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Attribution of Listeria monocytogenes human infections to food and animal sources inNorthern Italy

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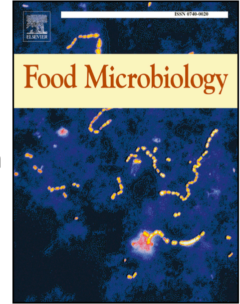

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Attribution of *Listeria monocytogenes* human infections to food and animal sources in Northern Italy

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Abstract

Listeriosis is a foodborne illness characterized by a relatively low morbidity, but a large disease burden due to the severity of clinical manifestations and the high case fatality rate. Increased listeriosis notifications have been observed in Europe since the 2000s. However, the reasons for this increase are largely unknown, with the sources of sporadic human listerioris often remaining elusive. Here we inferred the relative contributions of several putative sources of *Listeria monocytogenes* strains from listerioris patients in Northern Italy (Piedmont and Lombardy regions), using two established source attribution models (i.e. 'Dutch' and 'STRUCTURE') in comparative fashion. We compared the Multi-Locus Sequence Typing and Multi-Virulence-Locus Sequence Typing profiles of strains collected from beef, dairy, fish, game, mixed foods, mixed meat, pork, and poultry. Overall, 634 *L. monocytogenes* isolates were collected from 2005 to 2016. In total, 40 clonal complexes and 51 virulence types were identified, with 36% of the isolates belonging to possible epidemic clones (i.e. genetically related strains from unrelated outbreaks). Source attribution analysis showed that 50% of human listerioris cases (95% Confidence Interval 44-55%) could be attributed to dairy products, followed by poultry and pork (15% each), and mixed foods (15%). Since the contamination of dairy, poultry and pork products are closely linked to primary production, expanding actions currently limited to ready-to-eat products to the reservoir level may help reducing the risk of cross-contamination at the consumer level.

Keywords

Listeria monocytogenes, listeriosis, food safety, epidemic clones, source attribution, molecular epidemiology

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1 **Introduction**

2 *Listeria monocytogenes* is a bacterial foodborne pathogen that rarely causes severe disease
3 in healthy individuals. Indeed, clinical listeriosis mainly occurs in at-risk groups: pregnant wom-
4 en, elderly people, immunocompromised people, unborn babies, and neonates (Lomonaco, Nuce-
5 ra, and Filipello 2015). In Europe, the incidence of listeriosis is approximately 0.48 per 100,000
6 inhabitants, and infections can occur either in a sporadic or epidemic form (EFSA and ECDC
7 2018). Several wild and domestic animals can also acquire infection with *L. monocytogenes*,
8 particularly mammals and birds, which are also considered potential zoonotic reservoirs of the
9 pathogen (Vivant, Garmyn, and Piveteau 2013). Among mammals, ruminants are the most sus-
10 ceptible to listeriosis, and *L. monocytogenes* subtypes associated with human listeriosis cases
11 have been identified in bovine farms as well (Nightingale et al. 2004; Rocha et al. 2013). In
12 birds, listeriosis mainly occurs sporadically, and it is believed that birds may act as a potential
13 source for the infection in ruminants through the contamination of pastures and feed crops
14 (Dhama et al. 2013; Locatelli et al. 2013). While exposure to infected animals and contaminated
15 agricultural environments rarely appear to be directly linked to human infections, animal-derived
16 food products that are consumed raw or undercooked, refrigerated RTE stored for long periods,
17 as well as manure-contaminated fresh produce, often cause disease in humans (Nightingale et al.
18 2004; Lopez-Valladares, Danielsson-Tham, and Tham 2018). Moreover, unlike most foodborne
19 pathogens, *L. monocytogenes* can grow in conditions of fairly low moisture, high salt concentra-
20 tion, and most importantly, at refrigeration temperatures, thereby conferring ability to persist and
21 multiply in the food environment (Matthews, Kniel, and Montville 2017).

22 In case of human infection, the ubiquitous nature of *L. monocytogenes* and ability to sur-
23 vive for long periods outside the host, coupled with a relatively long incubation period, may

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24 hamper the identification of the source (Dhama et al. 2015). Indeed, by the time of listeriosis
25 diagnosis, food leftovers are very seldom available, and recalling the exact food consumption
26 history preceding the infection may also be difficult (Amato et al. 2017; Jackson, Iwamoto, and
27 Swerdlow 2010). Source attribution modelling based on microbial subtyping offers the oppor-
28 tunity to overcome these difficulties. Indeed, source attribution allows for the quantification of
29 the relative contributions of the main animal, food, and environmental sources of foodborne dis-
30 ease, and attributions can be estimated at different points along the food chain, including produc-
31 tion, distribution, and consumption (Pires et al. 2009).

32 Source attribution based on microbial subtyping relies on the characterisation of isolates
33 using different phenotyping or genotyping methods (Andreoletti et al. 2008). Human cases are
34 then probabilistically attributed to sources by comparing the subtype distributions of human
35 source strains through mathematical models (Mughini-Gras and van Pelt 2014). Two main fami-
36 lies of source attribution models are available: the so-called 'frequency matching' and 'popula-
37 tion genetics' models, each with several advantages and disadvantages, as discussed in a recent
38 opinion paper (Mughini-Gras et al. 2018). Overall, the source attribution approach has proven
39 useful in prioritising and guiding control strategies, allowing for the identification of the most
40 important reservoirs of specific pathogens (Boysen et al. 2014).

