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Food processing environments as reservoir of *Listeria monocytogenes* hypo- and hypervirulent clones: use of Whole Genome Sequencing and *in vitro* assays to characterize persistence

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Abstract

Listeria monocytogenes (*Lm*) is a significant foodborne pathogen causing human listeriosis, foodborne zoonoses with the highest hospitalization and fatality rate. This mainly due to its abilities to form biofilm, survive and grow under stressful conditions and resist disinfectants, and thus *Lm* can persist in food processing environments (FPEs) even for years with a continuous risk of food cross-contamination.

In this study the role of FPEs as reservoirs of hypo- and hypervirulent clones of *Lm* was investigated.

Whole Genome Sequencing (WGS) and bioinformatics analysis were used to assess the genetic relationships between the strains and to investigate their persistence and virulence profiles. Biofilm formation, sensitivity to Benzalkonium Chloride (BC) as well as adhesion and invasion abilities were assessed *in vitro*.

WGS analysis and in particular cgMLST, identified *Lm* clones persisting for up to four years in the same food producing plant (FPP) as well as clones contaminating different FPEs of Central Italy. Multidrug efflux-pumps genetic determinants (*sugE, mdrl, lde, norM, mepA*) were carried by various *Lm* Clonal Complexes (CCs). All the CC121 strains also harboured the *Tn6188_qac* gene specific for tolerance to BC. Strains belonging to CC3, CC7, CC9, CC31 and CC191 carried the stress survival islet SSI-1 while CC121 clones harbored the SSI-2. CC9 and CC121 strains presented high-level cadmium resistance genes (*cadA1C1*) carried by different plasmids and showed a strong biofilm production.

Preliminary results on *Lm* sensitivity to BC *in vitro* showed that strains belonging to a CC9 long term persistent cluster, despite not carrying specific genetic determinants for tolerance to BC, were less sensitive to low sanitizer concentrations than the other strains. Moreover, if compared with what was reported in recent studies on *Lm*, our results indicated a lower susceptibility to BC for the CC121 strains harbouring the Tn6188_*qac*.

An investigation of the virulence genetic profiles showed that all the CC9 and CC121 strains presented a premature stop codon in the *inlA* gene which was complete in the other isolated CCs. The *Listeria* Pathogenicity Island 1 (LIPI-1) was widespread in all the *Lm* isolates. CC1, CC3 and CC191 clones also harboured the LIPI-3.

The *in vitro* assessment of *Lm* virulence showed that the CC1, CC7, CC9 and CC121 tested strains isolated from food presented good adhesive and invasive abilities, with the CC7 clone showing the highest invasiveness and belonging to the epidemic cluster causing a severe listeriosis outbreak. All these findings represented a relevant risk for the consumers' health.

Hypovirulent CCs such as CC9 and CC121, more adapted to FPEs and able to persist after cleaning and sanitation, were the most frequently isolated in the FPP of Central Italy, representing a significant risk of food contamination. On the other hand, in this study hypervirulent clones (CC1 and CC2) were also detected in FPEs with situations in which they warningly persisted for long time in the same plant.

A systematic monitoring of *Lm* in FPEs should be included in Italian food safety surveillance programs performed by the Competent Authorities to improve the management of the pathogen in the food industry minimizing risk of food contamination and recurrence of severe outbreak.

Content

List of abbreviations

ALOA – Agar Listeria acc. to Ottaviani & Agosti

BC – Benzalkonium Chloride

BHI – Brain Heart Infusion Broth

CC – Clonal Complex

cgMLST – core genome Multi Locus Sequence Typing

DMEM – Dulbecco's Modified Eagle Medium

EC – European Commission

EPS – Extracellular Polymeric Substances

FAO - Food and Agriculture Organization

FBO – Food Business Operator

FPE – Food Processing Environment

FPP – Food Producing Plant

HGF – Hepatocyte Growth Factor

InlA – Internalin A

inlB – Internalin B

ISS – Istituto Superiore di Sanità

LGI – Listeria Genomic Island

LIPI – Listeria Pathogenicity Island

Lm – Listeria monocytogenes

MATE – Multidrug and Toxic Compounds Extrusion pumps

MBC – Minimum Bactericidal Concentration

MEC – Minimal Effective Concentration

MFR – Major Facilitator Superfamily pumps

MIC – Minimum Inhibitory Concentration

MLST – Multi Locus Sequence Typing

MST – Minimum Spanning Tree

NGS – Next Generation Sequencing

PFGE – Pulsed Field Gel Electrophoresis

PMSC – Premature Stop Codon

PSB – Phosphate Buffered Saline

QACs – Quaternary Ammonium Compounds

RS – Retail Store

RTE – Ready to Eat foods

SMR – Small Multidrug Resistance Efflux pumps

SNPs – Single Nucleotide Polymorphisms

SSI – Stress Survival Islet

ST – Sequence Type

TSYEA – Tryptic Soy Yeast Extract Agar

TSYEB – Tryptic Soy Yeast Extract Broth

UFC/CFU – Colony Forming Unit

WGS – Whole Genome Sequencing

WHO – World Health Organization

Introduction

Listeria monocytogenes: a foodborne pathogen of major concern worldwide

The gram-positive bacterium *Listeria monocytogenes* (*Lm*) is a significant foodborne pathogen of increasing public health concern. *Lm* causes human listeriosis, the foodborne zoonoses with the highest hospitalization (92.1%) and fatality (17.6%) rates [1]. When the infection occurs in vulnerable categories among humans such as the elderly, immunocompromised people, newborns and pregnant women, it manifests itself with invasive forms of the disease leading to sepsis, meningitis, encephalitis, abortion, stillbirth and death [2].

Listeriosis cases occur both sporadically and as outbreaks. In the last years, some relevant outbreaks of the disease, which have affected hundreds of people, occurred in South Africa [3], Germany [4] and Spain [5], renewing interest in the disease, both medically and in the media [6].

In 2019, in the EU the number of outbreaks caused by *Lm* (n = 21) increased by about 50% compared with 2018 (n = 14) and the related illnesses jumped from a total number of 748 outbreak cases reported in the EU between 2010 and 2018 (83.4 annual cases on average) to 349 cases in 2019 [1].

The main transmission route of *Lm* to humans is the consumption of contaminated food. Among the main foods implicated, Ready-to-Eat (RTE), i.e. meat products, smoked fish, milk products and minimally processed vegetables, have the greatest contribution to the burden of disease [7].

Numerous organisations worldwide such as World Health Organisation (WHO) and Food and Agricultural Organisation (FAO), among others have been applying the policy of "Zero Tolerance" for *Lm* in RTE processed foods to reduce the high risk of food contamination [3].

Currently there is no international agreement on 'acceptable levels' of *Lm* in foods. Some countries like USA adopt zero tolerance limits to ensure the protection of consumers against *Lm* in RTE products, while others apply a mixed policy with two different criteria depending on the food category [8]. In particular, the EU Member States, including Italy, refer to the Commission Regulation (EC) No 2073/2005 which divides RTE foods intended for adult people into two different categories: products able to support the growth of *Lm* and products not able to support the growth (products with pH $\leq$ 4,4 or aw $\leq$ 0,92, products with pH $\leq$ 5,0 and aw $\leq$ 0,94 and products with a shelf-life of less than five days). For the former category the food safety criteria required by the Regulation is the absence in 25g/ml of food (zero tolerance policy) while for the latter one, a tolerance level of 100 CFU/g is established (Figure

1). Moreover, there is a third category covered by the European Regulation which includes RTE foods intended for infants and those for special medical purposes. For this category is obviously adopted a zero tolerance policy for *Lm*.

Chapter 1. Food safety criteria

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (4)	*Listeria monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g (5)		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
		5	0	Absence in 25 g (7)		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life

Figure 1: EC Reg. No 2073/2015 and smi: Food safety criteria for *L. monocytogenes* in RTE foods.

According to the last European Union One Health Zoonoses Report [1], in 2019 for all food categories covered by the Regulation (EC) No 2073/2005 and sampled by the Competent Authorities, the level of unsatisfactory results remained low at retail (with a maximum of 2.1% in products of meat origin, fermented sausages) while at processing, this level was systematically higher.

