Food Microbiology 79 (2019) 116–122

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Molecular characterization of *Listeria monocytogenes* isolates from a small-scale meat processor in Montenegro, 2011–2014

Ivana Zuber[a], Brankica Lakicevic[b,*], Ariane Pietzka[c], Dubravka Milanov[d], Vesna Djordjevic[b], Nedjeljko Karabasil[e], Vlado Teodorovic[e], Werner Ruppitsch[f], Mirjana Dimitrijevic[e]

[a] Diagnostic Veterinary Laboratory, Dzordza Vasingtona bb, 81000, Podgorica, Montenegro
[b] Institute of Meat Hygiene and Technology, Kacanskog 13, 11000, Belgrade, Serbia
[c] Institute of Medical Microbiology and Hygiene/National Reference Laboratory for Listeria Division for Public Health, Austrian Agency for Health and Food Safety, Beethovenstraße 6 / A, 8010, Graz, Austria
[d] Scientific Veterinary Institute Novi Sad, Rumenacki put 20, 21000, Novi Sad, Serbia
[e] University of Belgrade, Faculty of Veterinary Medicine, Bulevar Oslobodjenja 18, 11000, Belgrade, Serbia
[f] Institute of Medical Microbiology and Hygiene Division for Public Health, Austrian Agency for Health and Food Safety, Währingerstrasse 25a, 1090, Vienna, Austria

ARTICLE INFO

Keywords:
Listeria monocytogenes
Meat products
cgMLST
AMR
Disinfectant resistance

ABSTRACT

The presence of *Listeria monocytogenes* was evaluated in a small-scale meat processing facility in Montenegro during 2011–2014. *L. monocytogenes* isolates from traditional meat products and environmental swabs were subjected to a) molecular characterization b) serotyping by both multiplex PCR and next generation sequencing (NGS) c) potential antimicrobial resistance (AMR) was assessed by extraction of specific genes from NGS data and d) screening for the presence of some disinfectant resistance markers. Overall, traditional meat products were contaminated, most likely from incoming raw materials, with 4 major specific STs of *L. monocytogenes* (ST515, ST8, ST21, ST121) representing 4 clonal complexes (CC1, CC8, CC21, CC121) identified during the four-year period. These strains belonged to serogroup IIa which predominated, followed by IVb (ST515, CC1). The strains from environmental swabs belonged, exclusively, to ST21 and were isolated from cutting board and floor swabs in 2011. Furthermore, we found Tn*6188*, a novel transposon conferring tolerance to BC, to be specific to sequence type ST121. In addition, antimicrobial resistance genes *mprF* and *fosX* were present in clonal complexes CC21 and CC121, while complexes CC8 and CC1 exclusively harbored the *mprF* antimicrobial resistance gene.

1. Introduction

Listeria monocytogenes is a facultatively anaerobic, gram-positive, non-spore-forming, psychrophilic, salt-tolerant, facultative intracellular pathogen of humans and animals, causing clinical manifestations that include gastroenteritis, encephalitis, meningitis, abortion and septicemia (Ruppitsch et al., 2015; Lakicevic et al., 2014a). Typically, pregnant women, the elderly, and immunocompromised individuals are at greatest risk (Hyden et al., 2016; Lakicevic et al., 2014b). Among all *Listeria* species, *L. monocytogenes* remains the only one of importance to human health with lineages I and II isolates (serotypes 4b, 1/2a, 1/2b) responsible for about 99% of all human cases of foodborne listeriosis (Orsi et al., 2011; Kasper et al., 2009). However, the genetic diversity, evolution and geographic distribution of *L. monocytogenes* clones remains largely unknown (Chenal-Franasque et al., 2011; Yin et al., 2015). The ability of *L. monocytogenes* to produce biofilms at low temperatures helps to facilitate persistent dissemination of this pathogen during food production (Allerberger et al., 2015). *L. monocytogenes* is transmitted from environmental sources outside the processing facility (incoming raw materials, animals, soil, dust and water) into the food processing environments (FPEs). Like other bacteria, *L. monocytogenes* can persist in biofilms on stainless steel surfaces and can be isolated from equipment, floors and cold storage areas over long periods of time. Having colonised an FPE, *L. monocytogenes* may spread throughout the facility via aerosols, personnel, food workflows, and contaminated contact materials leading to its persistent presence if sanitation procedures are insufficient (Alali and Schaffner, 2013). FPEs often display a multitude of niche environments that are challenging to effectively clean and sanitize. The problem is enhanced by inappropriate design of equipment, niche adaptation and biofilm

