**Listeria monocytogenes persistence in ready-to-eat sausages and in processing plants**

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**Abstract**

Listeria monocytogenes is of major concern in the fermented meat products and is able to persist in their processing environments. The aim of the present work was to evaluate the virulence profile and the persistence capacity of L. monocytogenes strains isolated in Sardinian fermented sausages processing plants. Food (ground meat, sausages at the end of acidification and ripening stage) and environmental samples (a total of n. 385), collected from 4 meat processing plants located in Sardinia (Italy), were examined to detect L. monocytogenes presence. All the L. monocytogenes isolates were identified by polymerase chain reaction (PCR) method. A subset of strains was also characterised by multiplex PCR-based serogrouping, using the lmo0737, lmo1118, ORF2819 and ORF2110 genes. Three different multiplex PCRs were used to obtain the virulence profiles by the rrl, hlyA, actA, prfA, intA, intB, iap, plcA, plcB and mpl marker genes. Furthermore, in vitro biofilm forming ability and resistance to disinfectants were carried out on microtiter plate. The overall prevalence was 31.5% in food, and 68.5% in environmental samples. The prevalent serotype resulted 1/2c (43%), followed by 1/2a (40%), 4b (8.6%), and 1/2b (8.6%). The amplification products of the virulence genes were found in all the isolates with the following prevalence: 77.1% hlyA; 100% rrl; 100% prfA; 97.1% iap; 65.7% intB; 88.6% intA; 100% plcA; 100% plcB and 74.3% mpl. As for biofilm forming ability, 37.1% of the strains were sensitive and resulted weak producer, but all the isolates were sensible to disinfectants showing a reduction of L. monocytogenes growth after each incubation time. More appropriate technologies and application of measures of hygienic control should be implemented to prevent the L. monocytogenes growth and cross-contamination in salsiccia sarda processing plants.

**Introduction**

Listeria monocytogenes environmental persistent colonisation of ready-to-eat meat processing plants has been reported by several authors (Gram et al., 2007). The pathogen may survive in meat processing plants because its capability to multiply at low temperatures, adapt to disinfectants and adhere to various surfaces; furthermore, the biofilm forming ability is an important cause for such persistence (Arevalos-Sánchez et al., 2012; Cruz et al., 2012; Fonnesbech Vogel et al., 2001). It is not still clear if the persistence ability of L. monocytogenes is the result of adaption of certain subtypes, of poor cleaning and disinfection procedures or tolerance to some of the used disinfectants (Gram et al., 2007). Efficiency in cleaning and disinfection process is essential in preventing L. monocytogenes contamination of meat products. However, the presence of organic debris may inactivate the disinfectant, hence, the bacteria may be exposed to only sublethal concentrations and survives (Cruz et al., 2012).

In Sardinian fermented sausages and their facilities L. monocytogenes was detected (Mazzette et al., 2006), and serotypes involved in sporadic outbreaks of listeriosis (1/2a, 1/2b, 1/2c and 4b) were reported. Longitudinal studies were previously carried out to evaluate the occurrence in this traditional ready to eat meat product, and to trace the sources and the routes in the processing plants. Moreover, the persistence ability of L. monocytogenes strains was demonstrated by molecular typing methods (Meloni et al., 2007, 2012a, 2012b).

The aim of the present work was to evaluate the virulence profile and the persistence capacity of L. monocytogenes strains isolated in Sardinian fermented sausages processing plants, during a short period of time, and to study the effectiveness of disinfecting products against the strains, in order to define more appropriate control strategies of the environment contamination.

**Materials and Methods**

A total of n. 385 samples taken from 4 Sardinian sausage processing plants, from 4 meat processing plants, representative of the positive samples, was selected from various sources. The different plants, and, within these, the environmental and food samples, and the sampling sessions (I, II), were considered. The subset of L. monocytogenes strains were submitted to the biomolecular and phenotypic investigations described in the following.

**Serotyping and molecular pathogenic profile**

Serotyping was performed as described by Doumith et al. (2004). The marker genes selected for the multiplex polymerase chain reaction (PCR) assay were lmo0737, lmo1118, ORF2819 and ORF2110. Moreover, three different multiplex PCRs were performed in order to detect ten virulence-associated genes using the protocols of Border et al. (1990) and Jaradat et al. (2002), with some modifications. Multiplex PCR 1 amplified the rrl, hlyA, actA and prfA genes, multiplex PCR 2 the intA, intB and iap genes and multiplex PCR 3 the plcA, plcB and mpl genes. The GoTaq Hot Start Master Mix (Promega Corp., Madison, WI, USA) was used for amplification of the target DNA fragments, as indicated in the manufacturer’s instructions. Primers sequences used are set in Table 1.

