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Genetic diversity of *Listeria monocytogenes* strains contaminating food and food producing environment as single based sample in Italy (retrospective study)

Vicdalia Aniela Acciari [a,b,*,1], Anna Ruolo [a,b,1], Marina Torresi [a,b], Lucilla Ricci [a], Antonella Pompei [a,b], Cristina Marfoglia [a,b], Francesca Maria Valente [a,b], Gabriella Centorotola [a,b], Annamaria Conte [a], Romolo Salini [a], Nicola D'Alterio [a,b], Giacomo Migliorati [a,b], Francesco Pomilio [a,b]

[a] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Campo Boario, 64100 Teramo, Italy*
[b] *National Reference Laboratory for Listeria monocytogenes, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Campo Boario, 64100 Teramo, Italy*

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ABSTRACT

Human listeriosis outbreaks are often associated with food products, which could be contaminated, at the same time, also by different clones of *Listeria monocytogenes.* This emphasize the need to type more than one *L. monocytogenes* isolate found in a single food or environmental sample.

The purpose of the present study was to evaluate the presence of different *L. monocytogenes* strains in food and food production environment in order to understand if there is need to type more isolates from the same sample in case of presence of *L. monocytogenes.* Between 2011 and 2015, at the Italian National Reference Laboratory for *L. monocytogenes,* for each positive sample, from two to twenty-three isolates of *L. monocytogenes* were collected. All the isolates were characterized by conventional serotyping and pulsed field gel electrophoresis (PFGE). Moreover, isolates from the same sample, having indistinguishable PFGE profile, were subjected to whole genome sequencing in order to perform core genome Multi Locus Sequence Typing (cgMLST).

Within each sample, more than one serotype and one pulsotype were found in 11.9% and 27.5%, respectively. For indistinguishable PFGE patterns the cgMLST analysis showed 96.2% of concordance demonstrating the added value of new sequencing technologies.

This study has demonstrated the need to select and type more than one *L. monocytogenes* colony in one food or food environmental sample to detect the diversity of *L. monocytogenes* strains and facilitate downstream investigations and effective source attribution in foodborne outbreak inquiry.

1. Introduction

L. monocytogenes is a pathogen transmitted by food products that may cause listeriosis, a severe infection in humans that has been recognized around the world as a serious public health problem. Currently in Europe the number of *L. monocytogenes* outbreaks is higher than in previous years, although confirmed cases of listeriosis are stable (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2021).

This disease may present either in its invasive form or as febrile gastroenteritis. The invasive form, which has a high mortality rate (20–30%), mainly affects the elderly and immunocompromised individuals, who may have different clinical symptoms such as septicemia, meningitis and meningoencephalitis (Montero et al., 2015). In infants and pregnant women, the invasive form can cause perinatal infections and spontaneous abortions, respectively. In contrast, febrile gastroenteritis caused by this pathogen is usually a self-limiting infection that generally affects healthy individuals after ingesting a large number of these bacteria from contaminated food products (Maurella et al., 2018).

Human listeriosis outbreaks are always associated with

* Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Campo Boario, 64100 Teramo, Italy.
E-mail address: v.acciari@izs.it (V.A. Acciari).
[1] Vicdalia Aniela Acciari and Anna Ruolo equally contributed to the drafting of the paper.

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contaminated food products, although L. *monocytogenes* is known to be common in the natural environment (Montero et al., 2015).

L. monocytogenes represents a threat for both food industry and consumers, due to its ability to tolerate extreme environmental conditions, such as dry environments, wide range of pH values and at a broad temperature range. This is made possible by *L. monocytogenes* ability to regulate the cytoplasmic membrane fluidity, modifying the lipid composition (Flegler et al., 2021). In addition, genetic factors responsible for the high adaptive capacity against physical–chemical factors confer at this pathogen the ability to produce biofilm, colonize and persist in food processing plant environment (Ricci et al., 2018).

The serotype 4b (PCR serogroups IVb) of *L. monocytogenes* is considered the serotype associated with 50% of human outbreaks, whereas 1/2a strains are more frequently isolated from food (Burall et al., 2017; Maury et al., 2016). This could be not only due to the particular genetic characteristics of strains, which provide them with adequate capabilities to resist or grow under different conditions, but also the result of the possible failure of selective enrichment to detect all strains in a food contaminated with strains having diverse serotypes (Zilelidou et al., 2016).

The main food sources attributed to human infections are milk and dairy products, vegetables, salads, fish and meat products (Ricci et al., 2018; EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2021; Kaptchouang Tchatchouang et al., 2020). Investigating the ecology and diversity *L. monocytogenes* in food products and in food production animals may yield valuable information on the potential sources of future human infections.