* Corresponding author.
E-mail addresses: ivana.zuber@udg.edu.me (I. Zuber), brankica.lakicevic@inmes.rs (B. Lakicevic), ariane.pietzka@ages.at (A. Pietzka), dubravka@niv.ns.ac.rs (D. Milanov), vesna.djordjevic@inmes.rs (V. Djordjevic), nedja@vet.bg.ac.rs (N. Karabasil), dekantat@vet.bg.ac.rs (V. Teodorovic), werner.ruppitsch@ages.at (W. Ruppitsch), mirjana@vet.bg.ac.rs (M. Dimitrijevic).

https://doi.org/10.1016/j.fm.2018.12.005
Received 17 June 2018; Received in revised form 29 November 2018; Accepted 4 December 2018
Available online 05 December 2018

formation leading to long-term persistence of the bacterium (Carpentier and Cerf, 2011; Lakicevic and Nastasijevic, 2017) and recurrent cross-contamination of food products (Ferreira et al., 2014).

During the investigation of a listeriosis outbreak, rapid and accurate subtyping methods are essential for identification of the infection source and subsequent elimination of the contaminated food (Pichler et al., 2011). Martín et al. (2018) highlighted that molecular typing of *L. monocytogenes* isolates has an important role in meat processing plants in order to trace the source of contamination and transmission routes. The ongoing evolution of sequencing technologies from Sanger sequencing to next-generation sequencing (NGS) enables analysis on a whole-genome level (Ruppitsch et al., 2015). Several studies using different bacteria have shown that whole-genome sequence (WGS)–based typing, using single nucleotide variant (SNVs) approaches (Turabelidze et al., 2013; Eyre et al., 2012) or gene-by-gene allelic profiling of core genome genes, frequently named core genome MLST (cgMLST) or MLST$^+$ (Mellmann et al., 2011; Maiden et al., 2013), are particularly attractive diagnostic tools for strain typing (FAO, 2016; Ruppitsch et al., 2015; Moura et al., 2016). These technologies for tracing the source of listeriosis, as well as the development and implementation of effective listeriosis prediction, monitoring, and risk assessment methods, are of great importance in the prevention and control both animal and human listeriosis (Yin et al., 2015).

This study aimed to: a) molecularly characterize selected *L. monocytogenes* strains obtained from Montenegrin meat processing establishment, b) determine the distribution of serogroups among isolates by multiplex PCR and NGS data, c) assess the presence of antimicrobial resistance (AMR) genes from NGS data and d) identify the presence of some disinfectant resistance markers (*bcrABC* cassette, *tetR*, *qacH*, *tnpABC*) (Müller et al., 2013).

2. Materials and methods

2.1. Meat processing plant

The commercial meat processing facility in Montenegro produced about 100 tons per year of traditional pork products, including dry-cured ham (*Njeguški pršut*), pork tenderloin, pancetta (thin dry bacon) and sausages. The products were manufactured using authentic traditional methods that included cutting the meat/soft fat and connective tissue trimming, salting, removing the water under pressure, beech wood smoking, drying and ripening in Lovcen mountain air for 9 months. These deli meat products (whole or sliced package) primarily sold on the domestic market. In total, 671 samples were taken during the slaughter and preparation of dry and smoked meat products from 2011 to 2014 in the last quarter of each year (Table 1). Samples from the processing environment (swabs from surfaces and drains) were also simultaneously collected and tested. These environmental samples (taken from easily and hardly accessible non-food and food-contact surfaces) were collected in the early morning hours, before the beginning of production process and after regular sanitation had been conducted the previous day (pre-operational collection of samples). The collected samples were transported to the laboratory within 2 h, in a cold bin at < 4 °C.

Table 1
The list of collected food and environmental samples during the four - year period.

Year	2011	2012	2013	2014	Σ
Dry sausage	18	20	34	18	90
Dry tenderloin	25	32	10	20	87
Pršut- dry cured ham	82	77	15	15	189
Dry pancetta	15	16	30	25	86
Dry neck	22	17	10	30	79
Swabs	40	58	22	20	140
Σ	202	220	121	128	671

2.2. Microbiological method

The food samples (an amount of 25 g), each consisting of 5 representative sample units collected from a production batch, were analyzed according to ISO 11290-1 (1996). Each environmental swab sample was taken from the total surface of 25 cm^2 and/or 100 cm^2 within the meat establishment. Wet-dry swabs (Dryswab™, MWE, UK) were used for the sampling according to ISO 18593:2004 (2004).