When testing against food safety criteria set out in the Regulation (EC) No 2073/2005 or in other food legislations provides unsatisfactory results, the product or batch of foodstuffs shall be seized and recalled.

Therefore, *Lm* presents a wide economic impact worldwide in terms of public health costs and food production losses [9].

Taxonomy and genetic diversity

The genus Listeria belongs to the phylum *Firmicutes* and currently includes 23 recognized species of small, rod-shaped Gram-positive bacteria [10–12]. Only two of these species, *Lm* and *L. ivanovii*, are considered pathogens with the first being the main pathogenic species of the genus [10,11].

To date, *Lm* is classified into four major evolutionary lineages (I, II, III, IV), 13 agglutination serotypes, and five molecular serogroups [13–15].

At least 95% of isolates from contaminated foods and clinical cases belong to serotypes 1/2a, 1/2b, 1/2c and 4b [15–17]. The serogrouping approach is time-saving as it is performed by

PCR and groups *Lm* serotypes into five serogroups: IIa (1/2a-3a), IIb (1/2b-3b-7), IIc (1/2c-3c), IVa (4a-4c) and IVb (4b-4d-4e) [13,15].

According to the conventional Multi Locus Sequence Typing (MLST) scheme, based on the sequence analysis of seven housekeeping genes (*acbZ, bglA, cat, dapE, dat, ldh and lhlA*), *Lm* isolates are classified into sequence types (STs), sharing seven alleles; strains sharing at least six alleles are grouped in the same clonal complex (CC) [18,19].

MLST analysis is to date a reference method for global epidemiology and population biology of bacteria, and its application to *Lm* effectively allows the rapid and inter-laboratory comparison of isolates [18,20]. The Bacterial Isolate Genome Sequence Database (BIGSdb; https://bigsdb.pasteur.fr/listeria/listeria.html) is a scalable, open source, web-accessible database system cured by the Institute Pasteur of Paris and developed for the storage and analysis of sequence data of bacterial isolates on the basis of MLST and other schemes [21]. Currently there are 4414 *Lm* profiles available in the BIGSdb, grouped into 2758 STs and more than 200 CCs (accessed on 26-06-2021). CCs commonly recognized as the most frequent are CC1, CC2, CC4, CC5, CC6, CC9 and CC121. Among them CC1, CC2, CC4 and CC6 are more frequently associated with clinical cases and have been reported as hypervirulent in a humanized mouse model of listeriosis. CC9 and CC121 instead, are mainly food-associated and are defined hypovirulent. The remaining CCs are defined intermediate clones [22–24].

Natural niches and reservoirs

Lm is ubiquitous and widespread in the natural environments. It can be detected in soil, water and plants and once ingested by herbivorous it is shed back into the environment by faeces [25]. Many wild and domestic mammals as well as birds, carry *Lm* in their faeces thus serving as important reservoirs of this microorganism either in symptomatic or asymptomatic cases [25].

Among farm animals, listeriosis primarily affects small ruminants and cattle. In these species, listeriosis can manifest itself in different clinical forms, ranging from a localized infection of the udder (mastitis), the eye (keratoconjunctivitis, uveitis) or gastroenteritis to the more severe invasive forms causing septicemia, rhombencephalitis, death and infection of the pregnant uterus leading to stillbirth or abortion [26,27].

Pigs are usually asymptomatic and can carry *Lm* in their intestinal content, tonsils and lymph nodes; in this animal species *Lm* could be detected from carcass swabs and tonsils [28].

Low-quality silage is considered the major source of contamination in farm animals but also the environmental source is possible. Indeed, the elimination of the pathogen through the faeces also makes the farm environment an important route for spreading contamination [26]. Moreover, contrary to other pathogens, typically associated with the enteric tract of animals, such as *Campylobacter*, *Salmonella,* and *E. coli*, once eliminated, it survives in soil for long time representing a source of contamination for crops and posing a problem for the microbiological safety of minimally treated and RTE vegetables [29].

Based on the above, *Lm* is a pathogen of great concern in the food industry as it can be found in almost all raw food materials both of animal origin or not (e.g., raw meat and fish, unpasteurized milk or uncooked, fresh vegetables) and it is able to survive and grow in food preservation conditions such as high salinity, acidity and refrigeration temperatures [30]. Once introduced in a food processing facility through raw materials, *Lm* can establish long-lasting colonization of niches persisting in the environment [31]. Therefore, not only unprocessed ingredients are sources of contamination in final food products but food can also be contaminated after processing through the FPE. Various contaminated food products, including vegetables, milk and dairy, red meat, poultry, seafood and diverse ready-to-eat (RTE) foods, such as salads and smoked fish, have been reported as sources of listeriosis infections [30].

Pathogenesis and virulence determinants

Systemic dissemination

Upon ingestion of contaminated food by the host, *Lm* traverses the intestinal epithelial barrier into the lamina propria and disseminates, via the lymph and the blood, towards the primary target organs such as liver, in which it replicates in hepatocytes, and spleen. In immunocompetent individuals, these initial stages are generally subclinical and self-limiting, unless a high bacterial dose is ingested, in which case febrile gastroenteritis may develop a few hours after ingestion of the contaminated food. If the primary infection is not adequately contained, as often occurs in immunocompromised people, the elderly and in pregnant women, bacteria are released into the bloodstream (bacteremia) through which they reach the secondary target organs mainly represented by the brain and the placenta [24,32]. The severity of the disease is thus mainly due to the characteristic ability of *Lm* to cross three host barriers: the intestinal barrier, the blood-brain barrier and the materno-fetal barrier [33].

Cell biology of infection

Lm is able to resist intracellular killing when phagocytosed by macrophages and to replicate within them. Moreover, it is well established that this pathogen also invades many types of cells which are normally non-phagocytic [25,32,33]. In all cell types, following entry, *Lm* is internalized into the vacuole from which, in most cases, it subsequently escapes by physically disrupting the vacuolar membrane through the activity of potent virulence factors. Once the bacterium is released into the cytosol, it can survive and replicate altering a plethora of host cell processes and also organelles [32]. *Lm* can also spread from one cell to another inducing, at one pole of the cell, the characteristic polarised actin-polymerisation that generates force to move the bacteria inside infected cells and between cells [32,34]. This phenomenon, also defined actin comet, allows *Lm* cells to move towards the plasma membrane where they induce the formation of pseudopods that invaginate into the neighbouring cell with the consequent release into a second infected cell [33,34]. In this way, by direct cell-to-cell spread, bacteria disseminate within host tissues, remaining protected from antibodies or complement.

Virulence factors involved in host infection

The pathogenicity of *Lm* is mediated by a wide array of virulence factors which allow it to infect, survive, and replicate in a variety of host cell types [35,36]. Thanks to the many studies conducted to investigate the adhesion, invasion, and/or virulence regulation of this pathogen, the role of different virulence factors (i.e., PrfA, ActA, InlA, InlB, InlC, LAP, Ami, p60, Auto) have been well characterized in different cell types or animal models together with the relative encoding genes [35,37]. Four Listeria pathogenicity islands (LIPI-1, LIPI-2, LIPI-3 and LIPI-4) have been identified so far [38–41]. LIPI-1, necessary for intracellular survival and spread, is present in all *Lm* strains and is composed by six genes including *prfA*, *actA*, *hly*, *mpl*, *iap*, *plcA* and *plcB*. The *prfA* gene encodes the protein regulatory factor (PrfA) which is required for the expression of the LIPI-1 genes as well as of the operon *inlAB*, described below [41,42]. Listeriolysin O (LLO) encoded by the *hly* gene is a pore-forming toxin that induces lysis of bacterium-containing phagocytic vacuole, resulting in the release of bacterial cells into the host cytoplasm. *plcA* and *plcB* encode the phosphatidylinositol-specific phospholipase C (PI-PLC) and zinc-dependent broad-spectrum phospholipase C (PC-PLC) respectively and are involved, together with LLO, in the escape of the pathogen from the vacuoles. *actA* is the genetic determinant of the surface protein actin A (ActA) involved,

through the actin polymerization process, in the intracellular motility and cell-to-cell spread of *Lm*. Finally, *mpl* encodes a zinc metalloproteinase which activates PC-PLC in order to initiate a new infection cycle [42,43].

