The biofilm formation ability of *Listeria monocytogenes* isolated from meat, poultry, fish and processing plant environments is related to serotype and pathogenic profile of the strains

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Abstract

In the present study, the relationships between serotype, pathogenic profile and *in vitro* biofilm formation of 106 *Listeria monocytogenes* strains, having no epidemiological correlation and isolated from different environmental and food sources, were analyzed. The quantitative assessment of the *in vitro* biofilm formation was carried out by using a microtiter plate assay with spectrophotometric reading (OD620). The isolates were also submitted to serogrouping using the target genes *lmo0737, lmo1118, ORF2819, ORF2110, prs*, and to the evaluation of the presence of the following virulence genes: *prfA, hlyA, rre, inlA, inlB, iap, plcA, plcB, actA and mpl* by multiplex PCRs. The 62% of the strains showed weak or moderate *in vitro* ability in biofilm formation, in particular serotypes 1/2b and 4b, frequently associated with sporadic or epidemic listeriosis cases. The 25% of these isolates showed polymorphism for the *actA* gene, producing a fragment of 268-bp instead of the expected 385-bp. The deletion of nucleotides in this gene seems to be related to enhanced virulence properties among these strains. Strains belonging to serotypes associated with human infections and characterized by pathogenic potential are capable to persist within the processing plants forming biofilm.

Introduction

*Listeria monocytogenes* is widespread in the environment including soil, water, sewage, vegetation, wild animal faeces, as well as on the farm and in food processing facilities.1,2 *L. monocytogenes* has been isolated from several processing environments (fish, meat, dairy products) and is responsible for numerous outbreaks associated with the consumption of ready to eat products.3 The pathogen is able to survive at a broad range of temperature (from 0 to 45°C) and pH (from 4.5 to 9.0), high salt concentrations (10%) and low aw values (0.92).4 *L. monocytogenes*, once introduced in the processing plants, is able to survive for long times under adverse environmental conditions and persists over time in niches as drains, walls, ceilings, storage tanks, hand trucks and conveyor belts, where food residues are accumulated.5-7 This can be explained with the ability of *L. monocytogenes* to form assemblages of surface-associated microbial cells, enclosed in hydrated extracellular polymeric substances and grow in biofilms on surfaces in contact or not with the food.5 The biofilm structure protects the microorganism from physical (scrubbing) and chemical (sanitizers and detergents) factors.8 It has been shown that different strains of *L. monocytogenes* can differ in their abilities to form biofilms.9 In the literature conflicting opinions can be found: several authors found a correlation between serotype, pathogenic profile and ability to form biofilm;10,11 on the contrary, other authors reported not such correlation.12,13 The presence of the pathogen on surfaces in contact without any contact with food increases the food safety risk.14,15 Thus, *L. monocytogenes* may become an important source of secondary contamination of food products and the effective control of its presence in the processing environments is a challenge for food processors.16 It is essential to characterize *L. monocytogenes* strains in order to carry out epidemiological studies and to trace the sources of contamination in the food chain.17 Serotyping has been widely used and although its discrimination power is poor, it remains the traditional and routinely used typing method in case of outbreaks.18 Among the 13 *L. monocytogenes* serotypes, only 1/2a, 1/2b, 1/2c and 4b have been associated with epidemic and sporadic cases of listeriosis in humans.19 In particular, serotypes 1/2a, 1/2b and 4b are responsible for 95% of human infections from which the majority of outbreaks are caused by strains of serotype 4b.20 In recent years, the proportion of human cases associated with strains of serotype 1/2a has increased.21,22 The molecular pathogenesis of *L. monocytogenes* is determined by multiple key virulence factors, such as internalins, haemolysins, phospholipases, actin polymerization protein and other minor virulence factors such as extracellular proteins (iap), anti oxidant factors, metal ion uptake systems and stress response mediators. The expression of these virulence factors is directly modulated by the regulator gene *prfA.*23 Recent studies have shown that the *prfA* gene has a significant positive impact on extracellular biofilm formation.24 Mutants lacking *prfA* were defective in surface-adherent biofilm formation. The objective of the present study was to evaluate the relationships between serotype, pathogenic profile and *in vitro* biofilm formation capacity of *L. monocytogenes* strains isolated from meat, poultry, fish and the environments of the respective processing plants.