2.3. Biofilm formation ability

All *L. monocytogenes* isolates were examined for their ability to form biofilms using the microplate assay (Borucki et al., 2003). Each strain were inoculated into the wells (4 wells for each strain) of sterile flat-bottom microtiter plates (Nunc, Rosklide, Denmark) and incubated for 72 h at 30 °C. Cut-off optical density (ODc) was defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows: non-biofilm producers (OD ≤ ODc); weak biofilm producers (ODc < OD ≤ 2 × ODc); moderate biofilm producers (2 × ODc < OD ≤ 4 × ODc) or strong biofilm producers (4 × ODc < OD) (Stepanovic et al., 2004). Optical density (OD) was measured spectrophotometrically (Lasystems Multiscan® MCC/340) using 595 filter. Based on the results obtained from the microplate assay, one isolate of *L. monocytogenes* (randomly selected), prepared in Tryptone soya broth (TSB; Oxoid Ltd., Basingstoke, UK) (incubated 72 h at 30 °C) was chosen for the visualization of biofilm by scanning electron microscopy. The sample was gold coated with a sputter coater (Sputter Coater, BAL TEC SCD 005, Liechtenstein) (working time 100 s, used current 30 mA) prior to SEM analysis (JEOL JSM 6390 LV, Japan).

2.4. Bacterial strains and DNA extraction

Isolate origin, biofilm formation ability (including mean OD ± SD) and period of isolation are listed in Table 2. All strains were cultured overnight at 37 °C on RAPIDL.Mono agar (Bio-Rad, Vienna, Austria) for species confirmation and were sub-cultured on Columbia blood agar plates (BioMérieux, Marcy l'Etoile, France) prior to high quality DNA extraction using the MagAttract HMW DNA Kit, according to the instructions of the manufacturer (Qiagen, Hilden, Germany).

2.5. Whole-genome sequencing, assembly and data analysis

Sequencing libraries were prepared using NexteraXT chemistry (Illumina Inc., San Diego, CA, USA) for a 2 × 300 bp sequencing run on an Illumina MiSeq sequencer. Samples were sequenced over a minimum coverage of 70-fold using Illumina's recommended standard protocols. The resulting FASTQ files were first quality trimmed and then de novo assembled using the Velvet assembler (Zerbino and Birney, 2008) integrated in Ridom SeqSphere software (Ruppitsch et al., 2015) (version 3.1; Ridom GmbH, Münster, Germany). Sequence reads were trimmed at their 5′- and 3′-ends until an average PHRED value of 30 was reached in a window of 20 bases. The assembly was performed with the Velvet assembler, with the k-mer values and coverage cutoffs automatically optimized for each genome, based on the average length of contigs with > 1000 bp. Contigs with an overall length less than 200 bp or an average coverage below five were discarded.

Assembled genomes were compared by a recently developed core genome MLST scheme using SeqSphere$^+$ as described previously (Ruppitsch et al., 2015). Minimum spanning tree was visualized in SeqSphere$^+$ and colored in InkScape v 0.91.

For serogroup determination, relevant information was extracted from WGS data as described previously (Hyden et al., 2016) and a five-plex PCR (Doumith et al., 2004).

Antimicrobial resistance genes were identified using The

Table 2
L. monocytogenes strains used in this study, indicating number of isolates testing, their origin, biofilm formation ability with mean OD ± SD and period of isolation.

ID	Sample code	Origin	Biofilm formation ability	Mean OD ± SD[£]	Collection year
1	152/1	Montenegrin dry sausage (pork)	strong[a]	0,724 ± 0029	2013
2	152/3	Montenegrin dry sausage (pork)	strong[a]	0,738 ± 0055	2013
3	256/2	Minced meat, from machine, for fill sausage	strong[a]	0,707 ± 0066	2013
4	256/3	Minced meat from Mixer	moderate[b]	0,386 ± 0032	2013
5	256/4	Montenegrin dry sausage (pork)	moderate[b]	0,467 ± 0042	2013
6	256/5	Montenegrin dry sausage (pork)	moderate[b]	0,577 ± 0024	2013
7	328/1	Dry pancetta - sliced	moderate[b]	0,542 ± 0051	2013
8	728/49	Montenegrin dry sausage (pork)	moderate[b]	0,417 ± 0056	2012
9	728/50	Dry pancetta – whole meat	moderate[b]	0,331 ± 0054	2012
10	728/51	Pork Prosciutto - sliced	moderate[b]	0,408 ± 0053	2012
11	1208	Dry pork Tenderloin	strong[a]	0,704 ± 0073	2013
12	2018/1-5	Dry pork Tenderloin	moderate[b]	0,472 ± 0057	2014
13	2019/1-5	Dry pancetta	moderate[b]	0,555 ± 0065	2014
14	2020/1-5	Dry pork neck	moderate[b]	0,380 ± 0026	2014
15	2052/43	Dry pork neck	moderate[b]	0,357 ± 0101	2014
16	3564/3	Montenegrin dry sausage (pork)	moderate[b]	0,404 ± 0056	2011
17	3565/7	Cutting board swab	moderate[b]	0,377 ± 0043	2011
18	3565/15	Floor swab – salting room	moderate[b]	0,361 ± 0049	2011
19	3356/1	Pork Prosciutto - sliced	moderate[b]	0,410 ± 0036	2011
20	3356/4	Pork Prosciutto - chopped	moderate[b]	0,333 ± 0019	2011

