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This is a pre print version of the following article:

Original

Survey of prevalence and seasonal variability of *Listeria monocytogenes* in raw cow milk from Northern Italy / Dalzini, Elena; Bernini, Valentina; Barbara, Bertasi; Paolo, Daminelli; Nadia, Losio Marina; Giorgio, Varisco. - In: FOOD CONTROL. - ISSN 0956-7135. - 60:(2016), pp. 466-470.
[10.1016/j.foodcont.2015.08.019]

Availability:

This version is available at: 11381/2795453 since: 2021-11-02T11:08:42Z

Publisher:

Elsevier Ltd

Published

DOI:10.1016/j.foodcont.2015.08.019

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note finali coverpage

(Article begins on next page)

23 July 2024

1 **Survey of prevalence and seasonal variation of *Listeria monocytogenes* in raw cow milk**
2 **from Northern Italy**

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25 **Abstract**

26 *Listeria monocytogenes* is an important food-borne pathogen causing meningitis, meningo-
27 encephalitis and abortion. Both sporadic and epidemic human listeriosis cases are associated
28 with the consumption of contaminated foods. To assess the potential risk to consumer health,
29 the presence of *L. monocytogenes* was investigated using qualitative and quantitative methods
30 in raw milk (bulk tank milk and milk for vending machine) collected from 2010 to 2013 in
31 Northern Italy (Lombardy and Emilia-Romagna regions). Overall, *L. monocytogenes* was
32 detected in 145 on 8716 of raw milk samples, with a prevalence of 1.66% (95% C.I. 1.4%-
33 1.7%). The prevalence ranged from 0.52% (95% C.I. 0.3%-0.9%) in 2012 to 2.7% (95% C.I.
34 2.0%-3.8%) in 2013, but no trend of increase was observed in four-years of investigation. The
35 pathogen was detected from 2.2% (95% C.I. 1.9%-2.6%) of bulk tank milk and from 0.5%
36 (95% C.I. 0.3%-0.8%) of milk for vending machine. A significative difference ($p < 0.05$) of
37 the prevalence data was observed between data collected in two different regions of Northern
38 Italy with an higher prevalence in Lombardy. In addition to the geographical area, the *L.*
39 *monocytogenes* presence was influenced also by the seasonal period of collection samples,
40 with peaks in spring and autumn. These results confirm the raw milk can be a source of
41 foodborne illness outbreaks if consumed without sanitizing treatments, but the low prevalence
42 and the low contamination levels (more than 80% of the contaminated samples contained <10
43 cfu ml⁻¹ of *L. monocytogenes*) proving the hygienic quality of the milk produced in Northern
44 Italy.

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49 **Key words:** Raw milk; incidence; *Listeria monocytogenes*; risk analysis.

50 **1. Introduction**

51 Milk and dairy products are assumed to be implicated in 2-6% of the bacterial foodborne
52 diseases (De Buyser et al., 2001) and, in particular, the presence of foodborne pathogens in
53 raw milk and bulk tank milk has been widely reported (D'Amico and Donnelly, 2010; De Reu
54 et al., 2004; Gaya et al., 1996; Hassan et al., 2000; Jayarao and Henning, 2001; Kousta et al.,
55 2010; Moshtaghi and Mohamadpour, 2007; Van Kessel et al., 2004; Waak et al., 2002). The
56 prevalence of pathogens in milk is influenced by numerous factors such as farm size, number
57 of animals on the farm, hygienic conditions, farm management practices, variation in sampling
58 and types of samples evaluated, differences in detection methodologies used, geographical
59 location, and season. However, in spite of the variation, all of these surveys clearly
60 demonstrated that milk can be a major source of foodborne pathogens of human health
61 significance (Oliver et al., 2005).