LIPI-2 is a 22 kb gene cluster composed of ten internalin genes as well as the sphingomyelinase *smcL* gene, involved in phagosome disruption [41,44,45]. This genomic region was first described as specific for *L. ivanovii* but in 2019 Yin et al., reported an atypical CC33 *Lm* strain, recovered from an ovine listeriosis outbreak in China, containing in its genome a partial LIPI-2 locus, including only *smcL* and the internalins i-*inlF* and i-*inlE* genes [41].

LIPI-3 is composed by eight genes (*llsAGHXBYDP*) and encodes a biosynthetic cluster involved in the production of Listeriolysin S (LLS), a haemolytic and cytotoxic factor that is known to be required for *Lm* virulence *in vivo* [40,46].

LIPI-4 is a cluster of six genes encoding a cellobiose-family phosphotransfer system (PTS) and is involved in neural and placental infection [22,47].

Internalins are a family of 25 surface proteins promoting *Lm* invasion of hepatic and intestinal epithelial cells during the infection process. Among them, Internalin A (InlA) and B (InlB), encoded by the *inlAB* operon, are considered the most relevant. They bind the eukaryotic cell membrane receptors, E-cadherin and Met, and the receptor of the hepatocyte growth factor (HGF), inducing the bacterial uptake through receptor-mediated endocytosis [37,42,44].

Many studies have previously reported multiple distinct mutations leading to a premature stop codon (PMSC) in the *inlA* gene. *Lm* isolates that carry a PMSC in the *inlA* gene produce a truncated form of InlA that is secreted rather than anchored to the bacterial cell wall [35,48–50]. These *Lm* show a reduced invasion efficiencies demonstrating attenuated ability to invade of Caco-2 human intestinal epithelial cells and low virulence levels in mammalian hosts [35,48,51]. Therefore, PMSC mutations in *inlA* could represent a molecular marker for *Lm* virulence attenuation [35,48].

In addition to InlA and InlB, InlC affects the rigidity of the cytoskeleton and innate immune signalling, InlP mediates placental invasion and InlJ is expressed solely in vivo, though its cellular receptor and tissue tropism remain to be identified [9].

Other proteins such as P60, fibronectin binding protein (FbpA), Auto, and Vip are suggested to have a role in mediating *Lm* entry into the host cell [52]. Previous studies have demonstrated the role of FbpA in liver and intestinal colonization [52–54]. In addition, the Listeria adhesion protein (LAP) and the autolysin Ami, promote adhesion of *Lm* to intestinal

cells and exploits epithelial defences allowing *Lm* to cross the intestinal epithelial barriers [52].

L. monocytogenes hypo- and hypervirulent clones

Lm is a genetically heterogeneous species in which isolates can be grouped into lineages, PCR serogroups, 7-genes MLST CCs and core genome MLST (cgMLST) sublineages. The heterogeneity of this species also concerns the pathogenic potential with the presence of hypovirulent and hypervirulent clones [22,23]. In their study, Maury et al. (2016) [22] identified clones epidemiologically associated either with food or with human central nervous system or maternal-neonatal listeriosis, also assessing the respective virulence in a humanized mouse model of listeriosis. Their results indicated that clones CC1, CC2, CC4 and CC6, all belonging to serotype 4b, were strongly associated with a clinical origin and were hypervirulent, whereas clones CC9 and CC121 were strongly associated with a food origin and were hypervirulent. Clones CC3, CC5, CC8+CC16, CC18, CC37 and CC155 were defined as intermediate between the two categories of highly prevalent clones [22] .

More in detail, they observed that among the hypervirulent clones, isolates belonging to CC1, CC4 and CC6 induced significantly more body weight loss and more efficiently infected the liver (CC1 and CC6) and brain (CC1, CC4 and CC6), demonstrating their neurotropism. In contrast, isolates belonging to CC9 and CC121 did not induce body weight loss following infection, were less invasive and were associated with bacteraemia without the involvement of central nervous system or foetal/neonatal.

Pan-genome studies have identified a number of accessory virulence-associated genes as specific to the hypervirulent clones and strongly associated with infectious potential at the population level. Such determinants include full-length *InlA*, LIPI-3, and gene clusters responsible for teichoic acid biosynthesis in serotype 4b strains [22,24,55]. Moreover, the LIPI-4, specific of CC4 clones, has been defined as the first *Lm* virulence factor specifically implicated in central nervous system or foetal/neonatal infections [22].

On the other hand, the main marker associated with the attenuated virulence of the hypovirulent clones, infecting mostly highly immunocompromised individuals, is the presence of PMSC leading to truncations in InlA [22,23]. These *inlA* mutations are observed in a significant proportion of *Lm* isolated from food and correlate experimentally with impaired entry into non-phagocytic cells (e.g., epithelial cells), offering a plausible explanation for the hypovirulent phenotype [22,23,51].

Laboratory assessment of L. monocytogenes virulence

Over the past two decades, several laboratory procedures have been developed and applied for evaluation of *Lm* pathogenic potential. Some of the initial methods consist of *in vivo* bioassays and *in vitro* cell assays. Despite their obvious limitations, these techniques have allowed the laboratory determination of *Lm* virulence making it possible to predict the risk of a strain to cause listeriosis and to better understand virulence mechanisms [56].

Methods for determining strains virulence include *in vivo* (animal models), *in vitro* (cell culture assays), and molecular methods (detection of virulence genes).

In vivo studies

Despite *Lm* naturally infects many animal species, the choice of the laboratory animal model to use for *in vivo* studies, is not simple and must take into consideration several aspects. An appropriate listerial animal model, as first requirement, should have comparable cell and tissue tropisms as humans as well as a similar physiology, immune response and pathophysiology of infection [57]. Another criteria to be used in the selection of practical animal models are size and cost of the animal, husbandry requirements, ability to reproduce in captivity and length of pregnancy [57]. According with all these findings, mice have been widely used as animal model to study virulence in *Lm* followed by the guinea pig [22,57–59].

Lm virulence is usually assessed by determining bacterial concentrations in liver and spleen at specific time points after infection or evaluating the 50 % lethal dose (LD 50). Routes of infection include oral, nasal, intraperitoneal, intravenous or subcutaneous routes. Among them the oral route is the most indicated as it closely mimics natural infection. Murine E-cadherin, in contrast to guinea pig E-cadherin, does not interact with inlA, which is important for listerial invasion of the intestine.

Therefore, a transgenic mouse line has been developed that expresses human E-cadherin to be used as listerial animal model [57,60].

In addition to mice, Zebrafish (*Danio rerio*) has become a popular model to study virulence of several pathogens including *Escherichia coli*, *Staphylococcus aureus* and *Aeromonas hydrophila* [61]. In particular Zebrafish larvae have proven to be effective in evaluation of listerial virulence genes, showing similar patterns of infection as mice [57,61].

Moreover, in recent years, also the use of larvae of the greater wax moth *Galleria mellonella* has emerged as a promising model for the assessment of *Lm* virulence [62]. The main advantages of this model are low cost, easy manipulation, ethical acceptability and the ability to incubate larvae at 37°C, the temperature of the human body that is required for the optimal

expression of many key virulence factors of *Lm*. Another important characteristic is that the innate immune system of *G. mellonella* resembles that of mammals, with enzymes, reactive oxygen species and antimicrobial peptides necessary to protect against bacterial infection [62].

Cell culture studies

Several mammalian cell lines have been used to study pathogenesis of Listeria species and their virulence *in vitro*. In particular, they have been used to measure adherence, invasion, intracellular replication, cell-to-cell spread, and plaque formation. Some examples of cell lines used include Caco-2 (human epithelial colorectal adenocarcinoma cells), HT-29 (human colon adenocarcinoma cells), Vero (kidney epithelial cell line), Hep-G2 (hepatocytes), Henle 407 (human embryonic epithelial cell line), A549 (lung alveolar basal epithelial cells), HEK293 (embryonic kidney cells), THP-1 (monocytes), L2 (mouse fibroblasts), J774 (murine macrophage cell line), PtK2 (male rat kidney cell line), and LLC-PK 1 and PK 15 (pig kidney epithelial cell line) [57]. Myeloid dendritic cells have also been used to study *in vitro* suppression of T cell functions after *Lm* infection [63,64]. However, among these cell lines, the Caco-2 has been the most widely used to evaluate the intestinal adherence and the invasion ability of *Lm* as well as to study its intracellular replication [35,57,65–67].