Materials and Methods

Selection of the bacterial strains

In this study, 106 *L. monocytogenes* strains recovered from meat, poultry, fish samples and the respective processing plants with no apparent epidemiological relations were examined. The strains were collected in a period from 2005 to 2010. 40% of the isolates were collected from swine (n.14) and poultry (n.13) carcasses, pork ground meat (n.7) and raw salmon (n.6). These isolates were grouped as *raw material* (RM). 3% of the strains (n.3) was isolated from semi-finished salmon (SFP), 15% from fermented sausages (n.11) and smoked salmon (n.4),
grouped as final products (FP). The remaining 42% came from the environments of swine slaughterhouse (n.4), fermented sausage (n.25) and smoked salmon (n.17) processing plants. In order to standardize the elaboration of these data, the environmental strains were grouped in two categories, according to the possibility to come in contact with food: surfaces without contact with food (SWCF) and surfaces with contact with food (SCF).

Characterization of the strains

Multiplex polymerase chain reaction-based serotyping

The isolates were submitted to a multiplex polymerase chain reaction (PCR) method to identify \textit{L. monocytogenes} serotypes.\textsuperscript{25} The target genes and the sequence of each primer (Roche diagnostics, Milan, Italy) are described in Supplementary Table 1. All amplification reactions were performed in a final volume of 100 \(\mu\)L, containing 2U of Taq polymerase (Roche diagnostics), 0.2 mmol of deoxyribonucleoside triphosphate (dNTP), and 50 mmol Tris-HCl, pH 8.3. All amplification reactions were performed in a Gene Amp 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed as follows: initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 40 min, 53°C for 1.15 min and extension at 72°C for 1.15 min, followed by a final extension period at 72°C for 2 min. The multiplex PCR products were resolved by electrophoresis on 1.5% agarose gel in 1X TAE and stained with ethidium bromide (0.1 mg/mL). The gels were observed and captured using the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

Multiplex polymerase chain reaction analysis of virulence factors

Three multiplex PCRs were standardized in order to detect the following 10 virulence associated genes: multiplex PCR 1: \textit{rml}, \textit{hlyA}, \textit{actA} and \textit{prfA}; multiplex PCR 2: \textit{inlA}, \textit{inlB} and \textit{iap}; multiplex PCR 3: \textit{pcaA}, \textit{pcaB} and \textit{mpl} by modifying the protocols of Border \textit{et al.}\textsuperscript{26} and Jaradat \textit{et al.}\textsuperscript{27} All amplification reactions were performed in a final volume of 50 \(\mu\)L, containing 2 \(\mu\)L of DNA, 5U of Taq polymerase (Roche diagnostics), 0.2 mmol/L of each deoxyribonucleoside triphosphate (dNTP), IX PCR buffer (1.5 mmol/L-MgCl\(_2\), 50 mmol/L-KCl, 10 mmol/L-Tris-HCl, pH 8.3). Supplementary Table 2 lists the concentration of each primer (Roche diagnostics) used in the three multiplex PCRs. All amplification reactions were performed in a Gene Amp 2700 Thermal Cycler (Applied Biosystems) programmed as follows: for multiplex PCR 1, denaturation at 94°C for 1.20 min, annealing at 55°C for 1.30 min and extension at 72°C for 2 min, followed by a final extension period at 72°C for 10 min. For multiplex PCR 2 and 3, cycles were as follows: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The amplified fragments were separated by 1.3% agarose gel electrophoresis (Roche diagnostics) in 1X. TAE buffer and stained with ethidium bromide (10 mg/mL). The gels were observed and digitalized by the Gel-Doc UV trans-illuminator (Bio-Rad).