Isolates derived by the selection of more than one colony in the same sample, can allow to highlight the presence of strains with different phenotypic and genotypic characteristics. Furthermore, strains detected from different sources and at different times, showing identical phenotypic and genotypic traits, suggest a common origin and can be defined as clones (van Belkum et al., 2007). It is well documented that mixed clones of *L. monocytogenes* may contaminate a single food sample. According to von Laer et al. (2009), the contamination caused by more than one *L. monocytogenes* strain, highlighted by the detection of different Pulsed-field-gel electrophoresis (PFGE) profiles in tested samples, may be linked to presence of *L. monocytogenes* from different sites of the food producing plants. Its results pointed out that several isolates from a single sample should be molecular typed in epidemiological and contamination studies (von Laer et al., 2009).

Even if not frequently, the isolation of more than one strain can also occur analyzing clinical samples (Tham et al., 2013). Such a phenomenon may not have any clinical relevance, although it may confuse the epidemiologist for surveillance, source attribution or foodborne outbreak investigation.

In 2011 in the United States, the first listeriosis outbreak caused by several distinct clones of *L. monocytogenes* occurred (Laksanalamai et al., 2012). The authors reported four different PFGE profiles isolated from patients, food producing environment, refrigerators and fruits.

From an epidemiological point of view, the findings of different clones of *L. monocytogenes* in the same sample emphasize the need for typing more than one isolates from a single food sample (Tham et al., 2013), particularly during foodborne outbreak investigation.

In recent years the improvement of laboratory methods able to increase the discriminatory capacity and phylogenetic analyses, such as whole genome sequencing (WGS) replaced the fingerprinting based on PFGE, that in the past was considered as the gold standard for *L. monocytogenes* subtyping (Brown et al., 2019; Ribot et al., 2019).

The purpose of the present study was to carry out a retrospective study, starting from the results of the molecular characterization of the isolates detected in one food sample, to demonstrate if the selection of one isolate per positive sample is sufficient or not to give comprehensive data for epidemiological purposes, such as foodborne outbreak investigation, prevalence and ecological studies.

2. Materials and methods

2.1. Strain collection

The isolates collected by the Italian National Reference Laboratory for *L. monocytogenes* (It LNR *Lm*) were used to build the database, dividing all samples into 4 ready to eat (RTE) food categories (dairy products, meat products, fish and fishery products, composite dishes) and food processing environmental (FPE).

The final dataset consisted of 1293 *L. monocytogenes* isolates (1076 from 325 food samples and 217 from 43 environmental samples). The number of the samples and isolates considered in this study was yielded from a randomized sampling, therefore the number of positive samples and isolates analyzed did not represent the effective percentage of contamination of *L. monocytogenes* in different matrices. In particular, were analyzed 413 isolates from 102 dairy products samples, 331 isolates from 115 meat products samples, 297 isolates from 97 fish and fishery products samples, 35 isolates from 11 composite dishes samples and 217 isolates from 43 FPE samples.

The number of the isolates collected for each sample, sent by official laboratories and collected at the It NRL *Lm,* ranged from two to twenty-three as reported in Table 1.

2.2. Serotyping

All the *L. monocytogenes* isolates were serotyped according to the method described in the United States Food and Drug Administration Bacteriological analytical manual (Bennet and Weaver, 2001), using commercial sera for somatic (O) and flagellar (H) antigens (Denkan Seiken Co. Ltd., Tokyo, Japan).

2.3. Pulsed-field gel electrophoresis

L. monocytogenes isolates were characterized by PFGE, using the PulseNet protocol (PulseNet-International, 2017) involving the restriction enzymes *Asc*I and *Apa*I as described by Acciari et al. (2016).

The analysis of PFGE gels was performed using BioNumerics software version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms for *Asc*I and *Apa*I macrorestriction profiles (MRPs) were generated based on the Dice correlation coefficient for similarity, and the unweighted pair group method with arithmetic means (UPGMA) was employed for cluster analysis. Band matching was carried out at optimization of 1% and position tolerance of 1% for both enzymes.

Starting from the single similarity matrices for each enzyme, the software created a single combined matrix (pulsotype), obtained from the average of the values of the single tests; each matrix was considered

Table 1
Number of *L. monocytogenes* collected by It NRL *Lm*, isolated from each single positive sample.

