Survey of prevalence and seasonal variability of Listeria monocytogenes in raw cow milk from Northern Italy

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

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**Abstract**

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

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

## 1. Introduction

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

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

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

## 2. Materials and methods

### *2.1. Samples collection*

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

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

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

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

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

### *2.3. Data analyses*

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

## 3. Results and discussion

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

The results are summarized in Table 1. The prevalence values in raw milk ranged from 0.52% (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 increase was observed in four-year investigation. Overall, *L. monocytogenes* was detected in 145 raw milk samples out of 8716, with a prevalence of 1.66% (95% C.I. 1.4%-2.0%). This result is mainly due ($p > 0.05$) to bulk tank milk contamination rather than to raw milk intended for vending machine. In fact, concerning bulk tank milk, *L. monocytoges* was found in 2.22% (95% C.I. 1.9%-2.6%) of samples (131/5897) in four years of survey (Table 2). These findings were in agreement with those reported in several studies carried out internationally and recently published, in which the prevalence of *L. monocytogenes* in raw milk has ranged from ‘not detected’ to 7.1%. In particular, the pathogen was “not detected” in Norway in 2011 (Jakobsen et al., 2011), and to the extent of 0.68% in New Zeland in 2012 (Hill et al., 2012), 2.12% in Turkey in 2006 (Aygun & Pehlivanlar, 2006), 2.61% in Algeria in 2007 (Hamdi et al., 2007), 5.5% in Finnish in 2013 (Ruusunen et al., 2013), 6.1% in North-West Spain in 2007 (Vilar et al., 2007) and 7.1% in USA in 2011 (Van Kessel et al., 2011). Previously, in 2005, the prevalence of *L. monocytogenes* in bulk tank milk has been reported to range from 1 to 12% (Oliver et al., 2005), therefore, a reduction of contamination samples seems to have been monitored in the last years. Anyway, the above data collected in different world areas underline the wide variability of the *L. monocytogenes* prevalence, which can be due to several factors, as geographical area, size farm, types of housings for the cattle. As further confirmation of this variability, the overall data we collected in this study show that even in the same geographical area there may be a different prevalence of *L. monocytogenes*.

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

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

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

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

To evaluate the seasonal influence on the *L. monocytogenes* presence in bulk tank milk, in Table 3 were reported the prevalence data for each year (from 2010 to 2013), broken down by each month. In four years, only in January on a total of 188 samples analyzed, milk samples positive for *L. monocytogenes* have never been found. Considering the seasonal variability, the *L. monocytogenes* prevalence was statistically lower ($p < 0.05$) during the winter season, with a prevalence of 0.8% (95% C.I. 0.3%-1.7%), in contrast with the spring prevalence of 3.04% (95% C.I. 2.3%-3.9%), the summer prevalence of 1.91% (95% C.I. 1.4%-2.6%) and the autumn prevalence of 2.33% (95% C.I. 1.6%-3.3%) (Table 3). Previous studies on *Listeria* spp. prevalence in raw milk reported some evidence of seasonal variation. Atil et al. (2011) in eastern Turkey observed a high prevalence in spring and winter; in France, Meyer-Broseta et al. (2003) reported peaks in winter. Ryser (1999) reported that seasonal variations in *Listeria* prevalence may be related to silage feeding, with higher prevalence in months when silage is fed to animals. In fact, *L. monocytogenes* could be present in silage, in which the pathogen can multiply if the silage has been inadequately fermented (pH above 5.0 to 5.5) (Husu, 1990). Seasonal differences in *L. monocytogenes* prevalence were observed in our monitoring, but more studies are needed to determine the validity and the causes of these differences. In fact, in the present study, the reduced number of samples found positive to *L. monocytogenes* in the winter season may be due to the lower number of samples analyzed (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. monocytogeses* 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 $ml^{-l}$
204 of *L. monocytogeses*. This trend is reflected from 2010 to 2012, while in 2013 the majority of
205 the positive samples contained <1 cfu $ml^{-l}$ 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 $ml^{-l}$ 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

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

## Disclosure

Authors declare that no conflict of interests exists.


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

| Year | Samples | Positive for *L. monocytogenes* (%) |
|---|---|---|
| 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%) |

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.
400

| Year | Bulk Tank Milk | | Raw Milk for vending machine | |
|---|---|---|---|---|
| | Samples | Positive for *L. monocytogenes* (%) | Samples | Positive for *L. monocytogenes* (%) |
| 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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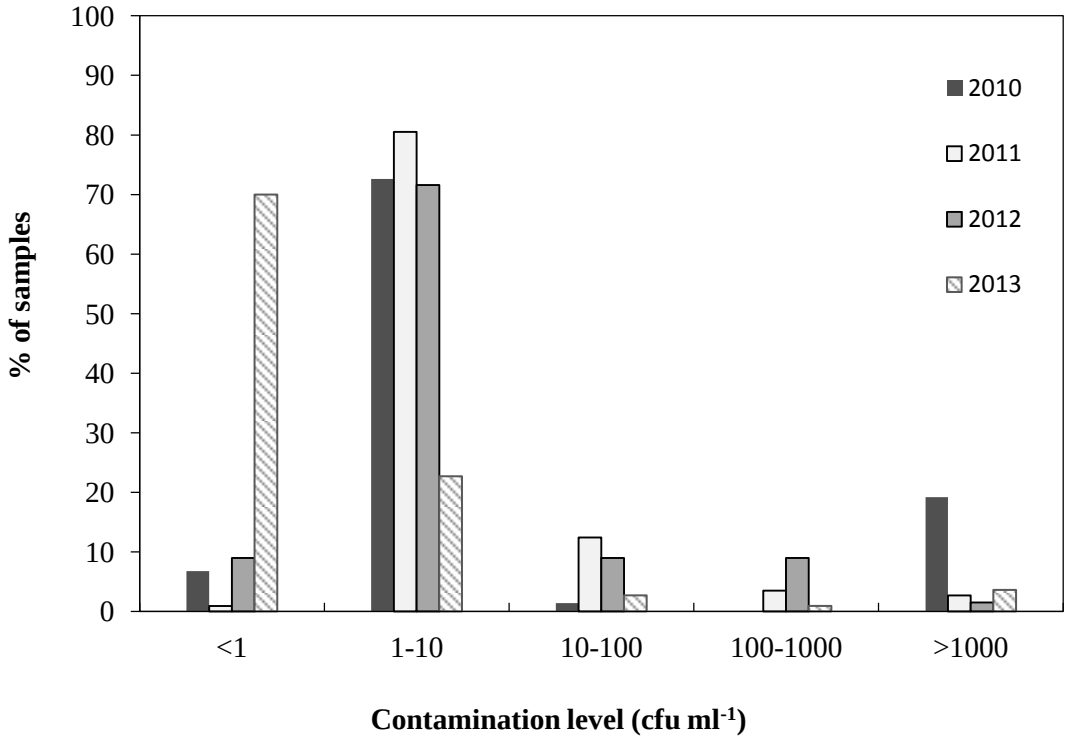
**Figure 1**. Counts of *L. monocytogenes* in bulk tank milk according the year of sampling, expressed as percentages of samples containing colony numbers ranging from < 1 to > 1000 cfu $ml^{-1}$.



## 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. monocytogeses* concentration (<10 cfu $ml^{-l}$ ).