62 In this context, *Listeria monocytogenes*, responsible of listeriosis, represents one of the most
63 serious food safety concerns. It has been isolated from many foods (Gianfranceschi et al.,
64 2003), among which milk and cheeses (Bernini et al., 2013; Dalmaso and Jordan, 2014;
65 Manfreda et al., 2005; Pintado et al., 2005; Torres-Vitela et al., 2012) and it has been involved
66 in numerous outbreaks occurring after consumption of contaminated milk and milk products
67 throughout the world (Linnan et al., 1988; Lyytikainen et al., 2000; Donnelly, 2001; Lunde'n
68 et al., 2004). In particular, dairy products contaminated with *L. monocytogenes* have been
69 implicated at almost the half of the reported listeriosis outbreaks in Europe (Lunden,
70 Tolvanen & Korkeala, 2004). The pathogen is widespread in nature and lives naturally in
71 plants and soil environments. Its ability to survive and grow over a wide range of
72 environmental conditions, including refrigeration temperatures, high salt concentration and
73 low pH, makes it a potential hazard in foods (Ryser, 2007). Moreover, *L. monocytogenes* into
74 food processing plants results in reservoirs that are difficult to eradicate: this is the case of

75 biofilms that are a constant issue in food processing environments (Oliver et al., 2005). So, in
76 addition to the risk associated to direct consumption, the introduction of raw contaminated
77 milk into dairy processing plants represents a risk to human health if milk is used
78 unpasteurized for cheese making or in case of cross contamination (Kousta et al., 2010).
79 Considering the threat represented by the pathogen in raw milk, a survey was conducted from
80 January 2010 to September 2013, involving a large number of samples collected in different
81 geographical area in the North of Italy. This research aimed to give a considerable overview
82 of *L. monocytogenes* presence in raw cow milk, intended both for cheese making and for
83 direct consumption, by evaluating the prevalence of the pathogen at farm level also in relation
84 to the seasonality.

85

86 **2. Materials and methods**

87

88 *2.1. Samples collection*

89 A total of 8716 raw cow milk samples were collected from January 2010 to September 2013 in
90 942 farms located in Lombardy and Emilia-Romagna regions, Northern Italy. All the samples,
91 consisting in 5897 samples of bulk tank milk intended for cheese making and 2819 samples of
92 milk intended for sale in automatic vending machines, were collected into sterile containers,
93 kept below 4 °C during transportation and analysed within 2 h after receipt. Samples were
94 collected in the frame of Food Business Operator's self-control programs or in the frame of
95 monitoring surveys officers of the Regional Veterinary Authority.

96

97 *2.2. Detection and enumeration of *L. monocytogenes**

98 All samples were tested for the presence of *L. monocytogenes* on 25 ml of raw cow milk by
99 means of qualitative methods.

100 The samples collected from 2010 to 2011 were examined qualitatively according to ISO
101 11290-1 (ISO, 1996). The samples collected from 2012 to 2013 were examined by a
102 biomolecular method (real-time PCR) (Biorad AFNOR BRD 07/ 10-04/05) (AFNOR, 2004) to
103 detect *L. monocytogenes* DNA. Samples testing positive were retested under a microbiological
104 protocol according to ISO 11290-1 (ISO, 1996). Typical colonies (n = 5) presumed to be
105 *Listeria* spp. were streaked from Agar Listeria acc. to Ottaviani & Agosti (ALOA) (Biolife
106 Italiana, Teramo, Italy) supplemented with ALOA enrichment-selective supplements (Biolife
107 Italiana) onto Tryptone Soya Yeast Extract Agar, TSYEA (Oxoid, Basingstoke, UK) and
108 plates were incubated at 37 °C for 24 h. By following the Gram's staining, catalase reaction
109 and tumbling motility were performed using the pure cultures obtained from TSYEA. The
110 isolates resulted positive to phenotypic tests were inoculated on 5% sheep blood agar (Oxoid)
111 to determine the Beta haemolytic reaction. For following confirmation, carbohydrate
112 utilization and CAMP tests were performed.

113 On samples found to be positive, the enumeration of *L. monocytogenes* was carried out
114 according to the method described by ISO 11290-2 (ISO, 1998) to evaluate the prevalence of
115 contamination level.