In vitro biofilm forming ability and resistance to disinfectants

The quantitative in vitro biofilm forming ability (BFA) was carried out on sterile 96-well polystyrene microtiter plates, using the method described by Stepanovic et al. (2004), modified. Each strain was inoculated into two molecular tests (Mureddu et al., 2011). Among these, a subset of n. 35 L. monocytogenes strains, representative of the positive samples, was selected from various sources. The different plants, and, within these, the environmental and food samples, and the sampling sessions (I, II), were considered. The subset of L. monocytogenes strains were submitted to the biomolecular and phenotypic investigations described in the following.
different microplates in order to assess biofilm formation after incubation at 30°C for 20 and 40 h. The optical density (OD) was evaluated using a Sunrise RC absorbance reader (Tecan, Mannedorf, Switzerland). In relation to the OD values, the strains of L. monocytogenes have been classified into 4 categories (Meloni et al., 2012a, 2012b).

A qualitative in vitro evaluation of the resistance to disinfectants (chlore and quaternary ammonium compounds), commonly used in the plants included in this survey, was performed, according to Fox et al. (2004) method, modified. For each strain, 5 mL of the overnight L. monocytogenes culture (37°C) on TSB was obtained [approximately 10^8 colony forming units (CFU)/mL]. The day of the experiment, starting from the disinfectant concentration used in each plant (stock concentration), two serial dilutions (10^-1, 10^-2) were prepared. A sterile microtiter ninety-six well plate, containing 150 µL of the disinfectant was inoculated with 10 µL of a 10^-1, 10^-2, 10^-3 and 10^-4 fold diluted overnight culture. A positive control (without inoculum) was used. Each plate was then placed in a Sunrise RC absorbance reader (Tecan, Sunris RC absorbance reader (Tecan, 40 h. The optical density (OD) was evaluated by measuring the OD at 620 nm, after four incubation time, for 0 up to 3 days (72 h).

Table 1. Sequence, concentration and size of the primers used to detect virulence genes.

| PCR | Primers | Sequence (5’-3’) | Primer concentration (µM) | Product size (bp) |
|-----|----------|------------------|---------------------------|------------------|
| 1   | *rr*     | F CAGCACGCCGCGTGATATC | 0.9 | 938 |
|     |          | R CTCCATAGGTTGACCTT |      |     |
|     | *hlyA*   | F CCTAAAGCCGCAATCGA | 702 |     |
|     |          | R AAGCCTCGAACCTGCTC |      |     |
|     | *actA*   | F GAGCAAAATCCCGGATGAA | 268/385 |     |
|     |          | R CDGCAGGAGGCCTGCTCC |      |     |
|     | *prfA*   | F CTGTTGAGGCTCTTCTTGTTAACATCG | 1060 |     |
|     |          | R AGCAACCTCGGACAATCTAACACT |      |     |
| 2   | *inlA*   | F CTTAGCAGCTCTTAACCCGAC | 0.9 | 255 |
|     |          | R TGCTATATTGTTATGCCCC |      |     |
|     | *inlB*   | F AAAGCAGAATTACATCGGA | 146 |     |
|     |          | R ACATAGCTTGGTGTTGCG |      |     |
|     | *iap*    | F CAAAGCTCAGCTTGGGACG | 131 |     |
|     |          | R TGACAGGCTTGTTAGACGA |      |     |
| 3   | *plcA*   | F CGGCGAACACAGCACGATAT | 0.9 | 129 |
|     |          | R CCGGGCTACACCTTTAAATGT |      |     |
|     | *plcB*   | F GGGAAATTCTGACACGTTT | 261 |     |
|     |          | R ATTTTCGTTAGCTGCCTT |      |     |
|     | *mpl*    | F TTGGTTCTGAATTTGAGATG | 502 |     |
|     |          | R TTTAAAAGGACCGGTGAAT |      |     |

PCR, polymerase chain reaction.