41 Multi-Locus Sequence Typing (MLST) and Multi-Virulence-Locus Sequence Typing
42 (MVLST) are sequence-based methods in which Single Nucleotide Polymorphisms (SNPs) in
43 fragments of a set of genes are used to determine allelic variants. MLST is based on a set of 7
44 housekeeping genes, while MVLST is based on a set of 6 virulence genes. MLST has been used
45 to study and describe the population structure and phylogeny of *L. monocytogenes*, while
46 MVLST has been used to identify Epidemic Clones (ECs) in outbreak investigations (Ragon et

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47 al. 2008; Amato et al. 2017; Lomonaco et al. 2013; Chen, Zhang, and Knabel 2005; Knabel et al.
48 2012). An advantage of using allele-based methods is the presence of a shared nomenclature
49 based on reference strains publicly available on dedicated databases (MLST,
50 http://bigsdb.pasteur.fr/Listeria/Listeria.html; MVLST,
51 https://sites.google.com/site/mvlstdatabase).
52 The aim of this study was to quantify the relative contributions of several putative sources
53 of human listeriosis cases in Northern Italy by using two established source attribution modelling
54 approaches based on MLST and MVLST data for clinical *L. monocytogenes* strains and strains
55 from beef, dairy, fish, game, mixed foods, mixed meat, pork, and poultry. To further describe the
56 strains circulating in the considered area the majority of the isolates were analysed with Whole
57 Genome Sequencing (WGS), and screened for Antimicrobial Resistance (AMR) genes and SNP
58 clustering through the NCBI Pathogen Detection pipeline.
59
60 **Materials and Methods**
61 ***Isolates collection***
62 A total of 634 *L. monocytogenes* isolates were available for this study. These included
63 218 isolates from human listeriosis patients and 416 from various food sources, divided into 8
64 categories (i.e. beef, dairy, fish, game, mixed food, mixed meat, pork, and poultry). Clinical iso-
65 lates were collected between 2005 and 2016 through a voluntary network of hospital laboratories
66 in two Northern Italy regions, i.e. Lombardy and Piedmont (Mammina et al. 2013; Filipello et al.
67 2015). The food isolates were collected between 2004 and 2015 during the routine surveillance
68 carried out by the Regional Animal Health and Food Safety Institutes (IZS) or in previous re-
69 search projects aimed at studying the epidemiology of *L. monocytogenes* along the food chain
70 carried out by the Department of Veterinary Sciences of the University of Turin.

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71 ***Molecular typing***

72 The whole genome sequences for 510 isolates, represented by food and environmental
73 (*n*=416) and clinical isolates (*n*=94), were obtained at the Center for Food Safety and Applied
74 Nutrition (CFSAN) of the US Food and Drug Administration (Lomonaco et al. 2018). DNA ex-
75 traction was performed using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), fol-
76 lowing manufacturer's instructions. DNA libraries were generated using the Illumina Nextera XT
77 DNA Library Preparation Kit. WGS was performed on a MiSeq or a NextSeq system using a
78 2×250 bp or a 2x150 bp paired-end MiSeq/NextSeq Reagent Kit, respectively (Illumina, San
79 Diego, CA, USA). MLST and MVLST data were extracted from the WGS data (Lomonaco et al.
80 2018). The remaining 124 clinical isolates were typed with MLST and MVLST as previously
81 described (Chen, Zhang, and Knabel 2005; Ragon et al. 2008). Sequence Types (STs) and Viru-
82 lence Types (VTs) were defined using the allelic sequences of the different loci schemes availa-
83 ble in the respective online databases (MLST, https://bigsdb.pasteur.fr/listeria/listeria.html and
84 MVLST, https://sites.google.com/site/mvlstdatabase/) and were used to assign isolates to Clonal
85 Complexes (CCs) (i.e. groups of isolates with at least 6 alleles in common with another member
86 of the same group) and to identify ECs. Both MLST and MVLST data were visualized using
87 Minimum Spanning Trees (MSTs), generated by the PHYLOViZ software (Francisco et al.
88 2012).

89 WGS data for the strains described herein is also available on the NCBI Pathogen Detec-
90 tion database (NCBI PD, https://www.ncbi.nlm.nih.gov/pathogens/), a centralized system inte-
91 grating WGS data for several bacterial pathogens obtained from different sources with the scope
92 of rapidly linking food or environmental isolates to clinical isolates to discover potential sources
93 of contamination and aid traceback/outbreak investigations. Single-linkage clustering (with SNP