N. of Isolates collected from a single sample	N. of positive environmental samples tested (N. of isolates)	N. of positive food samples tested (N. of isolates)
2	4 (8)	67 (134)
3	13 (39)	195 (585)
4	3 (12)	21 (84)
5	13 (65)	33 (165)
6	1 (6)	1 (6)
7	1 (7)	1 (7)
8	–	1 (8)
9	–	2 (18)
10	8 (80)	–
11	–	1 (11)
14	–	1 (14)
21	–	1 (21)
23	–	1 (23)
Total	43 (217)	325 (1076)

of equal importance, regardless of restriction enzyme. Pulsotypes were obtained combining *Asc*I and *Apa*I MRPs.

2.4. Whole genome sequencing

A subset of 230 isolates (127 from 39 food samples and 103 from 14 environmental samples) with indistinguishable pulsotype were selected for WGS.

DNA was extracted using the Maxwell 16® Cell DNA purification kit (Promega Italia Srl, Milan, Italy) according to the manufacturer's protocol. DNA quality and concentration were measured by NanoDrop® Spectrophotometer (ThermoFisher Scientific, Wältham, MA, USA).

Sequencing libraries were prepared with a Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA) according to the producers' instructions and sequenced in a NextSeq 500 Illumina platform, producing 150-bp paired-end reads (Illumina, San Diego, CA, USA).

An in-house developed pipeline was used for the generation of draft genome assemblies (Cito et al., 2018), which included steps for trimming with Trimmomatic v0.36 (Bolger et al., 2014) and quality control check of the reads (FastQC v0.11.5). De novo assembly of paired-end reads was performed with SPAdes v3.11.1 (Bankevich et al., 2012). Then, the genome assembly quality check was performed with QUAST v4.3 (Gurevich et al., 2013).

Multi Locus Sequence Type (MLST) and core genome MLST (cgMLST) profiles (1748 loci) were extracted from the assemblies using the tool available on the BIGSdb-Lm platform hosted by Pasteur Institute, France (http://bigsdb.pasteur.fr/listeria) (Moura et al., 2016).

Based on categorical differences in the allelic cgMLST profiles for each isolate, the Minimum Spanning Tree (MST) were built using the software online GrapeTree with parameters implemented in MSTree v2 ignoring missing values (Zhou et al., 2018).

2.5. Statistical analysis

Associations between food categories and percentage of positive samples for two or more pulsotypes was analyzed through a Bayesian approach using a Beta distribution and its 95% confidence intervals (Gupta and Nadarajah, 2004).

To evaluate association between pulsotypes and number of isolates per sample, Fisher exact test was calculated on the observed frequencies and significance per each condition (cell) was assessed (Fisher, 1954). Data analysis was performed using XLSTAT Version 2013.2.04 - Addinsoft 1995–2013.

3. Results

The final dataset consisted of 1293 *L. monocytogenes* isolates (1076 from 325 food samples and 217 from 43 environmental samples), as summarized in Table 1.

3.1. Serotyping

Serotyping revealed that the 1293 isolates were grouped in seven serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 4b and 4d), the main serotype detected was 1/2a (61.6%) followed by serotype 1/2b (14.5%) and serotype 1/2c (13.9%) (Table 2).

In 324 (88.1%) samples, only one serotype was detected. In 41 samples (11.1%) two different serotypes, while in three (0.8%) samples three different serotypes were present (Table 3).

Fig. 1 reports the number of different serotypes for each sample correlated to the number of isolates sent to It LNR *Lm.*

3.2. Pulsed-field gel electrophoresis

PFGE subtyping yielded 85 *Asc*I and 121 *Apa*I different MRPs. Combined analysis with both enzymes produced 166 combined pulsotypes, 148 (89.16%) of them were reported only in one food category, 15 (9.04%) pulsotypes were found in two categories and only three (1.8%) pulsotypes were found in three food categories.

Table 2
L. monocytogenes serotyping: number of strains for each serotype and sample origin.

Serotype	Total number of collected strains (%)	Number of strains from food samples (%)	Number of strains from environmental samples (%)
1/2a	797 (61.6)	633 (58.8)	164 (75.6)
1/2b	187 (14.5)	149 (13.8)	38 (17.5)
1/2c	180 (13.9)	177 (16.4)	3 (1.4)
4b	78 (6)	67 (6.2)	11 (5.1)
3a	39 (3)	39 (3.6)	0 (0.0)
4d	9 (0.7)	8 (0.7)	1 (0.5)
3b	3 (0.2)	3 (0.3)	0 (0.0)
Total	1293	1076	217

Table 3
L. monocytogenes serotyping: serotypes found in a single sample.

Number of serotypes	Number of food samples (%)	Number of environmental samples	Total number of samples(%)
1	284 (87.4)	40 (93)	324 (88.1)
2	38 (11.7)	3 (7)	41 (11.1)
3	3 (0.9)	0	3 (0.8)
Total	325	43	368

In 267 samples (72.55%) only one pulsotype was reported, while in 101 samples (27.45%) two or more pulsotypes were found. In Fig. 2, the distribution of pulsotypes as a function of the number of strains isolated from a single sample was represented.

