains grouped into dominant profile 9, corresponding to ST 2. From 6 strains of serogroup IIb, 5 were grouped to one profile 15, corresponding to ST 195. All analysed lineage I strains (serogroups IIb and IVb) showed the mutual similarity less than 60% and they were not grouped into one distinct cluster. Dendrogram of PFGE profiles complemented with relevant data on isolates identity, date of sampling, source and results of molecular serogrouping, PFGE profiling and MLST indication is given in Figure 1.

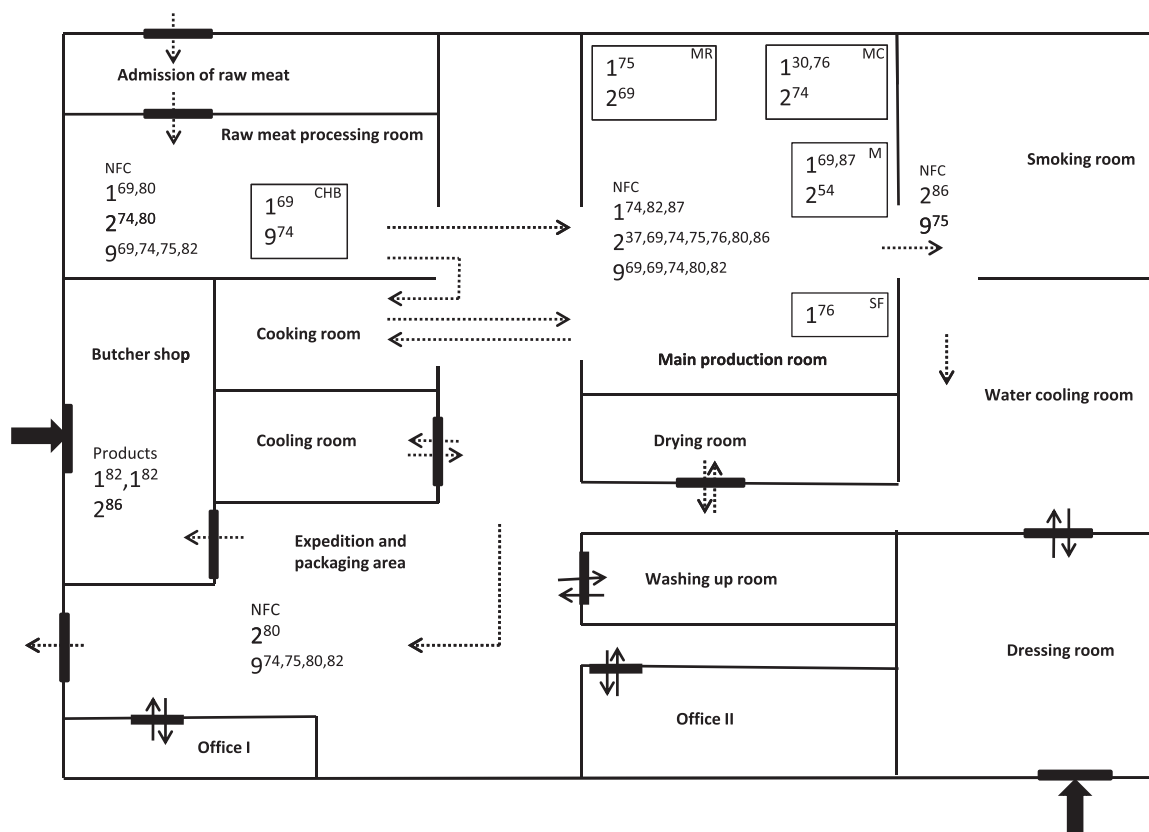
### 3.5 | Diversity and tracing of persistent *L. monocytogenes* isolates

PFGE profiling was used to determine the genetic variability of *L. monocytogenes* isolates and to trace the contamination along the meat-processing chain focusing to identification of potentially persistent contaminants. In fact, the strains with the undistinguishable PFGE profiles repeatedly isolated from the same places at different samplings over a longer period could be considered potentially persistent. During our four-year monitoring of the small-scale meat processing facility, accidental incidence of *L. monocytogenes* of relative high strain diversity, as well as persistent contamination was observed.