[£]The optical density values were displayed for Tryptone soya broth (TSB) incubated 72 h at 30 °C; ODc (TSB) = 0.161.
[a] Strong biofilm producer: OD > 0.644.
[b] moderate biofilm producer:0.322 < OD ≤ 0.644.

Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013).

2.6. Nucleotide sequence accession number

All raw reads generated were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) under the study accession numbers 3003770 and 3000198.

2.7. PCR screening for Tn6188 and bcrABC

In total, 20 *L. monocytogenes* strains were screened for the presence of transposon Tn*6188* conferning tolerance to benzalkonium chloride (BC). PCR primers targeting the *qacH* gene from Tn*6188* and the flanking *radC* gene, into which Tn*6188* is integrated, were designed based on available Tn*6188* and *L. monocytogenes* genome sequences (Müller et al., 2013). PCR conditions were as follows: 0.2 pmol/µl of each primer, 2 mM $MgCl_2$, 1 mM dNTP-Mix, 0.625U Platinum Taq DNA polymerase (Life Technologies). PCR cycling conditions were: initial denaturation for 5 min at 95 °C; 30 cycles of denaturation at 94 °C for 40s, annealing at 56 °C for 40s and elongation at 72 °C (for *qacH* 25s; for *radC* 165s); final elongation at 72 °C for 5 min. Negative controls (no DNA added) and positive controls (genomic DNA from *L. monocytogenes* 6179) were included in all PCR reactions. The presence and size of amplification products was checked with agarose gel electrophoresis using SYBR Safe (Life Technologies) or ethidium bromide (Merck) –staining.

These same 20 strains were also screened for the presence of the *bcrABC* resistance cassette using primers BcF5 and BcR targeting the *bcrABC* genes (Müller et al., 2013). PCR conditions were as described above using an annealing temperature of 62 °C and elongation for 45s. PCR products obtained were sequenced by LGC Genomics.

3. Results and discussion

Whole genome sequence based typing of all 20 *L. monocytogenes* isolates originating from the same Montenegrin meat establishment clearly grouped isolates into three different complexes and one singleton based on seven housekeeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA*) (Salcedo et al., 2003) (Fig. 1). Significantly, isolates within ST complexes are closely related indicating that these isolates persist since several years in the establishment. WGS also provided information on the antibiotic resistance gene profiles. The predicted proteins were analyzed by BLASTp against the Comprehensive Antibiotic Resistance Database (CARD) to predict potential antibiotic resistance. Complex 1, the largest complex, comprised isolates belonging to serogroup IIa (lineage II), cluster type 5746, sequence type (ST21) and clonal complex (CC21). Isolates of complex 1 differed by a maximum allelic difference of 3 genes and harbored both the antibiotic target-modifying enzyme (*mprF*) that changes cell wall charge, and an antibiotic target-modifying enzyme (*fosX*) that is a determinant of fosfomycine resistance. *L. monocytogenes* ST21 was linked to Montenegrin dry pork sausage, pork Prosciutto and environmental swabs and was isolated during 2011 and 2013 (Table 3). Two environmental *L. monocytogenes* isolates (out of 140), were genetically identical and identified from the cutting board and floor swabs during the 2011. According to Chenal-Franasque et al. (2011), strains of CC21 were isolated in Africa (Algeria, 1988), Europe (The Netherlands and Germany), North America (Canada, 1954), predominantly from human sources. No information is currently available regarding ST21 in meat products and food processing environments.

Complex 2 contained isolates belonging to serogroup IIa (lineage II), CT 5747 and 5750, sequence type (ST121), and clonal complex (CC121). Isolates of complex 2 differed by a maximum allelic difference of 10 genes and contained both the *mprF* and *fosX*, antimicrobial resistance genes. *L. monocytogenes* ST121, highly abundant in food and food production environments (Rychli et al., 2017), was linked to Montenegrin pork dry sausage, dry pancetta, pork tenderloin and dry pork neck with this recurrent sequence type identified in 2012, 2013 and 2014 (Table 3). Despite a high number of available *L. monocytogenes* genome sequences, only a few studies have focused on analyses of *L. monocytogenes* ST121 genomes (Holch et al., 2013; Ortiz et al., 2016; Schmitz-Esser et al., 2015). Henri et al. (2016) suggested that ST121 strains may be better adapted genetically to persist in food and food processing environments but that they are less virulent for humans due to mutations in the internalin A gene. This observation may be directly relevant for refining risk analysis models for better management of food safety. Additionally, the apparent expansion of CC121 may represent the impact of certain geographic regions (Bergholz et al., 2018).