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94 distance of 50 SNPs) is used to identify closely related sets of isolates and assign SNP cluster
95 accessions (i.e. PDS#). Individual phylogenetic trees are available for each SNP cluster, based on
96 maximum compatibility (Cherry 2017). Isolates that are not within 50 SNPs of any other isolate
97 are not assigned to a SNP cluster. The NCBI Pathogen Detection pipeline also provides data
98 about the AMR genotype listing the antimicrobial resistance genes that have been identified by
99 the NCBI AMR Finder process. As of April 1st, 2019, the NCBI PD database contains 26,567 *L.*
100 *monocytogenes* isolates, and the isolates analysed herein can be found under BioProject ID
101 PRJNA304956. Data on the NCBI PD is available for 508 of the 510 *L. monocytogenes* strains
102 typed with WGS under BioProject PRJNA304956 (Lomonaco et al., 2018). Two strains
103 (CFSAN045809 and CFSAN049182) were excluded from NCBI PD because their genome size
104 was considered too small and outside the accepted ranges. Overall, 514 isolates are listed under
105 BioProject PRJNA304956, with 6 strains (CFSAN044745, 044769, 046011, 046039, 046086,
106 049217) not included in the original publication (Lomonaco et al., 2018), and thus not consid-
107 ered herein.

108 ***Source attribution modelling***

109 Human cases were attributed to the putative sources by applying two different models in
110 parallel, the 'Dutch model' (Lapo Mughini-Gras, Franz, and van Pelt 2018) and 'STRUCTURE'
111 (Pritchard, Stephens, and Donnelly 2000). The Dutch model is a simple frequency-matching
112 model that compares the number of human cases caused by a specific subtype (i.e. ST or VT),
113 with the relative occurrence of that subtype in each source. This model was applied separately on
114 MLST and MVLST data, resulting in two model-data type combinations (MLST Dutch and
115 MVLST Dutch). STRUCTURE is a population genetics, Bayesian clustering model that uses
116 multi-locus genotype data to infer population structure and to assign individuals in a sample to

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117 populations. This model was applied separately to MLST, MVLST, and coupled
118 MLST+MVLST data (genotypic profiles defined by the combined 13 alleles), resulting in three
119 model-data type combinations (MLST STRUCTURE, MVLST STRUCTURE, and
120 MVLST+MLST STRUCTURE). For a more detailed description of the source attribution mod-
121 els, we refer to previous papers (Pritchard, Stephens, and Donnelly 2000; Lapo Mughini-Gras,
122 Franz, and van Pelt 2018).

123 ***Statistical analysis***

124 To assess differences in attributions over the different model-data type combinations (i.e.
125 MLST Dutch, MVLST Dutch, MLST STRUCTURE, MVLST STRUCTURE, and
126 MLST+MVLST STRUCTURE), the attributable proportions of cases were compared by exact
127 two-tailed binomial test for each model-data type combination. To evaluate the agreement be-
128 tween attributions, a correlation matrix between the 5 model-data type combination was calculat-
129 ed using the Pearson correlation coefficient (rho). For each model-data type combination, the
130 attributable proportions were ordered and ranked in ascending order. A median was calculated
131 for each food category taking into account each value and the median of the ranks was used to
132 provide an overall classification. All analyses were performed by open source software R (R
133 Development Core Team).

134

135 **Results**

136 ***MLST typing***

137 MLST results were available for 628 of the 634 isolates. MLST results were not available
138 for six isolates (378, 379, 409, 598, 600, 609; S1). Among the typed isolates, 596 isolates be-
139 longed to 40 different CCs, and 32 isolates belonged to 9 singleton STs (not belonging to any

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140 CC). The most significant group of clonal isolates was represented by ST9 (*n*=185 isolates,
141 29%), corresponding to 3 different VTs. (VT11, VT160, and VT162). In total 14 CCs accounted
142 for 95% of the isolates (Figure 1; S1).

143 ***MVLST typing***

144 MVLST results were available for all 634 isolates. In total, 51 different VTs were identi-
145 fied (S1), 17 isolates did not belong to any previously assigned VT and were therefore assigned
146 to new VTs (VT160-VT168). Overall, VT11 represented the most abundant group of isolates
147 (*n*=186, 29%), corresponding to ST9 (*n*=180) and ST204 (*n*=6). Overall, 36% (*n*=228) of the
148 isolates belonged to 9 ECs (Table 1). In particular, ECs represented 22% (*n*=90) of the food
149 chain isolates, and 64% (*n*=138) of the clinical isolates. The population structure of the isolates
150 typed with MVLST and the proportion of the different sources identified for each VT are de-
151 scribed in Figure 2.

152 W***GS analysis: antimicrobial resistance and SNP clusters***

153 Based on the NCBI Pathogen Detection browser, out of 508 isolates typed with WGS the
154 *tet*(M) gene coding for resistance to tetracycline was found in 5.3% (*n*=27), while one isolate
155 was listed with the *tet* gene. No presence of penicillin resistance genes was observed. Eighty-one
156 isolates (*n*=22 clinical and *n*=59 food/environmental) were not assigned to any SNP clusters,
157 while the remaining 427 isolates belonged to a total of 71 SNP clusters, as of April 1st, 2019
158 (Tables 2 and 3). About 32% (*n*=23) of the SNP clusters were "local", comprising only isolates
159 (*n*=73) from this study and not correlating with isolates from different countries/sources (Table
160 3). Of the 23 local SNP clusters, 16 only comprised food/environmental isolates (grouping from
161 2 to 8 isolates each), 6 only clinical isolates (grouping 2 or 3 isolates each), and 1 comprised both
162 clinical and food/environmental isolates. The latter (PDS000006278.4) grouped 3 isolates within