Three of 77 individual PFGE patterns classified as 1 (ST 9), 2 (ST 14) and 9 (ST 2) were attributed to 14, 18 and 17 strains, respectively, overall covering 63.6% isolates. These strains were considered persistent, being isolated repeatedly at five or six successive samplings during a period longer than one year, despite of regular cleaning and sanitation. They were isolated from several sampling sites in the production



**FIGURE 1** Dendrogram of Ascl/Apal-PFGE cluster analysis of 77 *L. monocytogenes* isolates with sample identification data, molecular serogrouping and sequence types. FC, Food-Contact samples; NFC, Non-Food-Contact samples; RMP, Raw Meat Product; RTE, Ready-To-Eat product



**FIGURE 2** Distribution of *L. monocytogenes* isolates of persistent AscI/ApaI-PFGE profiles (1, 2 and 9) in meat processing chain. Strains are identified by their PFGE profile, with the sampling event number given in superscript. NFC, Non-Food-Contact samples (floors, walls, washbasins, rags, protective overshoes); CHB, Chopping Block; MR, Mixer for Raw meat; MC, Mixer for Cooked meat; M, Mill for raw meat; SF, Sausage Filler

environment of raw-meat and cooked-meat processing areas, and from final products as well (Figure 1). Extremely high levels of *L. monocytogenes* contamination in 2013, being 47.3%, together with massive occurrence of the persistent PFGE subtypes were observed since summer 2013, probably because of inappropriate floor cleaning using full-pressure steam system and/or exchange of the employee responsible for production hygiene and safety control. Several attempts to improve the cleaning using other disinfectants were not successful. Only thorough mechanical cleaning and sanitation of all completely dismantled devices, all equipment and surfaces, non-food-contact areas (floors and washbasins, in particular) resulted in their reduction at the end of 2014 (Table 2).

Based on the subtyping of isolates by PFGE, spatial and temporal map of potentially persistent *L. monocytogenes* strains (PFGE types 1, 2 and 9) contamination was drawn (Figure 2). *L. monocytogenes* strain of PFGE profile 1 was sporadically detected in early single sampling event 30 (11-2010) in a single sampling site in the main production room. The massive appearance of strains with PFGE profile 1 was observed from sampling event 69 (07-2013) to 87 (12-2014), when the strains were spread to all processing rooms, food-contact equipment and surfaces as well as non-food-contact surfaces, resulting in contaminated final products in sampling event 82 (08-2014). Similar scenario was observed in the case of PFGE profile 2 with single detection in

sampling event 37 (06-2011) and massive appearance from sampling event 69 (07-2013) to 86 (11-2014), including the contaminated ready-to-eat product. On the other hand, *L. monocytogenes* strains of PFGE profile 9 were detected at first in sampling event 69 (07-2013) and then continuously to 82 (08-2014) in six successive samplings with massive prevalence almost exclusively associated with the floors in all processing rooms of the facility, from raw meat processing room to expedition and packaging area.

Distribution of the resting 28 sporadically isolated *L. monocytogenes* strains to 15 profiles indicated their high diversity. In connection to their occasional occurrence in different places along the production chain and in final products as well, it can be assumed that they originated in random external sources of contamination. Three of the sporadically identified PFGE profiles (6, 15 and 18) included 4 to 5 isolates identified in a maximum of three nonsuccessive samplings. Whereas 5 and 4 isolates of PFGE profiles 15 and 6, respectively, were associated almost exclusively with non-food-contact areas (mainly with floors), 1 of 4 isolates of PFGE profile 18 was also identified in a ready-to-eat product. However, single strains of 8 PFGE profiles were detected in food samples of raw meat and in one ready-to-eat product. Such contaminants were not repeatedly detected, as they were successfully eliminated from the production environment after the regular post-production process of sanitation.