Table 2. Virulence genes prevalence (%) of the L. monocytogenes strains (n. 35) in relation to the plants.

| Plant | Strains (n.) | actA | hlyA | rrn | prfA | iap | inlB | inlA | plcA | plcB | mLP |
|-------|--------------|------|------|-----|------|-----|------|------|------|------|-----|
| A     | 9            | 100  | 100  | 100 | 100  | 100 | 89   | 100  | 100  | 100  | 89  |
| B     | 10           | 100  | 90   | 100 | 100  | 100 | 90   | 100  | 100  | 100  | 70  |
| C     | 10           | 80   | 70   | 100 | 100  | 90  | 30   | 90   | 100  | 100  | 70  |
| D     | 6            | 100  | 100  | 100 | 100  | 67  | 100  | 100  | 100  | 100  | 100 |

Results

L. monocytogenes prevalence resulted as follows: 31.5% in food samples, 17.4% in surface in contact, and 11.4% in surface not in contact with meat. In particular, during the process, prevalence increased until the end of the acidification stage (60%), then decreased in the ripening period (20% in finished products), but the contamination levels were always below 100 CFU/g, as recommended in EC Regulation 2073/2005 (European Commission, 2005) for the food safety criteria in the RTE foods.

Serotyping and molecular pathogenic profile

The 1/2c was the prevalent serotype (43%) in all the plants, except A, followed by 1/2a (40%), that was detected in all the samples (surfaces and food), 1/2b (8.6%) and 4b (8.6%). The molecular analysis of the virulence factors (Table 2) showed that the amplification products of the analysed virulence genes were found in all the Listeria monocytogenes strains, with the following prevalence: 77.1% hlyA; 100% rrn; 100% prfA; 97.1% iap; 65.7% inlB; 88.6% inlA; 100% plcA; 100% plcB and 74.3% mpl (Table 2). Primers used for the detection of actA allowed the amplification of two different products (268 and 385 bp), showing a polymorphism for this gene. The 268 bp amplification band was detected in only 8.6% of isolates, while the 68.6% showed a 385 bp fragment.

In vitro biofilm forming ability and resistance to disinfectants

The 37.1% of the strains resulted able to in vitro biofilm formation after 40 h of incubation. All these strains belonged to the weak producer (WP) category, while none showed moderate (MP) or strong (SP) ability. The 50% of the strains able to produce biofilm belonged to serotype 1/2c, the 41.6% to 1/2a and the 8.3% to 4b. The results of the in vitro evaluation of the resistance to disinfectants is expressed in Table 3 as a reduction (+) and not reduction (-) of L. monocytogenes growth, on the basis of the OD value at 620 nm. All the strains showed
a reduction of \textit{L. monocytogenes} growth after each incubation time (24, 48 and 72 h). The \textit{L. monocytogenes} isolates were from environmental (n. 16) and products (n. 19) samples. Most of the strains belonged to 1/2c (n. 15) and 1/2a (n. 14) serogroups, while only 6 to 1/2b (n. 3) and 4b (n. 3).

In Table 4 the results of serotyping, biofilm forming ability and resistance to disinfectants of \textit{L. monocytogenes} strains are summarised in relation to the plant, batches and source. In plant A, \textit{L. monocytogenes} was detected both in food (except for the sausage at the end of ripening) and in environmental samples; all the isolates belonged to 1/2b and 1/2c serovars, and resulted NP biofilm and sensitive to disinfectants. In plant B, the strains were also isolated in food and in environmental sites, from both production batches. The 4b serovar profile (n. 1 strain) was isolated only in this plant from GM samples. Five strains isolated from food and environmental samples belonged to 1/2a resulted NP, while two strains isolated from the SWC were WP. In plant C, two strains from GM samples (batch I), belonging to 1/2a and 1/2c serovars, were WP; WP strains belonging to 1/2a serotype were also detected in AS, and both serotypes in surface in contact samples. The plant C had the higher production capacity. In plant D, four strains (batch I) belonged to 1/2c serovars, and isolated from food samples, were WP, in particular three were from GM and one from AS.

### Table 3. \textit{In vitro} qualitative reduction evaluation of \textit{L. monocytogenes} by disinfectant products.

| Disinfectant          | Plant | Concentration (%) | Temperature (°C) | Incubation time (h) |
|-----------------------|-------|-------------------|------------------|---------------------|
| Chlores substances    | GM    | 1-5%              | 24               | (+)                 |
|                       | GM    | 48                | (+)              |                     |
|                       | GM    | 72                | (+)              |                     |
| Quaternary ammonium compounds | GM | 2%               | 37               | (+)                 |
|                       | GM    | 24               | (+)              |                     |
|                       | GM    | 48               | (+)              |                     |
|                       | GM    | 72               | (+)              |                     |

### Table 4. Serotype, biofilm forming ability and resistance to disinfectants of \textit{L. monocytogenes} strains, in relation to the plants, batches and sources.