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163 11 SNPs, collected from a patient (blood) in 2014 and swabs from dairy plants collected in 2004
164 and 2014.
165 The remaining ~68% of SNP clusters (*n*=48) were "global", comprising 354 strains that
166 were similar to other 3,179 isolates in the database (Table 2). Overall, among all SNP clusters
167 detected herein, PDS000025311.40 was the largest, grouping a total of 517 isolates (246 clinical
168 and 271 food/environmental/other). The most predominant cluster observed among our isolates
169 was PDS000024241.19 (*n*=138), comprising ~75% of the 184 WGS-derived VT11 isolates, fol-
170 lowed by PDS000001093.24 (*n*=35), PDS000024645.27 (*n*=22), and PDS000025311.40
171 (*n*=20). Isolates belonging to the most common detected profile (i.e. VT11) were distributed in
172 5 global SNP clusters: VT9/ST11 isolates (*n*=151, 82%) in PDS000024241.19,
173 PDS000011669.6, PDS000025489.2, and PDS000024263.2; and all VT11/ST204 isolates (*n*=6,
174 3.2%) in PDS000024900.22. The remaining VT11 isolates were either in 5 local SNP clusters
175 (*n*=20, ~11%) (Table 3) or unclustered (*n*=7, 3.8%). In our study, 10 out of the 24 isolates
176 (~42%) from the production chain of Gorgonzola, a Protected Designation of Origin (PDO) blue
177 cheese, are grouped into SNP cluster PDS000001093.24 (*n*=58), which also contains isolates
178 from Gorgonzola, Taleggio, Blue Stilton and blue-veined and mold-ripened cheese isolates from
179 the US and Italy.
180 ***Source attribution***
181 All 5 combinations of models and type of data identified dairy products as the main
182 source of human listeriosis cases (maximum attribution 53%, 95% Confidence Interval [95%CI]
183 46.96-58.42; Figure 3 and 4; S2). Even if the attributions varied, the different sources ranked
184 similarly across the 5 model-data type combinations, with the exception of pork and poultry (Ta-
185 ble 4). Specifially, in the Dutch model, pork appears to be the second most important source

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186 (15% and 14% based on MLST and MVLST, respectively); while poultry appears to be more
187 important in STRUCTURE, especially when using MVLST (18%, 95%CI 15.23-21.51; S2).
188 We observed high agreement among the 5 model-data type combinations (Table 5), with
189 the lowest rho value (0.702, p<0.0001) observed between MVLST Dutch and MVLST STRUC-
190 TURE, and the highest rho value (0.997, p<0.001) between MLST STRUCTURE and MLST+
191 MVLST STRUCTURE. High rho values were also observed between the STRUCTURE and
192 Dutch models, with a rho value of 0.899 (p<0.0001) between MLST+MVLST STRUCTURE
193 and MLST Dutch. The high agreement among the different model-data type combinations sug-
194 gests a high goodness of fit. Increasing the number of loci in STRUCTURE by including 13 loci
195 for MLST and MVLST together did not influence the source attribution results significantly
196 (Figure 4).

197 **Discussion**

198 We characterized a large collection of *L. monocytogenes* isolates from human cases and
199 different putative food sources in Northern Italy and identified the most likely sources of human
200 listeriosis in that area. These results can support risk managers in prioritizing public health inter-
201 ventions. Source attribution using the microbial subtyping method is particularly important for
202 listeriosis, as not all strains have the same ability to cause disease (Nightingale et al. 2008).
203 In our study, source attribution was performed using 2 models (Dutch and STRUCTURE)
204 and 2 typing methods (MLST and MVLST), considering 8 different food sources. Moreover,
205 WGS was performed to obtain typing data, AMR data, SNP clusters, and comparison with more
206 than 26,000 isolates already present in the NCBI PD on-line databases. The screening of WGS
207 data for AMR genes showed that ~5% (*n*=27) of the isolates carried the tetracycline-conferring
208 resistance gene *tet*(M), a higher percentage than the 0.5% reported at the European level (Nielsen

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209 et al. 2017). Among our isolates, ~89% (*n*=24) of *tet*(M) positive isolates belonged to ST9/VT11
210 isolates, that were overrepresented, possibly explaining the higher proportion. As also reported in
211 other studies, *tet*(M) is the resistance gene most frequelntly detected in *L. monocytogenes* due to
212 the transfer through mobile genetic elements from other resistant Gram-positive bacteria (Hau-
213 bert et al. 2018). No isolates carried penicillin resistance genes, consistently with findings from
214 the European report (Nielsen et al. 2017).