It is known, that some PFGE-types appear to be exclusively associated with a single source, whereas other PFGE-types appear to be more widely disseminated and may be globally distributed (Fox et al., 2012). Our study is the first long-term survey in Slovakia with the application of molecular subtyping for hygienic and food safety purposes in meat processing environment with higher risk of *L. monocytogenes* contamination. Based on PFGE as well as MLST results we could conclude that two proposed persistent clones from our study, PFGE 1/ST 9 and PFGE 9/ST 2, belong to the strains frequently isolated also in other countries. *L. monocytogenes* strains belonging to ST 9 (PFGE 1) were isolated from human, animal and environmental samples (Listeria MLST database, <http://bigsd.dbweb.pasteur.fr/listeria/listeria.html>) and were identified also by Martín et al. (2014) as the most predominant allelic profile detected from meat-processing facilities in Spain during years, suggesting that the ST 9 is highly adapted to the meat-processing environment. The same *L. monocytogenes* types were isolated recently also from meat products or meat processing facilities in Switzerland (Ebner et al., 2015) or China (Wang et al., 2015), suggesting their potential global distribution.

Several studies focused on occurrence and tracing of persistent *L. monocytogenes* strains in various food production chains in European countries were performed in recent years. Eleven distinct pulsotypes including two frequently isolated and considered persistent *L. monocytogenes* strains were identified and four putative cross-contamination routes were confirmed in meat processing facility in Romania by investigating 226 samples over a 1-year period (Bolocan et al., 2015). Based on *L. monocytogenes* PFGE subtyping, predominant pulsotype was obtained and four colonization scenarios in newly-opened meat processing facility were formulated (Bolocan et al., 2016). Examination of *L. monocytogenes* in seafood processing facilities in the Republic of Ireland revealed only 2.5% prevalence in 508 samples analysed over two years, however, the results of the study showed the ability of smoked salmon to support the growth of *L. monocytogenes* (Leong, Alvarez-Ordóñez, Zaouali, & Jordan, 2015). The study of six European scientific institutions on environmental *L. monocytogenes* contamination in twelve food processing environments in the meat and dairy food sector (with 32% and 8.8% positivity from 2242 samples) highlighted that the food processing environments regarded for years as uncontaminated by *L. monocytogenes* may become contaminated and repeated environmental samplings could help to identify the potential sources of contamination (Muhterem-Uyar et al., 2015). The monitoring of *L. monocytogenes* presence in environmental sources in Czech Republic and molecular characterization of the recovered isolates confirmed clonal identity of environmental, food and human isolates indicating the external environment as a source of *L. monocytogenes* in food chain (Gelbíčová & Karpíšková, 2012).

*L. monocytogenes* strains isolated in our study, which were considered persistent, will be subjected to further characterization regarding their phenotypic and genotypic properties potentially supporting their persistence. In particular, further research of *L. monocytogenes* PFGE 2/ST 14, being ECIII and *comK* prophage-containing strain, is needed to confirm the proposed hypothesis on the function of the rapid adapta-

tion island (Verghese et al., 2011). Another question that has to be addressed is whether ECs of serotype 1/2a are better suited to survive in meat-production chains, and if this is the reason for apparent increase of serotype 1/2a outbreaks in recent years (Lomonaco et al., 2015).

## 4 | CONCLUSION

*Listeria monocytogenes* is a food-borne pathogen that can persist and cross-contaminate food products, thereby representing an important risk for the safety of the consumers. *L. monocytogenes* is the pathogen of great concern for the food producing companies and therefore, EU regulation requires the producers of ready-to-eat foods to examine the processing environment for *L. monocytogenes* contamination.

Sporadic incidences of *L. monocytogenes* with relative high strain diversity, as well as more significant massive persistent contamination by a couple of *L. monocytogenes* strains including potentially more virulent ECIII strains with integrated *comK* prophage was observed in meat processing facility. *L. monocytogenes* strains originating probably from external sources, caused contamination or, in the case of persistent strains, colonization of the production environment and, subsequently, contamination of final products. Presented results highlight the importance of using appropriate analytical tools, such as molecular typing methods, to trace the sources, distribution and routes of *L. monocytogenes*. In meat-processing facilities, most attention should be paid to contamination by persistent strains, to hygiene of the production chain and to development of improved process control strategies focused on effective prevention, not only to final products.

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