Complex 3 comprised isolates of serogroup IIa (lineage II), different cluster types (CT295, CT1358 and CT5748), sequence type ST8 and

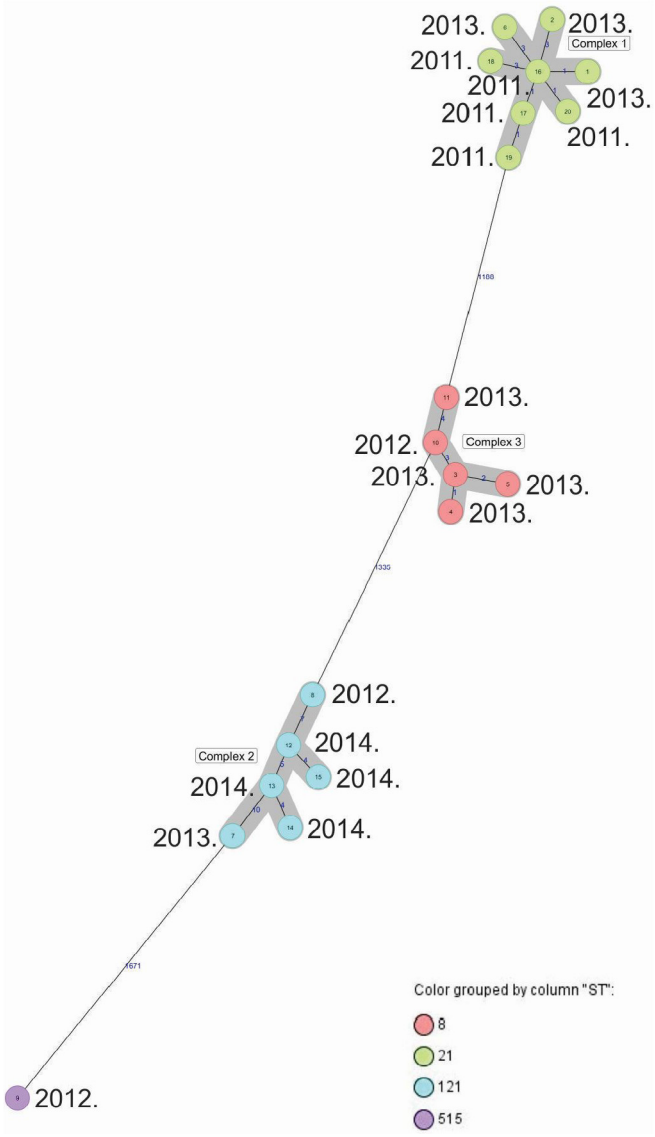

Fig. 1. Minimum spanning tree analysis based on cgMLST allelic profiles of 20 *L. monocytogenes* isolates from the Montegrin meat establishment. Each circle represents an allelic profile based on sequence analysis of 1701 genes. The numbers on the connecting lines illustrate the numbers of target genes with differing alleles. Isolates belonging to a cluster are called "complex" and these closely related genotypes (≤10 allele difference) are shown with a grey shadow. The different ST groups of strains are distinguished by the colors of the circles according to year of isolation.