215 In total, 40 CCs and 51 VTs were identified, with CC9 being the most prevalent type and
216 accounting for 43% of the food isolates and represented by all food sources (S1; Figure 2). On
217 the *Listeria* MLST Pasteur database, CC9 isolates (*n*=223, 6% of all isolates in the database)
218 originated from a wide variety of sources, including natural environment samples. None of the
219 CC9 isolates with available information on the Pasteur database (*n*=12) carried the *tet*(M) gene.
220 In our samples, CC9 mainly corresponded to VT11 and its Single Locus Variants (SLV – isolates
221 with *n*-1 alleles in common to the linked node; VT160 and VT162 in Figure 2). ST9/VT11 had
222 been previously identified as the most predominant and persistent type also in a study that inves-
223 tigated the presence of *L. monocytogenes* in meat processing plant in Spain (Martín et al. 2014),
224 and in a study carried out in a mushroom processing plant in the US (Murugesan et al. 2015).
225 Despite such a broad diffusion, it seems that ST9/VT11 isolates have a minor role in causing
226 clinical cases, as only 5 human clinical strains belongend to this genotype (2.3% of cases; S1),
227 and thus may be more adapted to survive in the environment. Indeed, CC9 has been observed as
228 significantly associated with food and food environment and with a particularly high prevalence
229 of truncated InlA variants, which are associated with hypovirulence (Moura et al. 2017; Nightin-
230 gale et al. 2008). The main cluster of clinical cases are instead represented by CC101 (*n*=50,
231 23%) and CC1 (*n*=31, 14.2%). In particular, CC101 is the major cluster of clinical cases, which

232 had been previously singled out in a 2014 study, where it stood out among different CCs for be-
233 ing the only one with a clear predominance of human isolates (Haase et al. 2014). A novel EC
234 associated with CC101, i.e. ECXI, was recently recognized as involved in two unrelated out-
235 breaks linked to the consumption of Ricotta salata (USA, 2012) and Taleggio cheese (Italy,
236 2011), both produced in Italy (Amato et al. 2017).

237 *L. monocytogenes* types found in foods and clinical isolates only partially overlap (Fig-
238 ures 1 and 2), strengthening the evidence that not all *L. monocytogenes* strains are equally capa-
239 ble of causing invasive disease. Overall, several studies have shown that lineage I *L. monocyto-*
240 *genes* strains are on average more virulent and more frequently associated with human clinical
241 cases than lineage II strains (Lomonaco, Nucera, Filipello, 2015; Pirone-Davies et al., 2018).
242 Such partial overlap was also observed in the local SNP clusters, with the majority (*n*=16,
243 69.5%) only grouping food/environmental isolates, followed by 26% comprising just clinical
244 isolates and only 4.3% currently containing both. Among the 81 isolates not currently included in
245 a SNP cluster, more than a half (*n*=45, 55.6%) were from food and food production enviroments,
246 while the rest was from clinical cases (*n*=22, 27%) or associated with agriculture (i.e. stools and
247 feeds, *n*=14, 17.2%). Additionally, a recent study showed that a significant proportion of *L.*
248 *monocytogenes* isolated from food production environments have reduced virulence (Van Stelten
249 et al. 2016). In light of these data, considering that current regulations in EU and US are based
250 on the sole detection of *L. monocytogenes*, it could be useful and more sustainable (e.g. given the
251 high economic impact due to recalls) to review a risk assessment process that incorporates strain-
252 specific virulence parameters, meaning the identification of virulence genes and their variants
253 that may be applied as markers either for disease-relevant strains or non-virulent strains (Wal-
254 land et al. 2015). For instance, internalin A and its truncated variants have often been identified

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255 as possible marker for reduced virulence (Van Stelten et al. 2016). Nevertheless, to date straight-
256 forward identification of such markers are still lacking, and inconsistent evidences have been
257 reported (Ferreira da Silva et al. 2017).
258 The different model-data type combinations used in the source attribution analysis
259 identifed dairy products as the main source of human listeriosis (28% to 53%) (Figures 3 and 4,
260 S2). Indeed, in Europe half of the reported outbreaks have been linked to dairy products (Lun-
261 dén, Tolvanen, and Korkeala 2004). In the Dutch model, pork appeared to be the second source
262 of listeriosis (Figure 3). This may be explained by the overrepresentation of pork isolates over
263 the other sources among the food isolates (28%; S1). This may influence the output, as the Dutch
264 model is a frequency matching based model. On the other hand, poultry appears to be a more
265 important source when using STRUCTURE, particularly with MVLST data (18%; Figure 4; S2).
266 The poultry category comprises both raw meat and cooked preparations and its impact in the
267 Dutch model may have been overshadowed due to the low number of isolates (n=13; S1). Given
268 this, STRUCTURE seems to be more reliable than the Dutch model in overcoming representa-
269 tiveness issues.
270 Because *L. monocytogenes* is highly susceptible to thermic treatment (i.e. cooking), source at-
271 tribution of the listeriosis cases is usually carried out only on ready-to-eat (RTE) products (Little
272 et al. 2010; Nielsen et al. 2017), as opposed to diseases like salmonellosis and campylobacterio-
273 sis that are studied also at the reservoir level (Pires et al. 2009; Boysen et al. 2014; Lapo Mug-
274 hini-Gras et al. 2018). Isolates collected at the reservoir level (i.e. non-RTE) were also included
275 in this study and possible associations were found, in particular with poultry (Figure 4, S2). This
276 finding underlines how controlling contamination at the reservoir level could be useful, in terms
277 of preventing cross-contamination that may occur both at the distribution (e.g. deli counters) and