The number of pulsotypes found within a single sample was correlated to the tested material. Fig. 3 reports the percentage of samples with two or more pulsotypes and the 95% of confidence intervals.

In particular, the number of pulsotypes found in the fish matrix was significantly lower than the number of pulsotypes in the dairy products and composite dishes. Even if slightly above 0.05, the significance is relevant also in comparison with meat ($p < 0.06$).

An association between a low isolates number with a low number of pulsotypes was observed as reported in Tables 4 and 5. In 324 of 368 positive samples, the number of the pulsotypes detected was one or two. In only 21 samples more than two pulsotypes were detected, with significance at the Fisher test ($p < 0.001$) (see Table 5). Lastly, in 19 samples more than five isolates were subtyped. Because of the low number of samples considered, the statistical were not calculated.

3.3. Whole genome sequencing

Results of the selected subset of isolates, submitted for WGS testing, were summarized in detail in Figs. 4 to 6.

All the strains detected in one sample were evaluated building a Minimum Spanning tree for each RTE food results in order to find additional genetic differences. The minimum number of called loci was 1714 (98%) of the 1748 included in the *Lm* cgMLST scheme. Samples with indistinguishable PFGE profile strains, showed less than seven allelic differences in 96.2% (<0.408% mismatched loci). Only two samples with indistinguishable PFGE profile showed eight and fifteen alleles differences.

4. Discussion

The present study reports typing of 1293 *L. monocytogenes* isolates from 368 RTE food and environmental samples. More than one strain was detected in 30.4% of tested samples, highlighting genetic variability, showing different serotypes, pulsotypes or WGS patterns.

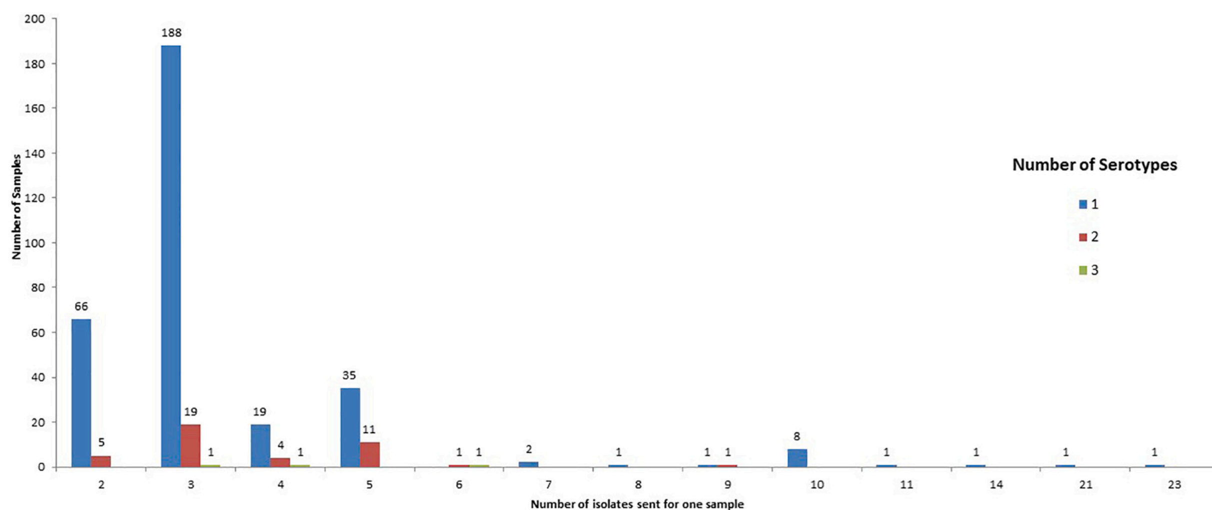

Fig. 1. Variability of the number of serotypes based on the number of strains analyzed for one sample.