116

117 2.3. Data analyses

118 The prevalence of *L. monocytogenes* in raw milk, calculated as proportion between positive
119 samples on total sample, was expressed in percentage values. Statistical analysis was
120 performed by Epi tools (<http://epitools.ausvet.com.au>): the confidence intervals (C.I.) of
121 proportions were calculated with using the binomial exact method and the statistical
122 significance of differences between proportions was evaluated by Chi-square (χ^2) test.

123

124 3. Results and discussion

125 From 2010 to 2013, 8716 raw milk samples, intended both for cheese making and for vending
126 machine, were collected in Northern Italy. Samples were taken from local farms within self-
127 control sampling programs and by the official veterinarians within state surveillance programs
128 and were investigated for the presence of *L. monocytogenes*.

129 The results are summarized in Table 1. The prevalence values in raw milk ranged from 0.52%
130 (95% C.I. 0.3%-0.9%) in 2012 to 2.73% (95% C.I. 2.0%-3.8%) in 2013, but no trend of
131 increase was observed in four-year investigation. Overall, *L. monocytogenes* was detected in
132 145 raw milk samples out of 8716, with a prevalence of 1.66% (95% C.I. 1.4%-2.0%). This
133 result is mainly due ($p > 0.05$) to bulk tank milk contamination rather than to raw milk
134 intended for vending machine. In fact, concerning bulk tank milk, *L. monocytogenes* was found
135 in 2.22% (95% C.I. 1.9%-2.6%) of samples (131/5897) in four years of survey (Table 2).

136 These findings were in agreement with those reported in several studies carried out
137 internationally and recently published, in which the prevalence of *L. monocytogenes* in raw
138 milk has ranged from ‘not detected’ to 7.1%. In particular, the pathogen was “not detected” in
139 Norway in 2011 (Jakobsen et al., 2011), and to the extent of 0.68% in New Zealand in 2012
140 (Hill et al., 2012), 2.12% in Turkey in 2006 (Aygün & Pehlivanlar, 2006), 2.61% in Algeria
141 in 2007 (Hamdi et al., 2007), 5.5% in Finnish in 2013 (Ruusunen et al., 2013), 6.1% in North-
142 West Spain in 2007 (Vilar et al., 2007) and 7.1% in USA in 2011 (Van Kessel et al., 2011).

143 Previously, in 2005, the prevalence of *L. monocytogenes* in bulk tank milk has been reported
144 to range from 1 to 12% (Oliver et al., 2005), therefore, a reduction of contamination samples
145 seems to have been monitored in the last years. Anyway, the above data collected in different
146 world areas underline the wide variability of the *L. monocytogenes* prevalence, which can be
147 due to several factors, as geographical area, size farm, types of housings for the cattle. As
148 further confirmation of this variability, the overall data we collected in this study show that
149 even in the same geographical area there may be a different prevalence of *L. monocytogenes*.

150 In Lombardy region, the pathogen was detected in 96 (2.6%; 95% C.I. 2.1%-3.1%) of 3721
151 samples analyzed and the prevalence was statistically highest ($p < 0.05$) compared to that
152 found in Emilia-Romagna region, where *L. monocytogenes* was detected in 26 (1.2%; 95%
153 C.I. 0.8%-1.8%) of 2176 milk samples (data not shown). The different prevalence values can
154 be due to the different treatment of the cattle usually practiced in the two Italian regions. In
155 Lombardy, the most of the cattle is fed with silages, the housing of cattle is indoors and these
156 practices, when combined with poor hygiene on the farm, may contribute to contamination of
157 milk, in agreement with Husu et al. (1990) and Sanaa et al. (1993). In contrast with this, many
158 researchers have identified the raw milk as a source of *L. monocytogenes*, but environmental
159 and fecal contamination during the transportation and storage of milk have also been reported
160 (Frece et al., 2010). Moreover, *L. monocytogenes* may also directly contaminate milk from
161 animals with mastitis (Hird & Genigeorgis, 1990).