\textbf{In vitro biofilm formation}

The quantitative assessment of the \textit{in vitro} biofilm formation was carried out on 96-well polystyrene microwell plates using the method described by Stepolovic \textit{et al.}\textsuperscript{28} with some modifications. Isolates were grown for 24 h in 2 mL of BHI broth. All the wells of a microwell plate were filled up with 230 \(\mu\)L of BHI broth. Afterwards, 21 wells per strain were filled up with 20 \(\mu\)L of culture. Each plate included 12 wells of BHI broth without inoculum, as negative control. Microtiter plates were incubated at 37°C for 20 and 40 h. At the end of the incubation the content of the wells was removed and the plates washed three times with 300 mL of sterile distilled water in order to remove loosely attached bacteria. The remaining attached bacteria were fixed with 250 \(\mu\)L of methanol per well, and after 15 min the wells were emptied and air dried. Each well was stained with 250 \(\mu\)L of Crystal violet for 5 min. After staining, the plates were washed under running tap water, then air dried and the dye bound to the adherent cells was resolubilized with 250 \(\mu\)L of 33% (v/v) glacial acetic acid per well. The plates were read spectrophotometrically (OD\(_{620}\)) using a Sunrise RC absorbance reader (Tecan, Maennedorf, Switzerland). The strains were divided up into four categories: no biofilm producers (NP= O.D. <0.5), weak producers (WP= O.D. \(\geq\)0.5 <1.0), moderate producers (MP= O.D. \(\geq\)1.0 <1.5) and strong producers (SP= O.D. \(\geq\)1.5).

\textbf{Statistical analysis}

The relationships between biofilm formation, serotype and pathogenic profile were evaluated by one-way analysis of variance (ANOVA) using the GLM procedures. The mean differences between serotypes and pathogenic profiles of the \textit{L. monocytogenes} strains in the \textit{in vitro} biofilm formation ability after incubation at 37°C for 20 and 40 h were evaluated using the LSD test. Significance was defined as P<0.05. Statistical analysis was conducted using Statgraphics Plus 5.1, software (StatPoint, Warrenton, USA).

\textbf{Results}

\textbf{Multiplex polymerase chain reaction-based Serotyping}

All the strains included in the study belonged to the \textit{L. monocytogenes} serotypes associated with epidemic and sporadic cases of listeriosis in humans (1/2a, 1/2b, 1/2c and 4b). Using multiplex PCR primers developed by Doumith \textit{et al.}\textsuperscript{25} 34% of the \textit{L. monocytogenes} isolates were recognized as 1/2a, 33% as 1/2b, 24% as 1/2c, 9% as 4b (Table 1).

\textbf{Multiplex polymerase chain reaction analysis of virulence factors}

Multiplex PCR-products of the 10 virulence-associated genes were obtained from all 106 \textit{L. monocytogenes} strains included in this study. Genotyping yielded 10 different pathogenic profiles (Table 2): the prevalent was n.3 (49%), 9 virulence associated genes, lack of \textit{inlB}) followed by n.1 (24%, 10 virulence associated genes, complete pathogenic profile) and n.2 (16%, 9 virulence associated genes, lack of \textit{mpl}). In general, PCR products of the virulence associated genes did not show polymorphism except for the \textit{actA} gene.\textsuperscript{28} Eighty-one strains (76%) showed the expected 385-bp ampiclon, whereas twenty-five strains (24%) showed the 268-bp ampiclon.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Source of isolation & N\(^{\circ}\) of strains & 1/2a & 1/2b & 1/2c & 4b \\
\hline
SWCF & 16 & 12.6 & 75 & 6.2 & 6.2 \\
SCF & 29 & 31 & 38 & 17.2 & 13.8 \\
RM & 37 & 43.3 & 8.1 & 40.5 & 8.1 \\
SFP & 9 & 33.3 & 44.4 & - & 22.3 \\
FP & 15 & 40 & 33.3 & 26.7 & - \\
\hline
Total & 106 & 34 & 33 & 24 & 9 \\
\hline
\end{tabular}
\caption{Prevalence of serotypes in the 106 \textit{L. monocytogenes} strains in relation to the source of isolation.}
\end{table}

\textit{SWCF;} surfaces without contact with food; \textit{SCF;} surfaces with contact with food; \textit{RM;} raw materials; \textit{SFP;} semifinished products; \textit{FP;} finished products.
Quantitative assessment of in vitro biofilm formation