clonal complex CC8. Isolates of complex 3 differed by a maximum allelic difference of 4 genes and possessed only the *mprF* antimicrobial resistance gene. *L. monocytogenes* ST8 was isolated from minced meat, Montenegrin dry pork sausage, pork Prosciutto, and pork tenderloin in 2012 and 2013 (Table 3). Only one isolate (number 9) belonged to CT5749, sequence type (ST515), and clonal complex (CC1) and was PCR-positive for serogroup IVb (Table 3). This singleton, belonging to lineage I, encodes *mprF* protein which is involved in resistance against cationic antimicrobial peptides (CAMP) and was isolated from dry pancetta (whole meat) in 2012 (Table 3). Strains belonging to CC1 and serotype 4b (clinical associated clone) are widely distributed globally, have a high risk of causing listeriosis (Yin et al., 2015). For example, serotype 4b *L. monocytogenes* strain F2365 was involved in the 1985 U.S. outbreak associated with Mexican-style soft cheese (Nelson et al., 2004), LL195 was involved in the 1983–1987 Switzerland outbreak associated with Vacherin Mont d'Or soft cheese (Weinmaier et al., 2013), and Scott A was involved in the 1983 U.S. outbreak associated with a clinical isolate (Bries et al., 2011). Clip80459 caused 85 deaths among 279 infected people associated with jellied pork tongue in France from 1999 to 2000 (de Valk et al., 2001). Interestingly, clonal complex CC1 is hypervirulent, strongly associated with central nervous system and materno-neonatal infections, whereas clone CC121 is associated with bacteremia and is more often isolated from highly immunocompromised patients (Maury et al., 2016). The same authors stated that most lineage I strains are overrepresented in human clinical infections and ruminant neurolisteriosis, while a majority of lineage II strains are associated with contaminated food and the environment. A recent landmark study, has revealed that *L. monocytogenes* CC1 strains harbour listeriolysin S (lls) and particular alleles of internalin (inl) F and inlJ which are not present in CCs commonly isolated from food and the environment (Rupp et al., 2017). Also, CC8 is globally distributed (Haase et al., 2014). In Switzerland, CC8 was the most prevalent clone during 2011–2013 (Althaus et al., 2014) and in Canada a CC8/ST120 clone caused both sporadic cases and outbreaks during 1988–2010 (Knabel et al., 2012).

Our results are partly similar to a recently conducted study in Serbia (Nastasijevic et al., 2017) where serotypes 1/2a, 1/2c and 4b, belonging to clonal complexes CC26, CC9 and CC1, were present in FPEs within a facility that manufacturers delicatessen meats made from pork. Notably, MLST-based phylogenetic analysis showed that serotype 1/2b and 4b strains are genetically related and have an “interval-type” evolution pattern, while serotype 1/2a and 1/2c strains have a “progressive-type” evolution pattern (Yin et al., 2015).

According to Kasper et al. (2009), 96% of all reported human listeriosis cases are caused by lineage I and II (serotypes 4b, 1/2a, 1/2b) isolates. The overwhelming preponderance of serotypes 4b, 1/2a, and 1/2b among clinical and food isolates, clearly points to differences in ability to survive in foods and/or cause disease (Bergholz et al., 2018).

All 20 *L. monocytogenes* strains were assayed for biofilm formation by using a microtiter plate assay. Intracomplex variabililty in biofilm formation was seen; but variation in biofilm-forming capacity at the serogroup level was not observed. The isolate (originated from dry sausage, sample code: 152/1), identified as a strong biofilm producer, was not able to form a biofilm on stainless steel (Fig. 2). This finding was previously confirmed by Midelet and Carpentier (2002) who reported greater attachment of *L. monocytogenes* to polyvinyl chloride and polyurethane compared to stainless steel. According to Chen et al. (2006), lineage I isolates were 100 times more likely to cause listeriosis than lineage II strains and are also more capable of forming biofilms (Borucki et al., 2003).

A total of 20 *L. monocytogenes* isolates were screened for presence/absence of a novel transposon Tn*6188* and *bcrABC* resistance cassette by PCR (Table 4). Overall, Tn*6188* was present in 6 strains belonging to sequence type (ST) 121, clonal complex (CC) 121 and were classified by PCR into serogroup IIa. The presence of resistance genes might contribute to the high prevalence of ST121 in food processing plant (Pasquali et al., 2018; Palma et al., 2017; EFSA, 2018).

Similar observation was reported by Rychli et al. (2017) who identified several candidate genes possibly involved in survival of ST121 *L. monocytogenes* strains in food and food production environments; like the transposon Tn*6188*, which confers increased tolerance towards various quaternary ammonium compounds. The authors concluded that Tn*6188* is particularly abundant among ST121 strains, which is in line with other recent studies (Ebner et al., 2015; Moura et al., 2016; Müller et al., 2013; Leong et al., 2015). However, we did not detect the *bcrABC* cassette, another mostly plasmid-borne genetic feature responsible for tolerance against quaternary ammonium compounds. These findings were confirmed by Rychli et al. (2017), with Meier et al. (2017) reporting benzalkonium chloride resistance in 12.3% of Swiss and 10.6% of Finnish *L. monocytogenes* strains. In both countries, BC-resistance was most prevalent among serotype 1/2c strains. The *bcr*ABC resistance cassette has been identified in a strain

Table 3
L. monocytogenes strains used in this study, indicating their origin, antimicrobial resistance, Accession number and sequence type according to year of isolation.