162 A low prevalence of *L. monocytogenes* was observed over the four years survey in raw milk
163 samples indented for vending machine (Table 2). The pathogen was detected in 0.50% (95%
164 C.I. 0.03%-0.8%) of samples (14/2819), but the prevalence ranged from 0% (95% C.I. 0%-
165 0.7%) in 2010 to 1.57% (95% C.I. 0.7%-3.1%) in 2011. A similar result was obtained by
166 Bianchi et al. (2013) who detected the *L. monocytogenes* in 1.6% of the milk samples for
167 vending machine during the monitoring survey from 2009 to 2011 in another Italian region
168 (Piedmont region, near Lombardy).

169 *L. monocytogenes* is widely extended throughout the environment (Fenlon et al., 1996). This
170 pathogen has the ability to survive in stress conditions and it is able to grow at low
171 temperatures in several food types as cooked meat (Daminelli et al., 2014), vegetables
172 (Sant'Ana et al., 2012), cold-smoked salmon (Beaufort et al., 2007), milk and cheese
173 (Schvartzman et al., 2010). To prevent the growth of the pathogen in raw milk, in Italy, where
174 the sale and the distribution of unpacked raw milk via automatic self-service vending

175 machines was authorized since 2007, the product must be maintained at constant temperature
176 between 0 °C and 4 °C, and the customers are instructed to boil the milk before consumption.
177 Even if the most important aspect remains the hygienic quality of the product, these practices
178 can contribute to improve the safety of the raw milk, as shown the low prevalence of *L.*
179 *monocytogenes* reported in the present study.

180 To evaluate the seasonal influence on the *L. monocytogenes* presence in bulk tank milk, in
181 Table 3 were reported the prevalence data for each year (from 2010 to 2013), broken down by
182 each month. In four years, only in January on a total of 188 samples analyzed, milk samples
183 positive for *L. monocytogenes* have never been found. Considering the seasonal variability,
184 the *L. monocytogenes* prevalence was statistically lower ($p < 0.05$) during the winter season,
185 with a prevalence of 0.8% (95% C.I. 0.3%-1.7%), in contrast with the spring prevalence of
186 3.04% (95% C.I. 2.3%-3.9%), the summer prevalence of 1.91% (95% C.I. 1.4%-2.6%) and
187 the autumn prevalence of 2.33% (95% C.I. 1.6%-3.3%) (Table 3). Previous studies on
188 *Listeria* spp. prevalence in raw milk reported some evidence of seasonal variation. Atil et al.
189 (2011) in eastern Turkey observed a high prevalence in spring and winter; in France, Meyer-
190 Broseta et al. (2003) reported peaks in winter. Ryser (1999) reported that seasonal variations
191 in *Listeria* prevalence may be related to silage feeding, with higher prevalence in months
192 when silage is fed to animals. In fact, *L. monocytogenes* could be present in silage, in which
193 the pathogen can multiply if the silage has been inadequately fermented (pH above 5.0 to 5.5)
194 (Husu, 1990). Seasonal differences in *L. monocytogenes* prevalence were observed in our
195 monitoring, but more studies are needed to determine the validity and the causes of these
196 differences. In fact, in the present study, the reduced number of samples found positive to *L.*
197 *monocytogenes* in the winter season may be due to the lower number of samples analyzed
198 (746 samples against over 1000 samples analyzed in each of the other seasons), or to other

199 variables such as the type of cattle feed (fresh grass or silage), the hygienic conditions of
200 breeding or the presence of cattle infected with mastitis.
201 The contamination levels of *L. monocytogenes* in bulk tank milk samples are reported in
202 Figure 1 as percentage of samples with pathogen concentration variable between <1 and
203 >1000 cfu ml⁻¹. Overall, more than 80% of the contaminated samples contained <10 cfu ml⁻¹
204 of *L. monocytogenes*. This trend is reflected from 2010 to 2012, while in 2013 the majority of
205 the positive samples contained <1 cfu ml⁻¹ of the pathogen, as shown in Figure 1. It is difficult
206 to compare the results from this study with those conducted in other countries, because most
207 studies express the results only qualitatively. However, a study by Meyer-Broseta et al.
208 (2003), shows that even in France, the level of contamination of bulk tank milk was very low,
209 generally less than 1 cfu ml⁻¹ of the *L. monocytogenes*.