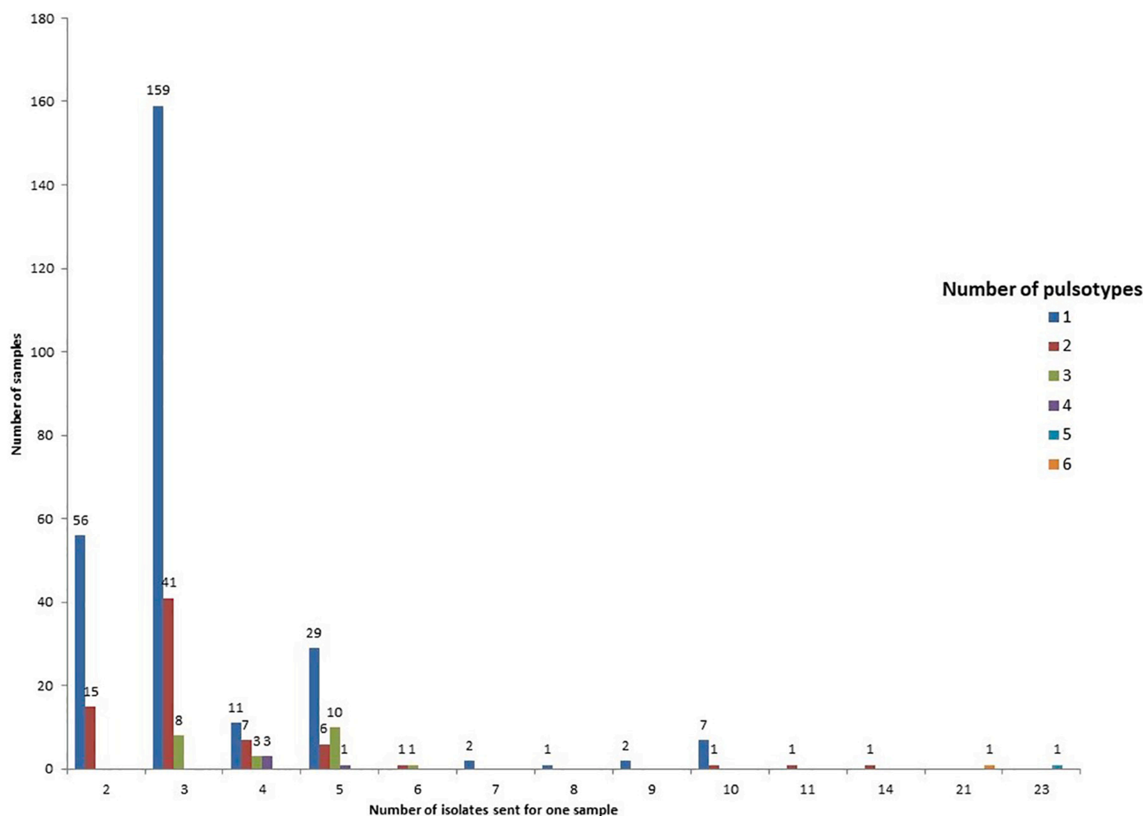

Fig. 2. Distribution of pulsotypes found in one sample.

The most common serovar detected was 1/2a, both in food (58.8%) and in environmental samples (75.6%) which is in agreement with previous studies in several countries (Braga et al., 2017; O'Connor et al., 2010). Usually the 1/2a strains are commonly detected in foods, and food environments, but it was reported also in listeriosis cases both in human and animal (Ricci et al., 2018; Orsi et al., 2011). Serotype 1/2a strains seem to be more resistant to bacteriocins produced by a range of lactic acid bacteria commonly present in foods, which probably confers an advantage in environments where bacteriocin-producing organisms are abundant (Buncic et al., 2001; Korsak et al., 2012). Moreover, the prevalence of this serotype in food environments could be due to that 1/2a strains seem to carry more plasmids that confer resistance to toxic metals and possibly other compounds found in the environment, than other isolates (Orsi et al., 2011).

Due to the low number of environmental samples considered in the study, it was not possible to evaluate the significance of the different distribution of serotypes between environmental and food strains.

Based on our data, 89.16% of pulsotypes have specific food or environmental origin. Moreover, results highlighted a higher number of pulsotypes found in milk samples than in fish product samples, showing greater genetic variability associated with dairy matrices.

Laboratories usually select a low number of isolates and, therefore, the estimation of the probabilities of losing isolates having different pulsotypes cannot be calculated. Based on the tested isolates, two