Molecular methods

As reported above, advances in genomics have enabled significant progresses in the identification of effective virulence target genes. Comparative genome sequencing investigates the differences in gene composition between hypervirulent and hypovirulent *Lm* strains, identifying genes responsible for listerial virulence, to be considered as virulence markers [22]. To date, there are several methods to detect virulence genes of interest in *Lm*, ranging from the traditional PCR-based technology to the Whole Genome Sequencing approach. For instance it is possible to develop different PCR assays comprising primers specific for various virulence genes of interest. Against this background, section below describes the great advances made using high-throughput sequencing and specifically outlines how quickly it is to obtain the entire sequence of the *Lm* genome and easily launch it in public databases containing specific patterns of dozens of *Lm*-specific virulence genes [68–70].

Colonisation of food processing environments by *L. monocytogenes*

Survival and persistence

Lm is widespread in the natural environment, animals and foods. The ubiquitous nature of this organism allows the introduction of *Lm* in FPPs, either with raw materials, through equipment or via employees. Once introduced, several factors increase the probability of a strain to establish long-lasting colonization of niches and to persist [31]. As previously reported, *Lm* can persist in FPPs even for years with an increased risk of food cross-contamination [31,55,71,72].

So far, there is no consensus on the definition of a persistent strain; however, it has been proposed to consider the persistent status when the same clone of *Lm* is repeatedly isolated over the time in the same FPE. Therefore, the main step in the study of persistence is the identification of high genetically related strains, recurrently isolated over the time from foods or surfaces in the same plant [68,71].

The environmental persistence of *Lm* is a complex and still poorly understood phenomenon that can be mediated by several concomitant and/or interacting mechanisms. The complexity of the transmission pathways of persistent and transient strains in FPEs makes the identification of the point of exposure source a critical task in risk management, public health preventions and food industry intervention [73].

A first aspect to consider is the ability of *Lm* to survive and grow under a wide range of environmental conditions, including those specifically used in food industry to limit or prevent microbial growth such as high salt concentrations (as high as 10-14% but survival up to 21%), large range of pH (pH 4.2 to 9.5), desiccation (low water activity), and low temperatures [31,74,75]. In particular, maintaining the cold chain is an essential parameter throughout the processing and distribution of food, protecting it from the growth of mesophilic microorganisms and thus extending its shelf life. However, the temperatures used for cold storage do not prevent the growth of psychrotrophic germs such as *Lm* and even if refrigeration decreases the bacterial growth rate, it does not inhibit it completely. *Lm* is able to grow at temperatures as low as −0.4°C but also survive in freezing temperature such as −18°C. Therefore the adaptation of this pathogen to low temperatures is of particular concern [75].

Additionally, the ability to form biofilms may enhance *Lm* survival and adaptation in FPEs, especially in niches that are difficult to reach during cleaning procedures. A biofilm consists of a sessile community of bacteria in which cells colonize a surface embedded in a matrix of extracellular polymeric substances (EPS) and present an altered phenotype compared to the relative planktonic cells [76]. Biofilm formation involves several stages. During the first one, a cell being at a specified distance from the surface (over 50 nm) begins to interact with it through gravitation and electrostatic forces and using flagella. The next stage, known as irreversible adhesion is induced by stronger cell-surface interactions and is characterized by a lesser cell-surface distance (less than 1.5 nm). Bacterial cells use adhesins to form a "key-lock" bond between the cell and the surface and secrete EPS that surround them. Once consolidated, the biofilm undergoes maturation consisting in volume increase and formation of a characteristic architecture [76]. During the biofilm formation the bacteria cell-cell communication plays a key role, with bacteria coordinating their activities through chemical signals that bind with receptors of own and neighbouring cells [76].

Biofilms of pathogenic bacteria, such as *Lm*, are a serious concern in many food industry sectors. Indeed, when bacteria are organized as biofilm, the self-produced EPS matrix gives them extra protection from harsh environmental conditions such as desiccation, nutrient deprivation, or disinfectant treatment [77]. *Lm* is able to form biofilm on several surfaces used in the food industry (stainless steel, polypropylene, glass or rubber), representing in this form a potential source of food contamination [77].

Further, the resistance to disinfectants is not necessarily given by the protective effect of the biofilm but also by intrinsic or acquired mechanisms which lead the cell in its planktonic state not to be inhibited by a specific concentration that usually inhibit the majority of other strains [78,79]. The main mechanisms involved in disinfectants resistance can be more or less specific and include membrane permeability, multidrug or specific efflux pumps and chemical transformation of toxic compounds [79].

One of the most reported resistances of *Lm* against biocides is that against quaternary ammonium compounds (QACs). These disinfectants, and in particular Benzalkonium Chloride (BC), are the most commonly used in food industry. Therefore the ability to survive these biocides contributes to the long-term persistence of some *Lm* strains in FPEs, despite sanitization [78,79]. However, the ineffectiveness of disinfectants may also be due to inappropriate sanitizing protocols such as insufficient cleaning before disinfection, disinfection of wet surfaces, dosage failure or incorrect use temperatures [78,79]. All these situations can often cause *Lm* to be frequently exposed to sub-inhibitory concentrations of

disinfectants. This is particularly true for disinfectants that are not fully biodegradable, such as QACs, which may persist in sewage for long periods with continuously fluctuating concentration gradients [80]. Repeated exposure to sub-inhibitory concentrations of QACs and prolonged environmental persistence of certain strains may facilitate the development of resistance over time as a kind of vicious circle [78,79].

In vitro assessment of biofilm forming abilities and disinfectants resistance

A variety of direct and indirect observation methods have been developed to study biofilm. Standard plate counts, roll techniques, and sonication are indirect methods that first detach the microorganisms from the surface and then count them. Other indirect methods (radiolabeled bacteria, enzyme-linked immunosorbent assay, biologic assays, stained bacterial films, and microtiter plate procedures) estimate the number of attached cells in situ by measuring some attribute for the attached organism [81,82].

Methods involving direct observation allow investigating the architecture of biofilm and include several microscopic techniques such as scanning electron microscopy, epifluorescence microscopy, and confocal laser scanning microscopy [76,81,82].

Currently, among the indirect methods, the microtiter plate assay is the most frequently used for biofilm investigation as it is high-throughput, inexpensive and does not need for advanced equipment apart from plate reader. In this method, an appropriately diluted culture, of about 10^8 CFU/ml for *Lm*, is introduced into individual wells and incubated under optimal conditions. In this regard, several authors have previously studied *Lm* biofilm formation using different parameters such as medium and temperature [81,83,84]. After incubation, the growth media is removed from the wells which are gently washed to remove weakly or not adhering bacteria. This is followed by crystal violet staining for some minutes in order to allow the dye to enter the attached cells. Subsequently the wells are de-stained using chemical agents such as acetic acid that remove the dye not adsorbed by the cells. In order to measure the biofilm attached biomass, the absorbance is determined using a microplate reader [76,82,83,85].

As for biofilm, several methods have been developed to evaluate the sensitivity of *Lm* to commonly used sanitizers both in its planktonic (bacterial suspension) and biofilm form [43,86–91]. The most commonly tested sanitizers are QACs, specifically BC, Peracetic Acid, Sodium hydroxide, Sodium hypochlorite and hydrogen peroxide [43,88,90–94].

Regarding bacterial suspensions, they are grown in specific nutrient broth, such as BHI (Brain Heart Infusion Broth) or TSYEB (Tryptic Soy Yeast Extract Broth), adjusted to a specific

concentration, usually of about 10^8 UFC/ml, and then mixed to serial 2- or 10-fold dilutions of the tested disinfectant. The contact time and temperature vary according to the specific protocol and the evaluation type to be made. At this regard, the Minimum Inhibitory Concentration (MIC) is the most commonly used approach. It represents the concentration of disinfectant at which there is the complete inhibition of bacterial growth and provides information on the tolerance or the ability of bacterial cells to grow in the presence of a specific biocide [43,86,87,91]. When this approach is used, the bacterial suspension is exposed to the disinfectants concentrations for an incubation time of about 20-48h [43,86,87,91]. After this time, the increase in concentration of bacterial cells is estimated, for example by measuring the optical density and the MIC is considered as the lowest concentration of disinfectant totally preventing growth [43,86,89].