210

211 **4. Conclusions**

212 During 2007-2009 in Italy there was an increase of notifications of listeriosis with the most
213 cases are reported in the Centre-North of Italy. This is probably attributable both to a real
214 increase of listeriosis in Italy and to surveillance implementation (Pontello et al., 2012).
215 However, statistically significant increasing trends in listeriosis notification rates from 2005 to
216 2009 were noted in Italy as elsewhere in Europe (EFSA/ECDC, 2011). In the present study the
217 raw milk was found positive to the presence of *L. monocytogenes*, confirming the milk as
218 potential source of the food borne disease, but the low prevalence and the reduced level of
219 pathogen concentration, when present, can highlight the hygienic quality of the milk produced
220 in Northern Italy. Furthermore, the collected data in the present study can be a useful tool for
221 the quantitative risk assessment study for human listeriosis linked to the consumption of raw
222 milk and cheese made from raw milk in Italy. Furthermore, the collected data in the present

223 study can be a useful tool for the quantitative risk assessment study for human listeriosis
224 linked to the consumption of raw milk and cheese made from raw milk in Italy.

225

226 **Disclosure**

227 Authors declare that no conflict of interests exists.

228

229 **Acknowledgements**

230 We sincerely thank Dr. Silvia Todeschi (Food Microbiology Department, IZSLER, Brescia,
231 Italy) for the technical assistance and for the informatics support development to the data
232 management.

233

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377 **Table 1.** Detection and prevalence of *L. monocytogenes* in raw milk collected in Northern
378 Italy according to the year of sampling.

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Year	Samples	Positive for <i>L. monocytogenes</i> (%)
2010	1728	20 (1.16%)
2011	3150	76 (2.41%)
2012	2519	13 (0.52%)
2013	1319	36 (2.73%)
Total	8716	145 (1.66%)

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398 **Table 2.** Detection and prevalence of *L. monocytogenes* in different categories of raw milk
 399 collected in Northern Italy according to the year of sampling.

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Year	Bulk Tank Milk		Raw Milk for vending machine	
	Samples	Positive for <i>L. monocytogenes</i> (%)	Samples	Positive for <i>L. monocytogenes</i> (%)
2010	1176	20 (1.70%)	552	0
2011	2639	68 (2.58%)	511	8 (1.57%)
2012	1317	9 (0.68%)	1202	4 (0.33%)
2013	765	34 (4.44%)	554	2 (0.36%)
Total	5897	131 (2.22%)	2819	14 (0.50%)

401 **Table 3.** Seasonal detection and prevalence of *L. monocytogenes* in bulk tank milk collected in Northern Italy according to the year of sampling.