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278 at the household level. Indeed, it is still poorly understood how *L. monocytogenes* circulates be-
279 tween animals, humans, and various environments (Walland et al. 2015). In particular, it has
280 been found that bovine farm environments have high prevalence rates of *L. monocytogenes*, in-
281 cluding subtypes linked to human listeriosis cases and outbreaks, and cattle appear to contribute
282 to the amplification and spread of *L. monocytogenes* in the farm environment (Nightingale et al.
283 2004). In Italy, Rocha et al. found 60% and 10% of *L. monocytogenes* isolated from bovine clini-
284 cal cases belonging to ECI and ECX, respectively (Rocha et al. 2013). Poultry is also a recog-
285 nized reservoir of *L. monocytogenes* and contaminated raw meat poses a concrete risk for the
286 human consumer (Dhama et al. 2013). In the US, several ECs were found in chicken processing
287 plants and listeriosis cases and outbreaks have been associated with consumption of undercooked
288 chicken and RTE poultry products (Lomonaco et al. 2013). Moreover, it is not clear whether
289 only specific *L. monocytogenes* subtypes are able to move from the reservoir to the hosts and
290 cause disease (Walland et al. 2015). Consequently, to improve our understanding of the ecology
291 of *L. monocytogenes*, it is important to study the prevalence of *L. monocytogenes* strains in all
292 different niches, such as the farm environment, livestock, raw materials, transport vehicles and
293 containers, manufacturing facilities (e.g. cheese plants) and humans. A recent study identified
294 eight genes significantly associated with food isolates across *L. monocytogenes* lineage II strains,
295 likely playing an important role in the survival and proliferation of *L. monocytogenes* in the food
296 environment. The authors indicated the need for futher studies on such genes as such knowledge
297 can help understand how *L. monocytogenes* adapts to the host and food environments (Pirone-
298 Davies et al., 2018).

299 Most other published source attribution studies (mainly on *Salmonella* and *Campylobac-*
300 *ter*) tend to have higher numbers of isolates (Kittl et al. 2013; de Knegt et al. 2016; Mughini-

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301 Gras et al. 2014; Boysen et al. 2014), and it has been reported that is preferable to have at least
302 100 isolates for each source analysed (Smid et al. 2013). Moreover, selection of isolates should
303 include contemporaneous sampling of isolates from sources and humans from a fixed geographic
304 area. In the current study, samples were collected over a fairly broad timeframe (13-year period,
305 2004-2016). While broad, such a timeframe was necessary to ensure that the strain collection
306 was as representative as possible within the scope of the study, given the low incidence of lister-
307 iosis.

308 **Conclusion**

309 Dairy products were identified as the most important source of human listeriosis in the
310 study area, highlighting the need for specific control measures to reduce *L. monocytogenes* con-
311 tamination in these products. To date, mainly RTE products have been included in source attribu-
312 tion studies of listeriosis. According to our results, implementing actions currently limited to
313 RTE products also at the reservoir level, may help reducing the risk of cross-contamination at the
314 distribution and household levels.

315 Considering the scarcity of data suited for source attribution of listeriosis, especially in It-
316 aly, this study represents a first stepping-stone for future research. Indeed, this is the first source
317 attribution study for listeriosis in Italy, and its routine application may help mitigating the impact
318 of the disease, both at a national and international level, by targeting the main sources. To reach
319 this goal, collaboration between the different competent authorites in a One Health perspective is
320 of paramount importance.

321

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514 **Figure 1** Minimum spanning tree of the 628 *Listeria monocytogenes* isolates typed with MLST.
515 Each circle represents a single Sequence Type (ST) indicated on the tree by the corresponding
516 number. Yellow nodes are group founders and black lines indicate Single Locus Variants (SLV –
517 isolates with *n*-1 alleles in common to the linked node). For each ST, isolates obtained from dif-
518 ferent sources are represented by the colours in the legend. The number and proportion of iso-
519 lates for each source are listed in brackets in the legend.

520 **Figure 2** Minimum spanning tree of the 634 *Listeria monocytogenes* isolates typed with
521 MVLST. Each circle represents a single Virulence Type (VT) indicated on the tree by the corre-
522 sponding number. Yellow nodes are group founders and black lines indicate Single Locus Vari-
523 ants (SLV – isolates with *n*-1 alleles in common to the linked node). For each VT, the colours
524 listed in the legend represent the proportion of isolates from the different sources. Grey slices
525 indicate isolates not assigned to any of the listed sources. The number and proportion of isolates
526 for each source are listed in brackets in the legend.

527 **Figure 3** Source attributions of listeriosis human cases with MVLST and MLST data using the
528 Dutch model (error bars denote 95% confidence intervals). Unknown bar represents clinical cas-
529 es caused by *Listeria monocytogenes* types not found in any source.

530 **Figure 4** Source attributions of listeriosis human cases with MVLST, MLST and
531 MVLST+MLST data using the STRUCTURE model (error bars denote 95% confidence inter-
532 vals).