Although the MIC is widely used to determine the susceptibility of a pathogen to serial dilutions of a sanitizer, evaluating its ability to grown in the presence of the agent, the determination of the bactericidal effect at the manufacturer's recommended concentration of a sanitizer is of practical interest to the food industry [88].

As stated by the UNI EN 1040:2006 and UNI EN 1276:2019, a product to receive the status of a sanitizer, must meet the standard effectiveness of 5-$\log_{10}$ CFU/ml reduction (99.999%) after a contact time of 5 minutes at room temperature. Therefore, an alternative approach used in studies on *Lm* resistance to disinfectants is the assessment of the required bactericidal effectiveness of different concentrations of sanitizers that are commercially available against *Lm* [88].

In these studies the most diluted suspension of the tested sanitizers to show a viable bacterial reduction of 5-log10 CFU/ml, after 5 minutes at 20-25°C, is defined as the minimal effective concentration (MEC) or the minimum bactericidal concentration (MBC) [88,95].

On the other hand, the assessment of *Lm* biofilm susceptibility to disinfectants is preceded by a biofilm formation phase using microtiter plate assays. Once the biofilm is formed it is exposed to different concentrations of the tested disinfectants for usually 5 minutes at room temperature including a negative control. At the end of incubation, neutralization solution is added to quench the antimicrobial activity of the sanitizer and the bacterial cells in the biofilm are scrapped and enumerated to calculate the log reduction of viable cells relative to the control [88,92,94].

Genetic determinants involved in environmental adaptation and persistence

Several genetic determinants involved in stress resistance of *Lm* have been identified. Among them, the Stress Survival Islet 1 (SSI-1) and Stress Survival Islet 2 (SSI-2) are known to play a role in survival within stressful conditions typically faced in FPEs such as low pH, high osmolarity, nisin (SSI-1) and alkaline and oxidative stresses (SSI-2) [96,97].

SSI-1 is a five-genes islet (lmo0444 – lmo0448) containing two genes encoding hypothetical proteins of unknown function (lmo0444 and lmo0445), a gene encoding a protein involved in bile tolerance (*pva*, lmo0446) and two genes encoding proteins involved in the glutamate-dependent acid resistance system (*gadD1* and *gadT1*, lmo0447 and lmo0448 respectively) [96,98]. It has been demonstrated that deletion of the entire SSI-1 in *Lm* led to reduced growth at high salt concentrations and at low pH, as well as reduced survival on hot dogs at 4°C [96] while deletion of *gadD1* led to a markedly reduced tolerance against both sublethal and lethal levels of the lantibiotic nisin [99]. Moreover, previous studies also reported a correlation between the presence of SSI-1 and greater surface adhesion and biofilm forming abilities in *Lm* strains [97,100].

SSI-2 consists of two genes - the transcription factor gene *lin0464* and the PfpI protease gene *lin0465*- and is present in the hypervariable genetic hot spot *lmo0443* to *lmo0449*, also harbouring SSI-1. SSI-2 is predominantly harbored by *Lm* strains isolated from food and FPEs and particularly by those belonging to ST121 (CC121). This islet is involved in a different stress response than SSI-1. Indeed, Lin0465 and, to a lesser extent, the transcription factor Lin0464, support survival under alkaline and oxidative stress. Of note, these conditions are faced by *Lm* during cleaning and sanitation procedures in the food processing environment as oxidizing agents such as hydrogen peroxide, chlorine dioxide, peracetic acid, and sodium hypochlorite are frequently applied as antimicrobials [101].

All these findings indicate that genomic islets such as SSI-1 and SSI-2 are part of the accessory genome conferring an improved adaptation to environmental variations.

Among the environmental adaptations of *Lm,* detoxification of heavy metals must also be considered [55,102]. These compounds exist in natural and anthropic environments in a variety of chemical forms and typically at low levels, although their concentrations can increase due to various anthropogenic interventions such as the use of disinfectants, soil fertilizers, and livestock feeding [103]. Among the heavy metals resistances, most studied and reviewed in *Lm* are those to cadmium and arsenic [104–106].

Cadmium-resistance is commonly mediated by the *cadAC* cassette, for which four distinct variants have been identified in *Lm*, three associated with mobile elements and one with chromosome [105,107]. More in detail, *cadA1C1* is associated with the plasmid-borne transposon Tn*5422*, *cadA2C2* is harbored by large plasmids such as pLM80 and *cadA3C3* has been located within an integrative conjugative element on the chromosome of *Lm* strain EGDe. On the contrary, The *cadA4C4* cassette has been recently identified in the chromosome of the *Lm* strain Scott A, on a 35-kb chromosomal island known as Listeria Genomic Island 2 (LGI2) [55,106,108].

Arsenic-resistance cassettes are composed by three (*arsRBC*) to five (*arsRDABC*) genes; two putative operons have been identified in *Lm* [109,110]. One of them consists of the *arsR1D2R2A2B1B2* cassette with two additional upstream genes *arsD1* and *arsA1* and has been identified on the LGI2 harbored by the CC2 strain ScottA upstream of the *cadA4* gene. The other cassette, *arsCBADR* [111], is associated with a Tn554-like transposon.

Copper export systems are also reported in *Lm* in which the operon *csoR-copA-copZ* has been identified [55,112].

The significance of heavy metals resistance determinants is shown by their wide distribution within *Lm* strains isolated from food, FPEs and humans affected by listeriosis. It is thus tempting to speculate that tolerance to these compounds may enhance the capacity of *Lm* to persist in the contaminated food or FPEs, but the specific mechanisms are still unknown [110].

Genetic factors exclusively involved in biofilm formation on abiotic surfaces by *Lm* are still relatively unknown. However, as reported above, previous studies have indicated that the presence of SSI-1 was strongly correlated with biofilm formation by *Lm* [97,100]. Moreover a study by Franciosa et al. (2009) [113] have suggested that truncation of the *inlA* gene, caused by PMSCs, significantly enhances biofilm formation, but this conclusion is still controversial and needs to be further investigated.

Finally, *Lm* resistance and tolerance to commonly used disinfectants, including QACs, can be mediated by intrinsic or acquired mechanisms coded by the bacterial genome that include drug efflux pumps [79]. Those strategies can be more or less specific. Actually, a number of genetic markers identified in *Lm* are known to play a role in resistance and tolerance to biocides. Among the multidrug efflux pumps determinants, multidrug resistance Listeria (*mdrl*) and Listeria drug efflux (*lde*) encode for pumps belonging to the Major Facilitator Superfamily (MFR), *sugE* for a Small Multidrug Resistance Efflux Pump (SMR) and *norM* and *mepA* for two Multidrug and Toxic Compounds Extrusion (MATE) pumps [80]. The

qacH gene instead, acquired by the transposon Tn*6188* [71,114], is a QAC-specific efflux determinant associated with the export of BC [80].

Adaptation to distinct ecological niches among major L. monocytogenes clones

As reported in several studies, hyper- and hypovirulent clones present adaptation to distinct ecological niches. Indeed, while hypervirulent clones such as CC1, CC2, CC4, and CC6 are known to colonize better the intestinal lumen than hypovirulent ones and to have a particular neural and placental tropism, reflecting their adaption to host environment, the hypovirulent ones, in particular CC9 and CC121, are more frequently isolated from food and show a great adaptation to FPEs [23,115,116]. Several studies have reported CC9 and CC121 strains persisting even for years in FPPs [31,55,117]. The main characteristics associated with the great adaptation to FPEs and a long lasting persistence of these clones are the presence of several stress resistance genes and the survival and great biofilm formation under sub-lethal BC concentrations [23]. Indeed, it has been reported that while the hypervirulent CC1, CC4, and CC6 clones only harbor genes that are common to all clones, with CC2 harbouring also genes involved in cadmium and arsenic resistance, CC9 and CC121 carry additional stress resistance determinants that may help them to adapt to highly diverse stress conditions.

The results of a number of studies have suggested that the occurrence of SSI-1 and SSI-2 is mainly associated with CC9 and CC121 respectively [22,23,55,101].