Sixty-two percent (62%) of the strains showed weak or moderate in vitro ability to form biofilm (Table 3). After 20 h of incubation (Figure 1), 75% of the strains was NP, 24% WP and 1% MP. At the end of 40 h of incubation (Figure 2), 49% of the strains was NP, while the prevalence of WP and MP increased up to 49 and 2% respectively. In agreement with Djordevic et al., ANOVA showed a statistically significant relationship between serotypes 1/2b-4b and in vitro biofilm production after 40 h (P < 0.05), also confirmed by the LSD test (Figure 3). Moreover, a statistically significant relationship was also found between pathogenic profile n.4 (9 virulence associated genes, lack of hlyA) and in vitro biofilm production after 20 and 40 h of incubation (P < 0.01). On the whole, the LSD test showed statistically significant differences (P < 0.05) between the mean values of the pathogenic profile n.4 associated with 1/2b and 4b serotypes and the other pathogenic profiles (Figure 4). The microtiter plate assay confirmed its utility as an indirect method of assessing the ability of L. monocytogenes strains to attach to abiotic surfaces, enabling researchers to rapidly analyze the adhesion of multiple bacterial strains within each experiment.

**Discussion and Conclusions**

As listeriosis is essentially caused by a food source contaminated along the food chain, it is important to investigate the molecular characteristics and persistence ability of L. monocytogenes strains recovered from different food sources or environments in order to design and implement more effective prevention strategies. In this study, we have characterized L. monocytogenes strains isolated from raw materials, finished products and environmental samples by serotyping and definition of the pathogenic profile (10 different virulence-associated genes). It is notable that 67% of the L. monocytogenes food and environmental isolates from Italy belonged to serotypes 1/2a (34%) and 1/2b (33%). A similar prevalence was reported by other studies carried out in France, China, Italy and Switzerland. Genotyping yielded 10 different pathogenic profiles, and surprisingly only 24% of the strains tested in this study were positive for all the considered virulence genes.

Table 2. Correlations between source of isolation, pathogenic profile and serotype.

| Source of isolation | N° of strains | Pathogenic profile | N° of strains and serotypes | Virulence associated genes |
|---------------------|--------------|--------------------|-----------------------------|---------------------------|
| SWCF                | 16           | 1                  | 5 (1/2b)                    | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 2                  | 8 (1/2b)                    | prfA, hlyA, iap, plcA, plcB, actA |
|                     |              | 5                  | 1 (4b)                      | prfA, hlyA, iap, plcA, plcB, actA |
|                     |              | 9                  | 1 (1/2a)                    | prfA, iap, plcA, plcB, actA |
|                     |              | 10                 | 1 (1/2a)                    | prfA, iap, plcA, plcB, actA |
| SCF                 | 29           | 1                  | 4 (2/1a, 2, 2a, 2/2c)       | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 2                  | 5 (2, 1/2b, 3, 4b)          | prfA, hlyA, iap, plcA, plcB, actA |
|                     |              | 3                  | 18 (8/1, 2a, 7, 2b, 3, 1a, 2c) | prfA, hlyA, iap, plcA, plcB, actA |
|                     |              | 5                  | 1 (4b)                      | prfA, hlyA, iap, plcA, plcB, actA |
|                     |              | 9                  | 1 (1/2a)                    | prfA, iap, plcA, plcB, actA, mpl |
| RM                  | 37           | 1                  | 11 (6/1a, 1, 1/2a, 2, 1b)   | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 2                  | 3 (4b)                      | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 3                  | 19 (10/1a, 1, 1/2b, 8, 1/2c) | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 7                  | 1 (1/2b)                    | prfA, iap, plcA, plcB, actA, mpl |
| SFP                 | 9            | 1                  | 2 (4b)                      | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 3                  | 3 (3, 1/2a, 1, 1/2b)        | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 4                  | 2 (4b)                      | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 6                  | 1 (1/2b)                    | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 7                  | 1 (1/2a)                    | prfA, iap, plcA, plcB, actA, mpl |
|                     |              | 8                  | 1 (1/2b)                    | prfA, iap, plcA, plcB, actA, mpl |
| FP                  | 15           | 1                  | 1 (1/2b)                    | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 2                  | 1 (1/2a)                    | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 3                  | 1 (1/2b)                    | prfA, hlyA, iap, plcA, plcB, actA, mpl |
|                     |              | 7                  | 1 (1/2a)                    | prfA, iap, plcA, plcB, actA, mpl |
|                     |              | 8                  | 1 (1/2b)                    | prfA, iap, plcA, plcB, actA, mpl |

SWCF, surfaces without contact with food; SCF, surfaces with contact with food; RM, raw materials; SFP, semifinished products; FP, finished products.