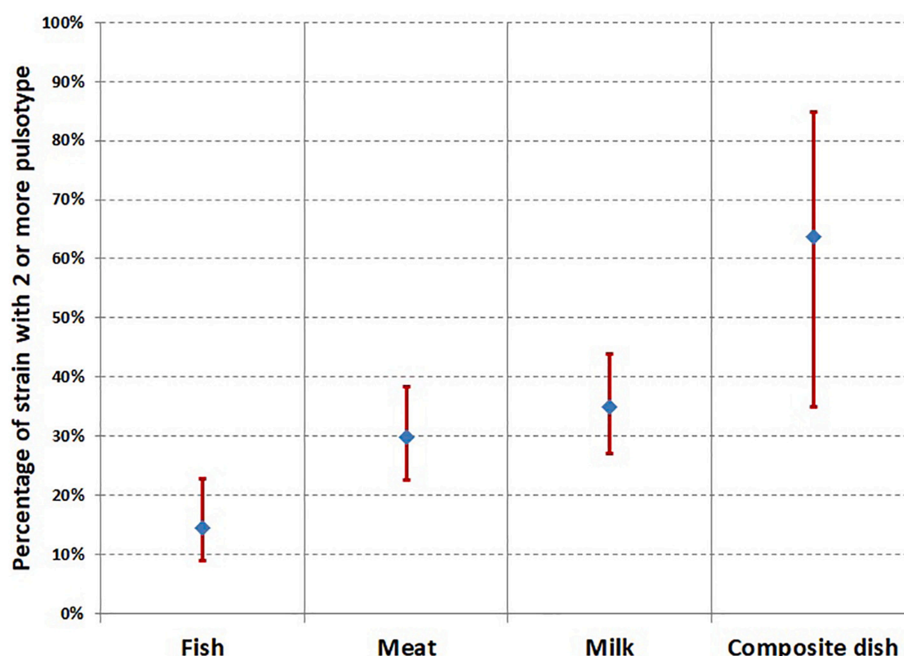

Fig. 3. Variability of number of identified pulsotypes according to the category of isolation.

Table 4
Association between tested isolates collected in a single sample and combined pulsotypes identified with PFGE.

Isolate collected in a single sample	Number of pulsotypes isolated in a single sample (in %)			
	1	2	3	4
2	56 (78.9)	15 (21.1)	N.A.	N.A.
3	160 (76.9)	40 (19.2)	8 (3.8)	N.A.
4	11 (45.8)	7 (29.2)	3 (12.5)	3 (12.5)
5	29 (63.0)	6 (13.0)	10 (21.7)	1 (2.2)
>5	12 (63.2)	4 (21.1)	1 (5.3)	2 (10.5)
Total[a]	256	68	21	4

N.A. Not applicable.
[a] Line >5 was not taken into account for calculation.

Table 5
Significance per cell (Fisher test). The table reports the results based on the expected ones.

Isolate collected in a single sample	Number of pulsotypes isolated in a single sample			
	1	2	3	4
2	>	>	N.A.	N.A.
3	>	<	<[a]	N.A.
4	<[a]	>	>	>[a]
5	<	<	>[a]	>

> more than expected.
< less than expected.
N.A. Not applicable.
[a] The asterisk denotes the significance per cell of the Fisher test.

different strains were found in 21.1% of samples if two isolates were characterized. Instead, in case of three isolates characterized, two and three different strains were found in 19.2% and in 3.8% of samples, respectively.

Of course, the results were biased by the number of isolates sent for characterization, showing association between low isolates number with a low number of pulsotypes.

Although, should be pointed out, instead, that the association between five isolates and three pulsotypes detected is much higher than the theoretical one.

New analytical methods, based on WGS, provided higher discriminatory alternative to PFGE and serotyping for the laboratory investigation of foodborne outbreaks. These methods are able to determine other variables within bacterial populations and highlight the differences between clones of the same species in a single tested sample.

The data available for the investigated interval of time, being only on serotype and PFGE cannot give a modern scenario, in which Next Generation Sequencing (NGS) is more sensitive and gives a better evidence of strains diversity.

Most of our isolates were clustered in 1/2a, 1/2b and 1/2c serotypes known to be less frequently associated with cases of listeriosis (Maury et al., 2016). However, *L. monocytogenes* is a pathogenically heterogeneous species, composed of hypervirulent and hypovirulent clones grouped in clonal complexes (CCs) and epidemic clones (ECs). The application of a WGS-based approach would have been useful to predict hypovirulent and hypervirulent phenotypes (Hurley et al., 2019).

Mixed populations of L. *monocytogenes* strains could be present in a single sample and ingestion of more than one strain by a consumer is a likely scenario (Zilelidou et al., 2016), resulting in an exposure of consumers to multiple strains. The occurrence of multiple strains in one food is an important aspect contributing to create potential mismatches between clinical isolates and infection sources during listeriosis outbreak investigations.

A higher number of isolates collected from a single sample and then tested by the laboratory could give the possibility to increase detection of strains genetically different increasing the probability to find a match in case of foodborne outbreak investigation (Ryser et al., 1996).

For environmental samples, the issue has a minor relevance since usually a large number of sample are taken (from 15 to 60 samples or more). If a large number of positive samples are detected, also the selection of one isolate for positive sample could give the opportunity to detect multiple clones.