The occurrence of specific determinants for resistance to BC is mainly associated with CC9 and CC121 [23,118,119]. Several studies reported that none of the tested CC1 and CC4 isolates harbored BC tolerance genes, whereas all CC121 and several CC9 strains harbored *qac* on Tn6188 [119–122].

Regarding heavy metals resistance genes, *cadA1C1*_Tn*5422* is predominant in CC121 and CC9 while genes encoding arsenic resistance are mainly detected in CC9 strains (carried on Tn*554*) but also in CC2 strains (located on the chromosomal island LGI2) [119].

Finally, Maury et al. (2019) [23] reported in their study that upon increasing BC concentrations CC9 and CC121 produced significantly more biofilm than CC1, CC2, CC4, and CC6 showing that biofilm formation by these hypovirulent clones can also occur in presence of high BC concentrations.

In conclusion, all these findings show that also in terms of ecological niches there are two distinct patterns among major *Lm* clones: hypervirulent clones that are host-associated and exhibit a low adaptation to FPEs and hypovirulent clones with low adaptation to the host but

persisting efficiently in FPEs owing to efficient biofilm formation and tolerance to disinfectants and stress conditions [23,119].

Whole Genome Sequencing: the new era of listeriosis surveillance

Next-generation sequencing (NGS) technology, or high-throughput sequencing, combined with different bioinformatics pipelines, has become a powerful tool for detection, identification, and analyses of human pathogens. In particular, whole genome sequencing (WGS) by NGS provides the most comprehensive overview of a bacterial strain with the highest possible microbial subtyping resolution compared to the other typing methods. WGS allows having in a single test all the information related to the genome of a microorganism (molecular subtyping, resistance profiles, virulence factors) extracting them in silico from the sequence data [123,124].

To date, WGS represents the most powerful tool available to the public health authorities for the surveillance of foodborne diseases with a significant increase of the speed with which threats are detected and a more detail in which the threats are understood. This allows quicker and more targeted interventions and has important implications in high-burden diseases such as listeriosis [123].

Actually, WGS is used in foodborne outbreak investigations and source attribution studies as well as for the exploration of strain resistome (known genes associated with drug resistance) and virulome (set of genes encoding virulence factors) [125].

However, the main application in the surveillance of listeriosis is undoubtedly *Lm* strain comparison or cluster analysis. Indeed, the evaluation, with high discriminatory power, of genetic relationships between different clinical and food isolates, allows individuating epidemic clusters, linking cases to an outbreak and identifying the source of contamination. Core genome MLST (cgMLST) is a highly reproducible gene-by-gene method that enables strain comparison across laboratories by using standardized schemes of alleles [68,126]. The Institute Pasteur scheme of 1748 core alleles is used worldwide for the cgMLST analysis of *Lm* strains with a threshold of 7 different alleles (similarity cut-off of 99.6%) to include more strains in the same genetic cluster [55,68]. The power of cgMLST in identifying national and multi-country listeriosis outbreaks has been proven several times [69,127,128]. In addition to cgMLST, the Single Nucleotide Polymorphisms (SNPs) analysis presents an increased discriminatory power and is based on mapping raw sequence reads against a reference

genome to call variations in both genes and intergenic regions [126]. However, for this method there is not yet a shared and standardized threshold for *Lm* cluster definition.

As described above, in addition to strain comparison, WGS allows carrying out multiple investigations on a bacterial strain. Different bioinformatics software can be used to investigate the virulence profiles of *Lm* isolates as well as the presence of genes for survival under stress conditions, tolerance to disinfectants and heavy metals resistance.

Several online public databases have been implemented for in silico screening of those determinants starting from the whole genome sequence. The most used database and analysis tool for *Lm* is hosted by the Institute Pasteur (https://bigsdb.pasteur.fr/listeria/listeria.html) which contains several gene schemes such as those related to virulence, antibiotic resistance and tolerance to stress, metals and disinfectants [55,69,70]. Other public databases that can be used for the analyses of virulence factors and resistance genes are Virulence Factor database (VFDB), CARD [129], and Resfinder [130] which can be queried individually or together by the software ABRicate v0.8.10 [131].

If the sequence of the entire genome is available, it is also possible to find the presence of prophages and mobile elements such as plasmids. Openly accessible databases aimed at these analyses are PHASTER (https://phaster.ca/), for the rapid identification and annotation of prophage sequences within bacterial genomes and PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), for the identification of plasmids in whole-genome sequences [132,133]. The identification and characterization of phages' and plasmids' sequences is also important to investigate the location of genes of interests such as those associate to different kinds of resistance, evaluating the possibility of their horizontal gene transfer.

WGS has rapidly transformed food-borne epidemiology of *Lm* and other pathogens making disease surveillance faster and more effective [134]. In addition, all the bioinformatics tools available for the analysis of WGS data, offer unprecedented potential for a wide strains characterization that can be applied to the study of persistence and adaptation abilities of *Lm* in FPEs. In this context, cluster analysis methods such as cgMLST, allow to identify highly genetically related strains, recurrently isolated over the time from foods or surfaces in the same processing plant and so considered persistent [2,55,71,135]. Once persistent clusters have been identified, it is possible to investigate the presence of stress and disinfectants resistance genes as well as their virulence profile.

Lm characterization in terms of virulence profiles and survival biomarkers is of great importance for all the strains isolated from food and surfaces in a specific food processing

plant. This allows identifying the main mechanisms promoting the contamination of a plant by the pathogen, in order to provide useful recommendations to Food Business Operators (FBOs) improving the management of the pathogen in the food industry [55].

Aim of the thesis

The main goal of this thesis was to investigate the role of FPEs as reservoir of hypo- and hypervirulent clones of *Lm*, improving knowledge about persistence and virulence characteristics of *Lm* strains associated with small-scale FPPs of Central Italy and identifying genetic biomarkers that can be used to predict their adaptation and long-term survival in food-processing facilities. All this, was aimed at providing new tools for better designing effective strategies for the removal or reduction of resident *Lm* in FPEs and to improve surveillance of human listeriosis using a combination of de novo whole-genome analyses.

More in detail, the specific objectives were to: (i) use WGS and bioinformatics analysis to identify the main circulating hypo- and hypervirulent CCs, (ii) evaluate the genetic relationships between the *Lm* strains identifying persistent clones, (iii) characterize the isolates identifying in silico key genomic features contributing to stress response and persistence in FPEs, along with virulence potential and (iv) use *in vitro* assays to assess their biofilm forming-ability, sensitivity to BC and adhesion and invasion abilities.

Papers

Included in the thesis

This Thesis is based on the following proceeding papers and original research papers, reported in their chronological order and referred to in the text by their roman numeral.

Proceedings Papers

I. **Guidi Fabrizia***, Blasi Giuliana, Centorame Patrizia, Torresi Marina, Duranti Anna, Acciari Vicdalia Aniela, Schiavano Giuditta Fiorella, Amagliani Giulia, Pomilio Francesco, Brandi Giorgio. *Listeria monocytogenes* persistence in food processing environments: whole Genome Sequencing and in *vitro* assessment of disinfectants resistance and biofilm forming ability; *Journal of Preventive Medicine and Hygiene*, 2019 Dec; 60(4 Suppl 3): E1–E85. https://doi.org/10.15167/2421-4248/jpmh2019.60.4s3

II. P. Centorame, L. Iacone, R. Salini, A. Ciarulli, **F. Guidi**, F. Pomilio. Biofilm production by Listeria monocytogenes strains: detection with colorimetric analysis. *European Journal of Public Health*, Volume 30, Issue Supplement_5, September 2020, ckaa166.238, https://doi.org/10.1093/eurpub/ckaa166.238

Original Research Papers

I. **Guidi, F.***; Orsini, M.; Chiaverini, A.; Torresi, M.; Centorame, P.; Acciari, V.A.; Salini, R.; Palombo, B.; Brandi, G.; Amagliani, G.; Schiavano, G.F.; Massacci, F.R.; Fisichella, S.; Domenico, M.D.; Ancora, M.; Pasquale, A.D.; Duranti, A.; Cammà, C.; Pomilio, F.; Blasi, G. Hypo- and Hyper-Virulent *Listeria monocytogenes* Clones Persisting in Two Different Food Processing Plants of Central Italy. *Microorganisms* **2021**, *9*, 376. https://doi.org/10.3390/microorganisms9020376

II. Gabriella Centorotola, **Fabrizia Guidi***, Guglielmo D'Aurizio, Romolo Salini, Marco Di Domenico, Donatella Ottaviani, Annalisa Petruzzelli, Stefano Fisichella, Anna Duranti, Franco Tonucci, Vicdalia Aniela Acciari, Marina Torresi, Francesco Pomilio, Giuliana Blasi. Intensive environmental surveillance plan for *Listeria monocytogenes* in food producing plants and retail stores of Central Italy: prevalence and genetic diversity. – *Foods* **2021**, *10*, 1944. https://doi.org/10.3390/foods10081944

III. Giuditta Fiorella Schiavano, Annalisa Petruzzelli, Amagliani Giulia, **Fabrizia Guidi**, Mauro De Santi, Collins Njie Ateba, Veronica Mele, Francesco Pomilio, Giuliana Blasi, Antonietta Gattuso, Di Lullo Stefania, Rocchegiani Elena, Giorgio Brandi.