ID	Origin	AMR	ARO Category	ARO Accession	Sequence Type	Collection year
1	Montenegrin dry sausage (pork)	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2013
2	Montenegrin dry sausage (pork)	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2013
3	Minced meat, from machine, for fill sausage	mprF	Antibiotic target modifying enzyme, gene altering	3003770	8	2013
4	Minced meat from Mixer	mprF	Antibiotic target modifying enzyme, gene altering	3003770	8	2013
5	Montenegrin dry sausage (pork)	mprF	Antibiotic target modifying enzyme, gene altering	3003770	8	2013
6	Montenegrin dry sausage (pork)	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2013
7	Dry pancetta – sliced	mprF	Antibiotic target modifying enzyme, gene altering	3003770	121	2013
8	Montenegrin dry sausage (pork)	fosX	Deteriminant of fosfomycin resistance	3000198	121	2012
9	Dry pancetta – whole meat	mprF	Antibiotic target modifying enzyme, gene altering	3003770	515	2012
10	Pork Prosciutto - sliced	mprF	Antibiotic target modifying enzyme, gene altering	3003770	8	2012
11	Dry pork Tenderloin	mprF	Antibiotic target modifying enzyme, gene altering	3003770	8	2013
12	Dry pork Tenderloin	fosX	Deteriminant of fosfomycin resistance	3000198	121	2014
13	Dry pancetta	fosX	Deteriminant of fosfomycin resistance	3000198	121	2014
14	Dry pork neck	mprF	Antibiotic target modifying enzyme, gene altering	3003770	121	2014
15	Dry pork neck	mprF	Antibiotic target modifying enzyme, gene altering	3003770	121	2014
16	Montenegrin dry sausage (pork)	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2011
17	Cutting board swab	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2011
18	Floor swab – salting room	mprF	Antibiotic target modifying enzyme, gene altering	3003770	21	2011
19	Pork Prosciutto - sliced	fosX	Deteriminant of fosfomycin resistance	3000198	21	2011
20	Pork Prosciutto – chopped	fosX	Deteriminant of fosfomycin resistance	3000198	21	2011

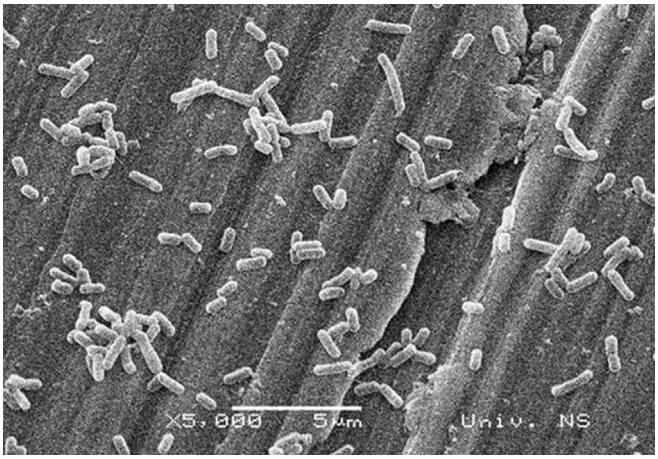

Fig. 2. Scanning Electron Microscopy (SEM) – Single cells and diplo-forms producing V or Y shapes strain on stainless steel surface (bar 5 μm) after incubation of *L. monocytogenes* isolate in TSB for 72 h days at 30 °C (JEOL JSM 6390 LV, Japan).

related to the 1998/1999 hot dog associated listeriosis outbreak in the USA (Elhanafi et al., 2010). Its presence in nonpathogenic *Listeria* spp. indicates the possibility of these strains as reservoirs of BC and other resistance determinants for *L. monocytogenes* as a result of conjugative transfer (Katharios-Lanwermeyer et al., 2012). In one clone (CC8) that includes strains implicated in the 2008 deli meat outbreak in Canada, tolerance to quaternary ammonium disinfectants was found to be mediated by *ermE,* harbored on a chromosomal island. Expression of the *emrE* gene is upregulated in the presence of BC, raising the concern of possible adaptation and persistence of *Listeria* strains harboring this gene in the food processing environment (Kovacevic et al., 2015).

Tn*6188* is related to *Tn554* from *Staphylococcus aureus* and other Tn*554*-like transposons such as Tn*558*, Tn*559* and Tn*5406* found in various *Firmicutes*. Tn*6188* comprises 5117 bp, is integrated chromosomally within the *radC* gene and consists of three transposase genes (tnpABC) as well genes encoding a putative transcriptional regulator (*tetR*), quaternary ammonium compound resistance protein (QacH), and a small multidrug resistance protein family transporter (SMR) (Müller et al., 2013). Genes and gene cassettes conferring tolerance to quaternary ammonium disinfectants and to phage appear to have been acquired from other bacteria. Strains harboring these genes may have enhanced fitness and persistence in manufacturing facilities (Buchanan et al., 2017).