The interaction among different strains in one sample could change their growth rate; it could enhance or inhibit growth leading potentially

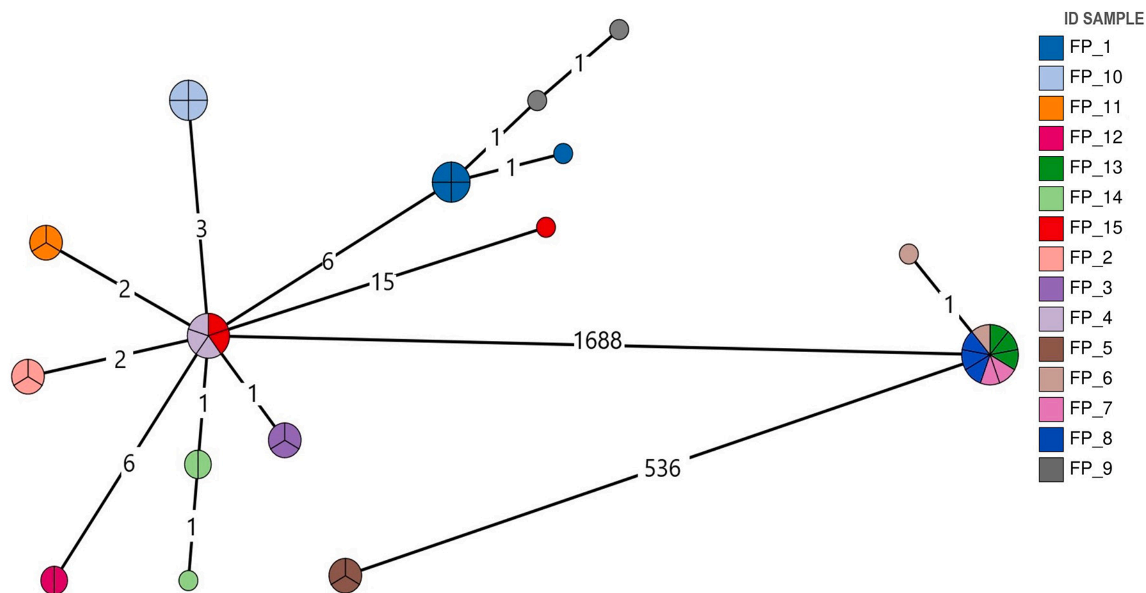

Fig. 4. Cluster analysis of Lm strains isolated in fish product: Minimum Spanning tree were built using the software GrapeTree based on cgMLST allelic profiles. The nodes are colored according to the sample (FP = fish product). The number reported in the branches indicate the allelic differences existing between the isolates.

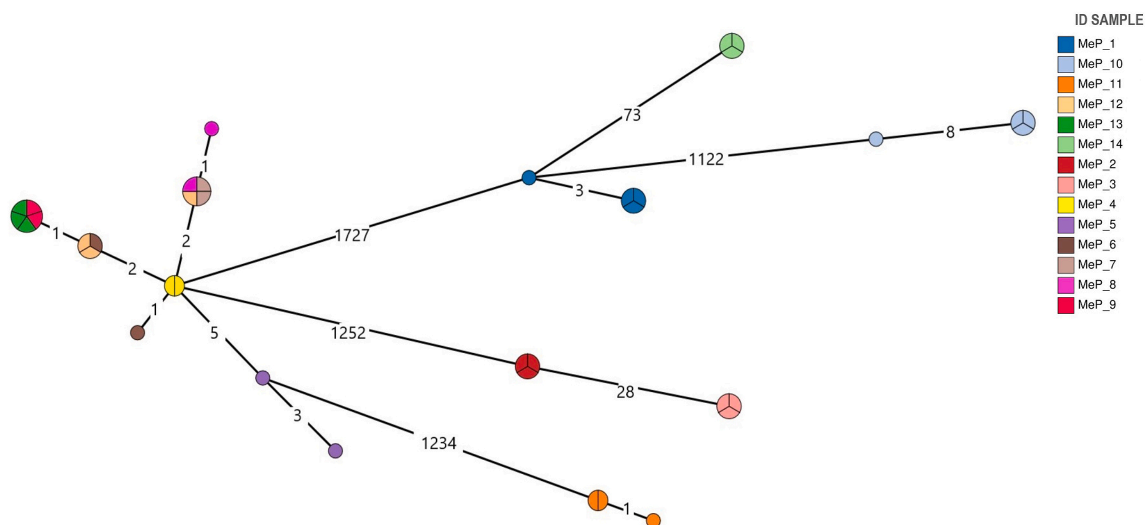

Fig. 5. Cluster analysis of Lm strains isolated in meat product: Minimum Spanning tree built using the software GrapeTree based on cgMLST allelic profiles. The nodes are colored according to the sample (MeP = meat product). The number reported in the branches indicate the allelic differences existing between the isolates.

to biased recovery during enrichment procedures (Bruhn et al., 2005; Gorski et al., 2006). Furthermore, the presence of more than one L. *monocytogenes* strain in one food sample can lead to an increased infection rates due to synergistic effects on the potential virulence (Ricci et al., 2018).