Whole Genome Sequencing analysis of *Listeria monocytogenes* virulence profiles and cell adhesion/invasion assessment *in vitro*. *In preparation*

Not included in the thesis

Original Research Papers

- Marina Torresi, Anna Ruolo, Vicdalia Aniela Acciari, Massimo Ancora, Giuliana Blasi, Cesare Cammà, Patrizia Centorame, Gabriella Centorotola, Valentina Curini, **Fabrizia Guidi**, Maurilia Marcacci, Massimiliano Orsini, Francesco Pomilio, Marco Di Domenico. **A** Real Time PCR screening assay for rapid detection of Listeria monocytogenes outbreak strains. Foods 2020, 9(1), 67; https://doi.org/10.3390/foods9010067
- **Fabrizia Guidi *,** Alexandra Chiaverini, Antonella Repetto, Cinzia Lorenzetti, Gabriella Centorotola, Viviana Bazzucchi, Barbara Palombo, Antonietta Gattuso, Francesco Pomilio and Giuliana Blasi. Hyper-virulent *Listeria monocytogenes* strains associated with respiratory infections in Central Italy. Frontiers in Cellular and Infection Microbiology, 11:765540. https://doi.org/10.3389/fcimb.2021.765540
- Alexandra Chiaverini* , **Fabrizia Guidi**, Marina Torresi, Vicdalia Aniela Acciari, Gabriella Centorotola, Alessandra Cornacchia, Patrizia Centorame, Cristina Marfoglia, Giuliana Blasi, Alexandra Chiaverini, Giacomo Migliorati, Sophie Roussel, Francesco Pomilio and Yann Sévellec. Phylogenetic analysis and Genome Wide Association Study applied to an Italian *Listeria monocytogenes* outbreak. Frontiers in Microbiology. *In press*

Posters and conference proceedings

- **Guidi Fabrizia**, Orsini Massimiliano, Centorame Patrizia, Torresi Marina, Duranti Anna, Pomilio Francesco, Acciari Vicdalia Aniela, Blasi Giuliana. *Listeria monocytogenes* persistence in a small-scale meat plant of Central Italy: biofilm-forming ability, stress and disinfectants resistance genes. 20th International Symposium on Problems on Listeria and Listeriosis (ISOPOL), September 24 to 27, 2019, Toronto (ON).
- Foglini M., Blasi G., Pomilio F., **Guidi F.**, Acciari V. A., Gattuso A., Fiore A., Duranti A. Operation tools applied in management of a human listeriosis case in Central Italy. 20th International Symposium on Problems on Listeria and Listeriosis (ISOPOL), September 24 to 27, 2019, Toronto (ON).

- **Guidi F.,** Acciari V.A., Orsini M., Orecchioni F., Pocognoli A., Di Pasquale A., Rinaldi A., Pomilio F., Blasi G. *Whole Genome Sequencing* per lo studio di un pulsotipo di *Listeria monocytogenes* 4b persistente**.** Atti del XIX Congresso Nazionale S.I.Di.L.V. Matera, 23-25 ottobre 2019

Disclaimer

Proceeding paper I

The aim of this study was to use a combined approach based on both WGS and *in vitro* assays for characterizing *Lm* strains isolated over the years in a small-scale meat processing plant of Central Italy in order to identify persistent clones and to evaluate biofilm forming ability and resistance to Benzalkonium Chloride.

Listeria monocytogenes persistence in food processing environments: whole Genome Sequencing and *in vitro* assessment of disinfectants resistance and biofilm forming ability

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Introduction

Listeria monocytogenes (*Lm*) is the causative agent of listeriosis, an invasive disease primarily affecting immunocompromised people, the elderly, children and pregnant women, with high hospitalization (98.6%) and fatality rates (13.8%) [1]. The disease is most commonly caused by eating contaminated food, in particular ready-to-eat. The ability of some strains to persist, even for years, in food processing environments can increase the risk of food contamination. Persistence can results from *Lm* survival after disinfection, thanks to protective biofilm formation and disinfectants and stresses resistance mechanisms or from the repeated reintroduction through raw materials [2,3]. The identification of recurring highly genetically related isolates (Whole Genome Sequencing, WGS and core genome MLST, cgMLST) is necessary to define a strain as persistent in a plant [4]. The aim of this study was to evaluate persistence and resistance to commercial sanitizers commonly used in food processing environments, in *Lm* strains isolated within the laboratory activity of IZSUM (Istituto Zooprofilattico Sperimentale Umbria e Marche). Our approach was based on both WGS and *in vitro* assays.

Materials and methods

32 *Lm* strains were isolated between 2014-2018 in a meat processing plant of Marche region (Central Italy). The assembled genomes were analyzed by cgMLST, according to the Institute Pasteur scheme (1748 loci), using the BIGSdb-Lm database (http://bigsdb.pasteur.fr/listeria) to obtain the allelic profiles. The Minimum spanning tree (MSTv2) was edited by the GrapeTree software [5]. The same bioinformatic platform was used to detect disinfectants resistance genes and Stress Survival Islands (SSI). We tested the strains' ability to form biofilm after 48h at 30°C, using an *in vitro* crystal-violet microtiter plate assay according to the protocol described by Di Bonaventura et al. [6] with minor modifications. To assess strains' sensitivity to quaternary ammonium compounds (QAC) disinfectants the MEC (Minimal Effective Concentration) method was used [7].

Results

The cgMLST analysis showed three main clusters and a single strain clustering outside. Two clusters resulted persistent in the plant, one for 4 years and the other for 2. All the strains were able to produce biofilm at 30°C. Lm belonging to the same cluster showed different biofilm-forming ability suggesting it was a phenotypic feature. All the strains carried *sugE*, *MdrL* and *Lde* genes, involved in tolerance to QAC. In some strains, all belonging to the same cluster, was also detected the Tn6188 transposone, conferring resistance to Benzalkonium chloride, a specific QAC [8]. Moreover all the *Lm* carried the SSI-1 or SSI-2 suggesting their improved ability to grow in stress conditions. Preliminary results about MEC determination indicated sensitivity to Benzalkonium chloride at concentrations used in food processing environments.

Conclusions

The meat processing plant studied was widely affected by *Lm* persistence with two clusters repeatedly isolated over the years. All circulating strains carried genes for QAC resistance and stress tolerance. These features could explain their long term persistence in the plant. However, despite the presence of genetic determinants for QAC resistance, preliminary results showed that the commercially used concentration of Benzalkonium chloride was effective. Our results confirmed that cgMLST could represent a useful tool in monitoring Lm persistence in food processing environments; combining this WGS approach with the *in vitro* assessment of biofilm producing capability and the evaluation of disinfectants and stresses resistance could allow the effective surveillance and control of Lm contamination in food associated environments.

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Proceeding paper II

The purpose of this study was to compare three different staining methods and two different wavelengths for the *in vitro* assessment of *Lm* biofilm forming ability in order to identify the best conditions to use.

Biofilm production by *Listeria monocytogenes* strains: detection with colorimetric analysis

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Keywords: Biofilm, *Listeria monocytogenes*, Colorimetric analysis.

Background

In literature, there are no standardized laboratory methods to detect formed biomass by colorimetric analysis. The purpose of this study was to compare three staining methods and two different wavelengths for determination of biofilm formation of *Listeria monocytogenes (Lm)* strains.