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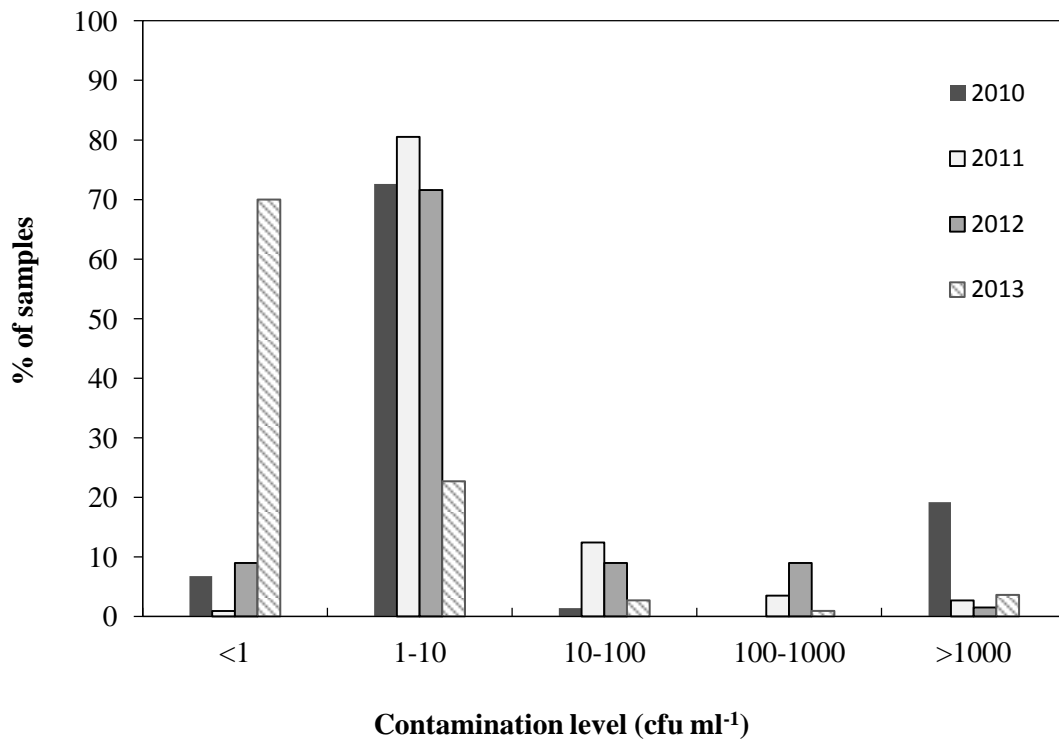
Season	Month	Sample positive / Sample analyzed (%)					
		2010	2011	2012	2013	Total for Month	Total for Season
Winter	December	0/58 (0%)	2/106 (1.89%)	0/45 (0%)	nc	2/209 (0.96%)	6/746 (0.8%)
	January	0/47 (0%)	0/49 (0%)	0/70 (0%)	0/22 (0%)	0/188 (0%)	
	February	0/108 (0%)	1/51 (1.96%)	3/158 (1.9%)	0/32 (0%)	4/349 (1.15%)	
Spring	March	2/174 (1.15%)	7/233 (3%)	0/215 (0%)	21/212 (9.91%)	30/834 (3.6%)	56/1844 (3.04%)
	April	0/74 (0%)	8/225 (3.56%)	0/79 (0%)	12/167 (7.19%)	20/545 (3.67%)	
	May	1/112 (0.89%)	5/218 (2.29%)	0/76 (0%)	0/59 (0%)	6/465 (1.29%)	
Summer	June	4/84 (4.76%)	5/297 (1.68%)	0/80 (0%)	0/73 (0%)	9/534 (1.69%)	37/1934 (1.91%)
	July	2/116 (1.72%)	8/463 (1.73%)	5/212 (2.36%)	1/148 (0.68%)	16/939 (1.7%)	
	August	2/129 (1.55%)	10/121 (8.26%)	0/162 (0%)	0/49 (0%)	12/461 (2.6%)	
Autumn	September	6/119 (5.04%)	12/485 (2.47%)	1/154 (0.65%)	0/3 (0%)	19/761 (2.5%)	32/1373 (2.33%)
	October	3/97 (3.09%)	5/273 (1.83%)	0/35 (0%)	nc	8/405 (1.98%)	
	November	0/58 (0%)	5/118 (4.24%)	0/31 (0%)	nc	5/207 (2.42%)	

nc: data not collected.

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404 **Figure 1.** Counts of *L. monocytogenes* in bulk tank milk according the year of sampling,
405 expressed as percentages of samples containing colony numbers ranging from < 1 to > 1000
406 cfu ml⁻¹.

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Highlights

1. Survey study of *Listeria monocytogenes* in raw cow mil in Northern Italy.
2. Low level of milk contamination with *L. monocytogenes*.
3. Seasonal differences in *L. monocytogenes* prevalence (lower in winter).
4. Overall, more than 80% of the contaminated samples contained low *L. monocytogenes* concentration (<10 cfu ml⁻¹).