While competition of *L. monocytogenes* with other bacteria, including other *Listeria* species, has been described, little is known about *L. monocytogenes* inter-clone interactions. Recent studies have demonstrated different recovery rates of *L. monocytogenes* clones during the selective enrichment process, as a result of strains competition (Zilelidou et al., 2015).

5. Conclusion

Previous studies have already demonstrated the occurrence of multiple *L. monocytogenes* clones in one food sample. This scenario, if we are not able to detect the multiple occurrence, can complicate the downstream investigations and the effective source attribution.

Furthermore, strains could interact and change the contamination scenario in the food, due to their genetic and phenotypic diversity. Some strains present in foods may be missed during detection and such probability should be taken into consideration during outbreak investigation.

To date, we are not able to suggest the number of isolates to be tested in order to obtain the exact distribution of different strains in a single sample. Nevertheless, this study highlights the importance to select and characterize more than one isolate from each positive sample. These results should be taken into consideration particularly when attempting to identify *L. monocytogenes* strains of clinical importance during food-borne outbreak investigations of listeriosis, improving the sensitivity of surveillance activities and increasing the probability of source attribution.

In our dataset, we performed WGS only in strains showing indistinguishable PFGE profiles. Results showed high correspondence, only in

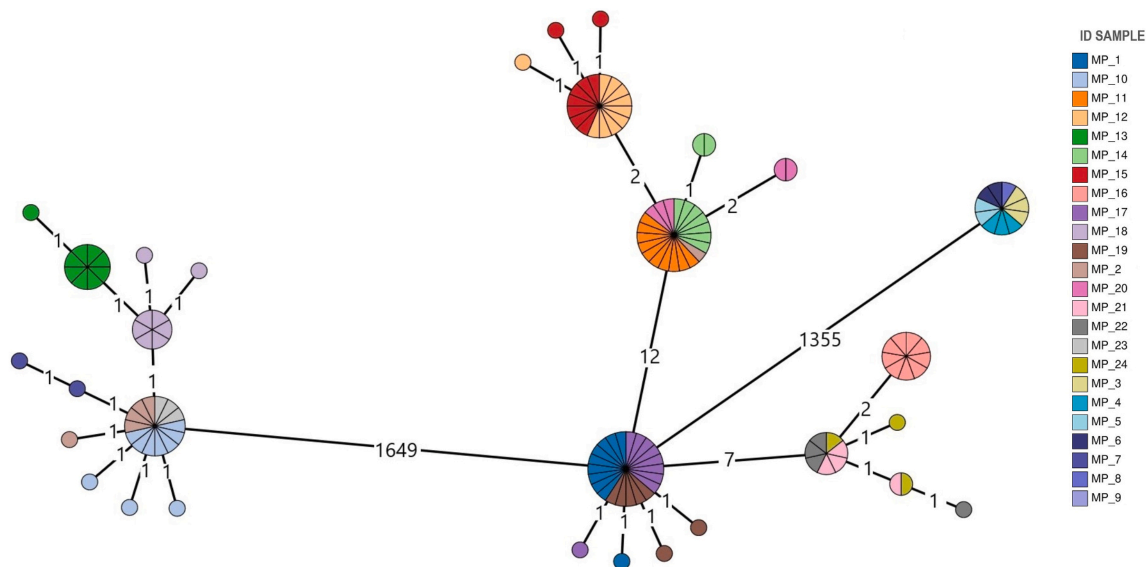

Fig. 6. Cluster analysis of Lm strains isolated in milk product: Minimum Spanning tree built using the software GrapeTree based on cgMLST allelic profiles. The nodes are colored according to the sample (MP = milk product). The number reported in the branches indicate the allelic differences existing between the isolates.

two samples difference between isolates was greater than seven alleles. Future studies with an increased number of isolates typed by WGS will probably help to understand how many *L. monocytogenes* isolates need to be selected and molecular typed in case of a positive sample in order to obtain a comprehensive scenario.

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CRediT authorship contribution statement

AR, FP, VAA: Conceptualization.
AP, CM, GC, MT: Investigation.
AC, RS: Statistical analysis.
LR: Validation.
AR, FMV, VAA: formal analysis and writing-original Draft.
VAA, FP: Writing- Review & Editing.
GM, NDA: Supervision.
All authors corrected and approved the manuscript.

Declaration of competing interest

None.

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