Table 3. Formation of biofilm in the 106 L. monocytogenes strains in relation to the source of isolation.

| Source of isolation | N° of strains | Biofilm formation (%) |
|---------------------|--------------|-----------------------|
|                     |              | 20 h                  | 40 h                  |
|                     |              | NP WP MP SP NP WP MP SP |
| SWCF                | 16           | 68.8 25 6.2 - 50 37.5 12.5 - |
| SCF                 | 29           | 82.8 17.2 - - - 34.4 65.6 - - |
| RM                  | 37           | 78.4 21.6 - - - 62.2 37.8 - - |
| SFP                 | 9            | 66.7 33.3 - - - 22.3 77.7 - - |
| FP                  | 15           | 66.7 33.3 - - - 60 40 - - |
| Total               | 106          | 75 24 1 - - 49 49 2 - - |

SWCF, surfaces without contact with food; SCF, surfaces with contact with food; RM, raw materials; SFP, semifinished products; FP, finished products; NP, no biofilm producers; WP, weak producers; MP, moderate producers; SP, strong producers.
In general, PCR products of the virulence-associated genes did not show polymorphism except for the \textit{actA} gene\textsuperscript{27}. The \textit{actA} gene has been found to be important for the spread of \textit{L. monocytogenes} to neighboring cells and maintenance of infection\textsuperscript{23}. Twenty-five strains (25\%) showed polymorphism producing a fragment of 268-bp instead of the expected 385-bp. The deletion of nucleotides in this gene seems to be related to enhanced virulence properties among these strains\textsuperscript{34}. On the contrary, other authors did not observe statistical correlations between the ownership of the 268-bp \textit{actA} and the ability to invade HeLa cells \textit{in vitro}\textsuperscript{32}. Several authors reported polymorphism for other virulence-associated genes, such as \textit{hlyA}, \textit{iap} and \textit{inlA}, \textit{inlB}\textsuperscript{35,36}. However, in this study, we did not identify any polymorphism in the PCR products of the other virulence associated genes. As reported by Franciosa \textit{et al.}\textsuperscript{37}, the low \textit{actA} PCR product was related to the serotype of the strains (1/2b). On the whole, 52\% of the isolates showed weak or moderate \textit{in vitro} ability to form biofilm, in particular strains isolated from SWCF as floor drains. Floor drains can be a critical site to the control of contamination of the processing plant environment: decontamination is especially challenging because, when entrapped in a biofilm, \textit{L. monocytogenes} is afforded unusual protection against available disinfectants and treatments\textsuperscript{5,38,39}. By means of statistical analysis, the relationships between biofilm formation, serotype and pathogenic profile were evaluated. ANOVA showed statistically significant differences in terms of \textit{in vitro} biofilm formation (Figures 3 and 4): strains belonging to the evolutionary lineage 1 (serotypes 1/2b and 4b) were characterized by a nearly complete pathogenic profile (9 virulence associated genes, lack of \textit{hlyA}) and by an \textit{actA} product of 268-bp. These strains showed better ability to form biofilm \textit{in vitro}. From a risk analysis perspective it is important to investigate the molecular characteristics and the ability of \textit{L. monocytogenes} to persist in the food processing environments\textsuperscript{32}. In this study, \textit{L. monocytogenes} strains isolated from critical sites in terms of control of processing environment contamination (floor drains) and belonging to serotypes associated with human infections, were characterized by pathogenic potential and were capable to form biofilms on abiotic surfaces. The polystyrene surfaces used for this \textit{in vitro} experiment approximately mimics some of the plastic materials used in the processing plants. Further testing with other plastic and steel specimens are needed in order to
better understand the mechanism of in vivo biofilm formation and persistence within the processing plants. These findings should help the Food Business Operators when designing and implementing more effective strategies to manage and control the presence of the pathogen in the food processing environments.

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