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Table 1. Number of *L. monocytogenes* isolates belonging to each of the currently identified Epidemic Clones (ECs), among the all the strains collected from clinical cases and 8 different food sources.

	Epidemic Clones (ECs)									
Source	**I**	**II**	**IV**	**V**	**VI**	**VII**	**VIII**	**X**	**XI**	**Total**
Human	30	6	8	17		15	10	2	50	**138**
Beef				1						**1**
Dairy	13	7	4	1	1	4	2	1	8	**41**
Fish				2		1				**3**
Game	2						3			**5**
Mixed food			4	2	2	1	1			**10**
Mixed Meat	1		1	2						**4**
Pork	1		6	7	3		1	1		**19**
Poultry			1	3						**4**
Unknown		1		1			1			**3**
Total	**47**	**14**	**24**	**36**	**6**	**21**	**18**	**4**	**58**	**228**

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Table 2. List of the 48 "global" SNP cluster, comprising 354 isolates from this study and correlating with 3,178 isolates from different countries/sources avilable on the NCBI PD database (as of April 1st, 2019). The number of environmental/food/other and clinical isolates, is indicated as those originating from this study over the overall number (i.e. #/#). Bold font was used to highlight SNP clusters grouping only isolates from Italy. SNP clusters are determined by the NCBI Pathogen Isolates pipeline and several information are listed for each: Virulence Type (VT), Epidemic clone (EC), Sequence Type (ST), accession number and analysed version, overall number of isolates and specific from this study, and overall number of environmental/food/other and clinical isolates.

						Number of isolates (from this study/overall)		
Sequence Type (ST)	**Clonal Complex (CC)**	**Virulence Type (VT)**	**Epidemic Clone (EC)**	**SNP Cluster Accession ID and Version (as of April 1st, 2019)**		**Total in SNP cluster**	**Environ./ food/other**	**Clinical**
ST1	CC1	VT20	ECI	PDS000003341	.13	2/4	0/0	2/4
				PDS000003348	.26	1/18	1/6	0/12
				PDS000006160	.21	8/9	4/4	4/5
				PDS000041947	.5	1/105	0/25	1/80
ST2	CC2	VT21	ECIV	PDS000024430	.11	9/107	7/42	2/65
				PDS000024474	.2	1/3	0/0	1/3
				PDS000024705	.8	3/30	3/24	0/6
ST3	CC3	VT14	ECVIII	PDS000006340	.10	3/5	1/3	2/2
				PDS000007098	.4	2/4	0/1	2/3
				PDS000009528	.3	1/2	0/0	1/2
				PDS000009530	.3	1/2	0/1	1/1
ST5	CC5	VT63	ECVI	PDS000032961	.1	1/2	1/2	0/0
ST6	CC6	VT19	ECII	PDS000024682	.26	1/273	0/73	1/200
				PDS000024688	.2	2/4	0/0	2/4
				PDS000043734	.1	1/2	1/2	0/0
				PDS000024930	.2	1/5	1/1	0/4
				PDS000024684	.9	9/53	5/14	0/39
		VT163					3/14	1/39
ST7	CC7	VT56	ECVIII	PDS000024618	.8	4/38	4/16	0/22

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ST8	CC8	VT59	ECV	**PDS000003019**	.6	1/3	1/3	0/0
				PDS000025311	.40	20/517	17/271	3/246
ST9	CC9	VT11	*	PDS000024241	.19	138/324	136/297	2/27
				PDS000011669	.6	6/9	6/9	0/0
				PDS000025489	.2	4/6	4/6	0/0
				PDS000024263	.2	3/4	3/3	0/1
ST204	CC204			PDS000024900	.26	6/199	6/172	0/27
ST18	CC18	VT118		PDS000025244	.1	2/4	0/1	2/3
ST19	CC19	VT84		PDS000006154	.4	1/14	1/3	0/11
ST29	CC29	VT74		PDS000024749	.4	6/9	1/2	5/7
				PDS000024751	.2	1/3	1/2	0/1
ST32	CC32	VT93		PDS000037504	.2	1/6	1/1	0/5
ST388	CC388			PDS000025477	.5	1/10	1/2	0/8
ST37	CC37	VT61		PDS000032941	.18	4/174	1/111	3/63
ST38	CC101	VT80	ECXI	PDS000001213	.20	10/31	8/15	2/16
ST101				PDS000024823	.11	1/74	0/55	1/19
ST59	CC59	VT119		PDS000011242	.8	1/15	1/8	0/7
ST121	CC121	VT94		PDS000024645	.27	22/430	22/403	0/27
				PDS000024656	.28	7/457	4/424	1/33
		VT109					2/424	0/33
ST155	CC155	VT45		PDS000005514	.13	9/27	0/5	9/22
				PDS000006382	.27	1/128	1/102	0/26
ST217	CC217	VT62		PDS000024967	.21	2/128	2/20	0/108
ST224	CC224	VT124		PDS000009525	.4	1/3	0/2	1/1
ST296	CC88	VT8		PDS000003204	.81	1/128	1/104	0/24
ST325	CC31	VT113		PDS000001093	.24	35/58	30/53	5/5
ST394	CC415	VT2		PDS000009385	.6	1/10	0/9	1/1
ST398	CC398	VT100		PDS000024700	.1	13/14	12/13	1/1
ST425	CC90	VT151		PDS000042587	.1	1/6	0/0	1/6
ST451	CC451	VT140		PDS000024708	.17	1/69	0/29	1/40
ST562	CC562	VT166		PDS000004800	.42	3/7	3/6	0/1
TOTAL						354/3533	269/2345	58/1188