4. Conclusion

Due to the fact that several clones exist in Montenegrin meat establishment source identification should be significantly enhanced with the aid of an effective, reliable and powerful core genome MLST method which is beneficial to the food industry and may help to improve consumer safety. The Montenegrin meat processing company yielded 4 different specific STs (ST515, ST8, ST21 and ST121) representing 4 major clonal complexes (CC1, CC8, CC21 and CC121). All of these strains belonged to molecular serogroup IIa (lineage II), except one (ST515, CC1), which belonged to serogroup IVb (lineage I) and was isolated from dry pancetta in 2012. Tn*6188* - a novel transposon conferring tolerance to BC, was found in 6 of the 20 strains, all of which belonged to specific sequence type ST121. These six strains were isolated from pancetta, sausage, tenderloin and pork neck during 2012, 2013 and 2014. Different meat products, including minced meat, were contaminated with *L. monocytogenes* ST8 in 2012 and 2013. Interestingly, the isolates from environmental swabs (cutting board and floor swabs) and from Montenegrin pork, dry sausage and pork Prosciutto belonged exclusively to ST21. In contrast, *bcrABC* was not detected in any of the strains tested. Two antimicrobial resistance genes, *mprF* and *fosX,* were present in clonal complexes CC21 and CC121, while complexes CC8 and CC1 exclusively harbored antimicrobial resistance gene *mprF.* Based on these findings, contaminated incoming raw materials led to contamination of the final Montenegrin meat products, suggesting that animals might be carrying these strains. Collectively, these results could help food processors and food agency investigators more quickly identify those *Listeria* strains that are likely to possess enhanced tolerances to certain stresses and persist long-term in food processing environments.

Acknowledgements

This research was supported by the Ministry of Defence, Republic of Serbia, under the research grant MFVMA/1/17-19, and by the Ministry of Education, Science and Technological Development of the Republic of Serbia, under the research grant No. 31034.

The authors would like to thank Dr. Elliot Ryser, Gordana Terzic for assistance in figure preparation and Snezana Vucinic for help and support in this project.

Table 4
The connection between Core Genome Multilocus Sequence Typing, AMR and some disinfectant resistance markers.

ID (NRL Graz)	ID	ST	Cluster Type	Serogroup	CC	Profile	abcZ	bglA	cat	dapE	dat	ldh	lhkA	bcrA	bcrB	bcrC	tetR	qacH	tnpC	tnpB	tnpA
MRL-17-01214	1	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01215	2	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01216	3	8	295	IIa	CC8	5, 6, 2, 9, 5, 3, 1	5	6	2	9	5	3	1	–	–	–	–	–	–	–	–
MRL-17-01217	4	8	1358	IIa	CC8	5, 6, 2, 9, 5, 3, 1	5	6	2	9	5	3	1	–	–	–	–	–	–	–	–
MRL-17-01218	5	8	1358	IIa	CC8	5, 6, 2, 9, 5, 3, 1	5	6	2	9	5	3	1	–	–	–	–	–	–	–	–
MRL-17-01219	6	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01220	7	121	5747	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01221	8	121	5750	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01222	9	515	5749	IVb	CC1	3, 1, 1, 39, 3, 1, 3	3	1	1	39	3	1	3	–	–	–	–	–	–	–	–
MRL-17-01223	10	8	5748	IIa	CC8	5, 6, 2, 9, 5, 3, 1	5	6	2	9	5	3	1	–	–	–	–	–	–	–	–
MRL-17-01224	11	8	1358	IIa	CC8	5, 6, 2, 9, 5, 3, 1	5	6	2	9	5	3	1	–	–	–	–	–	–	–	–
MRL-17-01225	12	121	5750	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01226	13	121	5747	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01227	14	121	5750	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01228	15	121	5750	IIa	CC121	7, 6, 8, 8, 6, 37, 1	7	6	8	8	6	37	1	–	–	–	+	+	+	+	+
MRL-17-01229	16	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01230	17	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01231	18	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01232	19	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–
MRL-17-01233	20	21	5746	IIa	CC21	7, 7, 3, 10, 5, 6, 1	7	7	3	10	5	6	1	–	–	–	–	–	–	–	–

ST: Sequence Type; *CC*: Clonal Complex; *abcZ* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (Succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (lactate deshydrogenase), *lhkA* (histidine kinase), *bcrABC* (benzalkonium chloride resistance cassette), *tetR* (transcriptional regulator), *qacH* (Quaternary ammonium compoundresistance protein), *tnpABC* (transposase), + present; - absence.

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