| Plant | Source | Batch | Serotype | BFA | Disinfectant resistance |
|-------|--------|-------|----------|-----|-------------------------|
| A     | GM     |       | 1/2b (1) | NP  | (-)                     |
|       | AS     |       | 1/2b (2) | NP  | (-)                     |
|       | RS     |       | 1/2c (1) | NP  | (-)                     |
|       | SC     |       | 1/2c (3) | NP  | (-)                     |
|       | SWC    |       | 1/2c (1) | NP  | (-)                     |
| Total | n. 9   |       |          |     |                         |
| B     | GM     |       | 1/2a (1) batch I | WP (1) | (-) |
|       | AS     |       | 4b (1)   | WP  | (-)                     |
|       | RS     |       | 1/2c (1) batch | WP  | (-)                     |
|       | SC     |       | 1/2a (1) batch II | WP  | (-)                     |
|       | SWC    |       | 1/2a (2) | WP  | (-)                     |
| Total | n. 10  |       |          |     |                         |
| C     | GM     |       | 1/2c (1) batch I | WP (1) | (-) |
|       | AS     |       | 1/2c (1) | WP  | (-)                     |
|       | RS     |       | 1/2c (1) batch I | WP  | (-)                     |
|       | SC     |       | 1/2c (1) batch II | WP  | (-)                     |
|       | SWC    |       | 1/2c (1) | WP  | (-)                     |
| Total | n. 10  |       |          |     |                         |
| D     | GM     |       | 1/2c (1) batch I | WP (1) | (-) |
|       | AS     |       | 1/2c (1) | WP  | (-)                     |
|       | RS     |       | 1/2c (1) batch I | WP  | (-)                     |
|       | SC     |       | 1/2c (1) batch II | WP  | (-)                     |
|       | SWC    |       | 1/2c (1) | WP  | (-)                     |
| Total | n. 6   |       |          |     |                         |

BFA, biofilm forming ability; GM, ground meat; AS, sausage at the end of acidification; RS, sausage at the end of ripening; SC, surface in contact; SWC, surface without contact.

### Discussion and Conclusions

The results underline the overall presence of \textit{L. monocytogenes} in different samples of Sardinian fermented sausages and environmental niches. In addition to the primary contamination by raw meat, cross contamination by surfaces in contact with meat should be considered as source of \textit{L. monocytogenes}. An inhomogeneous decrease of the pathogen prevalence was observed in ripened sausages (29.1%), highlighting, in agreement with other authors (Thévenet et al., 2005), the presence of strains able to survive during sausages fermentation. Even if the contamination level was always low, the presence of \textit{L. monocytogenes} in the ripened products confirms that the hurdles of microbial growth (mainly the a, decrease) should properly be applied during the ripening in order to prevent the \textit{L. monocytogenes} growth.

The evaluation of biofilm production showed a low short-time persistence (3-4 months) capacity of the \textit{L. monocytogenes} strains included in the study. Most of the isolates were unable, and only 37.1% showed a weak \textit{in vitro} ability to biofilm formation, in particular strains from food (61.5%) and, less frequently, from surfaces with (trolleys) and without (floor drains) contact with meat. Harborage niches, as the floor drains, can be critical sites for the processing plant environment and food product contamination (Tomkin, 2002). Decontaminating floor drains is essential to prevent the \textit{L. monocytogenes} biofilm formation, and a potential resistance against available disinfectants and treatments (Gram et al., 2007). About this topics, disinfectant resistance has been studied among groups of persistent and non persistent strains, but no clear link between persistence and increased disinfectant resistance was seen (Kastbjerg et al., 2009; Holah et al., 2002). Some authors showed that a reorganisation or regeneration mechanism of the lipidic membrane may occur (Fox et al., 2011), and others highlight that no strains show unique properties that lead to persistence, but harborage sites in food industry premises and equipment can persist (Carpentier et al., 2011). In our study all the strains resulted sensitive to all the tested disinfectants. Other authors had previously reported that the recommended concentrations of commercial sanitisers are higher
than required (Cruz et al., 2012). Although the results are preliminary and related to a small number of strains, the microtiter plate assay seems to be useful as an indirect way of assess the disinfectant resistance of *L. monocytogenes* strains.

The molecular findings revealed the presence of all the considered virulence genes, suggesting that all the isolates could be potentially dangerous for public health. No correlation between serotype and virulence profile was observed, neither between serotypes and plants. This is not in agreement with previous studies, where some *L. monocytogenes* serotypes appeared to be unique for a processing plant and should be considered as plant-specific (Fugett et al., 2007).

The fermented pork meat RTE products, as dry and semi-dry sausages, have been rarely implicated in food-borne disease. However, more risks should be linked to the consumption of these products, mainly in the manufacturing of traditional products, where an empirical application of the hurdles technology and lack of measures of hygienic control often occurs.

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