* includes 21 strains carrying *tet*(M) (overall this SNP cluster includes two more *tet*(M)-carrying strains from Italy, which were not included in Lomonaco et al., 2018)

includes 3 strains carrying *tet*(M)

$ includes 1 strain carrying *tet*

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Table 3. List of the 20 "local" SNP cluster, comprising isolates (*n*=73) correlating only with other Italian isolates originating from the current study (as of April 1st, 2019). SNP clusters are determined by the NCBI Pathogen Isolates pipeline and several information are listed for each: Sequence Type (ST), Clonal Complex (CC), Virulence Type (VT), Epidemic clone (EC), accession number and analysed version, overall number of isolates and specific from this study, and overall number of environmental/food/other and clinical isolates. The SNP clusters are divided into three groups, those only grouping environmental/food/other isolates, those grouping only clinical and those grouping both. Bold font was used to highlight the same VT/ST observed in different groups, while * was used to indicate isolates carrying the *tet*(M) gene.

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Type of isolates grouped	Sequence Type (ST)	Clonal Complex (CC)	Virulence Type (VT)	Epidemic Clone (EC)	SNP Cluster Accession ID	Version (as of April 1st, 2019)	# of env./food/other isolates	# of Clinical isolates
Only environmental /food / other isolates	**ST1**	**CC1**	**VT20**	**ECI**	PDS000016512	.1	2	0
					PDS000016511	.1	5	0
					PDS000006159	.3	3	0
	ST2	CC2	VT21	ECIV	PDS000005749	.4	3	0
	ST3	**CC3**	**VT14**	**ECVIII**	PDS000009529	.3	4	0
	ST5	**CC5**	**VT63**	**ECVI**	PDS000016519	.1	3	0
	ST9	CC9	VT11	*	PDS000006163	.4	8	0
			VT11		PDS000024252	.1	5	0
			VT162		PDS000024740	.1	4	0
			VT11		PDS000024741	.1	3	0
			VT11		PDS000025500	.1	2	0
			VT160			.1	1	0
			VT11		PDS000024296	.1	2	0
	ST36	**CC36**	**VT75**		PDS000024703	.1	3	0
	ST427	CC29	VT74		PDS000006155	.5	5	0
	ST663	ST663	VT62		PDS000024699	.1	2	0
					PDS000024702	.1	2	0
Only clinical isolates	**ST1**	**CC1**	**VT20**	**ECI**	PDS000024707	.1	0	2
	ST5	**CC5**	**VT63**	**ECVI**	PDS000016343	.1	0	3
	ST7	CC7	VT56	ECVII	PDS000016346	.1	0	2
	ST14	CC14	VT125		PDS000016335	.1	0	2
	ST54	CC54	VT79		PDS000016380	.1	0	2
	ST398	CC398	VT100		PDS000024922	.1	0	2
Both env./food/other and clinical isolates	**ST3**	**CC3**	**VT14**	**ECVIII**	PDS000006278	.4	2	1
TOTAL							59	14

* isolates carrying the *tet*(M) gene

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Table 4. Median of ranks and the ranks (in descending order) for each of the 8 food sources and each of the 5 model-data type combination considered herein.

	Dutch		STRUCTURE			
Source	**MLST**	**MVLST**	**MLST**	**MVLST**	**MLST + MVLST**	**Median**
Dairy	1	1	1	1	1	1
Poultry	5	7	2	2	2	2
Mixed food	3	4	3	3	3	3
Fish	6	6	4	5	4	5
Mixed meat	4	3	5	6	5	5
Game meat	7	5	6	4	6	6
Pork	2	2	7	7	7	7
Beef	8	8	8	8	8	8

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Table 5. Pearson correlation coefficient (rho) matrix to calculate the agreement between attributions obtained with the 5 model-data type combination considered herein The lowest and highest rho values are marked in bold.

		Dutch		STRUCTURE		
		MLST	**MVLST**	**MLST**	**MVLST**	**MLST + MVLST**
Dutch	**MLST**	1	*	*	*	*
	MVLST	0.979	1	*	*	*
STRUCTURE	**MLST**	0.918	0.85	1	*	*
	MVLST	0.762	**0.702**	0.934	1	*
	MLST + MVLST	0.899	0.828	**0.997**	0.953	1

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Highlights

- Up to 53% of listeriosis cases in Northern Italy are attributable to dairy products
- 37% of isolates were Epidemic Clones, strains involved in more than one outbreak
- Poultry accounted for up to 18% listeriosis cases
- Including isolates at the reservoir level may identify cross-contamination events

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Conflict of Interest and Authorship Conformation Form

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- ✓ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

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