Methods

Three strains of *Lm* isolated from different origin were tested using 96 well polistirene plates at 12°C and 30°C, after incubation the wells were subjected to washing, detaching and staining with crystal violet (CV) at 0.2% and 2% (Panreac EU) in 95% ethanol and with Gram's crystal violet solution (Merck KGaA, Germany). The absorbance at 492nm and 540nm wavelengths was read using a spectrophotometer (SIRIO S, Seac, Firenze, Italia).

Results

The strains incubated at 12 °C displayed production of biofilm when stained with CV 2% and with Gram's crystal violet solution, both at 492 and 540 nm (with better evidence at 540 nm). If CV 0.2% was used to stain and reading at both optical densities there was evidence of weak or no biofilm production.
At 30 °C, the biofilm production was displayed at both temperature and with all the stains. For all the strains and for all the conditions tested, the absorbance was greater but not proportional using the Gram's crystal violet solution, versus the CV 0,2% and CV 2%, and absorbance was higher at 540nm versus at 492nm.

Conclusion

Results confirmed the lack of reproducibility of each of the method used to detect and quantify the biomass produced during a biofilm formation test *in vitro* and the absence of ratio between the different results obtained using different CV concentration and wavelengths for reading.

Main messages

- Biofilm production at 12°C could not be adequately detected staining the wells with CV 0,2%. Absorbance could be influenced by the solvent in the stain used (ethanol, methanol or phenol or mixtures).

- To obtain data for assessment of biomass formation, being the method characterized by poor reproducibility, the laboratory should use at least the same stain and wavelength.

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Original Research Paper I

The main goal of this study was to improve knowledge about persistence and virulence characteristics of *Lm* strains associated with small-scale FPPs of Central Italy, in order to support Food Business Operators in contrasting *Lm* persistence in their establishments, to minimize the risk of food contamination and to avoid recurrence of severe outbreaks of listeriosis. More in detail, the single objectives were to: (i) use WGS and bioinformatics analysis to assess the genetic relationships between the strains identifying persistent clones, (ii) characterize the isolates identifying genetic determinants contributing to stress response and persistence in FPEs, as well as to virulence potential and (iii) assess the biofilm forming-ability *in vitro*.

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Article

Hypo- and Hyper-Virulent *Listeria monocytogenes* Clones Persisting in Two Different Food Processing Plants of Central Italy

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Abstract: A total of 66 *Listeria monocytogenes* (*Lm*) isolated from 2013 to 2018 in a small-scale meat processing plant and a dairy facility of Central Italy were studied. Whole Genome Sequencing and bioinformatics analysis were used to assess the genetic relationships between the strains and investigate persistence and virulence abilities. The biofilm forming-ability was assessed in vitro. Cluster analysis grouped the *Lm* from the meat plant into three main clusters: two of them, both belonging to CC9, persisted for years in the plant and one (CC121) was isolated in the last year of sampling. In the dairy facility, all the strains grouped in a CC2 four-year persistent cluster. All the studied strains carried multidrug efflux-pumps genetic determinants (*sugE*, *mdrl*, *lde*, *norM*, *mepA*). CC121 also harbored the Tn*6188* specific for tolerance to Benzalkonium Chloride. Only CC9 and CC121 carried a Stress Survival Islet and presented high-level cadmium resistance genes (*cadA1C1*) carried by different plasmids. They showed a greater biofilm production when compared with CC2. All the CC2 carried a full-length *inlA* while CC9 and CC121 presented a Premature Stop Codon mutation correlated with less virulence. The hypo-virulent clones CC9 and CC121 appeared the most adapted to food-processing environments; however, even the hyper-virulent clone CC2 warningly persisted for a long time. The identification of the main mechanisms promoting *Lm* persistence in a specific food processing plant is important to provide recommendations to Food Business Operators (FBOs) in order to remove or reduce resident *Lm*.

Keywords: *Listeria monocytogenes*; persistent clusters; biofilm; environmental stresses resistance; QAC-resistance; hypo-virulent clones; hyper-virulent clones; WGS; bioinformatics analysis

1. Introduction

Listeria monocytogenes (*Lm*) is a major foodborne pathogen causing human listeriosis, the most severe zoonoses with the highest hospitalization (97.0%) and fatality (15.6%) rates [1]. Invasive forms of the disease are particularly dangerous for the elderly, immunocompromised people, newborns and pregnant women, leading to sepsis, meningitis, encephalitis, abortion and stillbirth [2]. *Lm* is widespread in the natural environment, animals and food, especially ready-to-eat such as deli meat, dairy products, smoked fish and salads. Once introduced in a food processing facility, several factors increase the probability of a strain to establish long-lasting colonization of niches and to persist [3]. *Lm* is able to survive and grow under a wide range of environmental conditions, including refrigerating temperatures. Stress resistance genetic determinants have been selected in *Lm*, conferring resistance to environmental stresses, such as low pH, high osmolarity, bile and nisin (Stress Survival Islet 1, SSI1) and to alkaline and oxidative stresses (Stress Survival Islet 2, SSI2) [4].

Among the environmental adaptations of *Lm,* resistance to heavy metals must also be considered [5]. Cadmium-resistance is commonly mediated by the *cadAC* cassette, for which four distinct variants have been identified in *Lm,* three associated with mobile elements and one with chromosome [6,7]. In particular, *cadA1C1* is associated with the plasmid-borne transposon Tn*5422*, *cadA2C2* is harbored by large plasmids such as pLM80 and *cadA3C3* is associated with an integrative conjugative element on the chromosome of *Lm* EGDe. The *cadA4C4* cassette, instead, has been recently identified in the chromosome of the *Lm* strain Scott A, on a 35-kb chromosomal island, termed Listeria Genomic Island 2 (LGI2) [8,9]. Arsenic-resistance cassettes are comprised of three (*arsRBC*) to five (*arsRDABC*) genes and two putative operons have been identified in *Lm* [10,11]. One consists of the *arsR1D2R2A2B1B2* cassette with two additional upstream genes *arsD1* and *arsA1* and initially identified on the LGI2 harbored by the CC2 strain ScottA upstream of the *cadA4* gene. The other cassette, *arsCBADR* [12], is associated with a Tn554-like transposon. Copper export systems are also known in *Lm* and the operon *csoR-copA-copZ* has been identified [13].

Additionally, the ability to form biofilms may enhance *Lm* persistence, especially in niches that are difficult to reach during cleaning procedures [3]. Genetic factors involved in biofilm formation on abiotic surfaces by *Lm* are still relatively unknown. However, previous studies revealed that truncated forms of Internalin A (InlA), produced by a premature stop codon (PMSC) mutation in the *inlA* gene, are associated with an increased ability to form biofilm [14,15]. These truncations also result in less virulence, as InlA is a major *Lm* virulence factor.

Further, resistance and tolerance to commonly used disinfectants including the quaternary ammonium compounds (QACs) contribute to the long-term persistence of such strains despite sanitization. Resistance to these biocides can be mediated by intrinsic mechanisms coded by bacterial genome that include drug efflux pumps [16]. These strategies can be more or less specific. Actually, a number of genetic markers identified in *Lm* are known to play a role in resistance and tolerance to biocides. Among the multidrug efflux pumps determinants, multidrug resistance Listeria (*mdrl*) and Listeria drug efflux (*lde*) encode for pumps belonging to the Major Facilitator Superfamily (MFR), *sugE* for a Small Multidrug Resistance Efflux Pump (SMR) and *norM* and *mepA* for two Multidrug and Toxic Compounds Extrusion (MATE) pumps [17]. The *qacH* gene instead, acquired by the transposon Tn*6188* [18,19], is a QAC-specific efflux determinant associated with the export of Benzalkonium Chloride (BC), a QAC largely used in the food industry [17].

Lm persistence in food processing environments (FPEs) increases the risk of food contamination and represents a major concern for food industry and food safety that needs to be studied in depth [17]. So far, there is no consensus on the definition of a persistent strain; however, it has been proposed to consider the persistent status when the same subtype of *Lm* is repeatedly isolated over the time in the same FPE [20]. Therefore, the main step in the study of persistence is the identification of highly genetically related strains, recurrently isolated over the time from foods or surfaces in the same plant [18].

Whole Genome Sequencing (WGS) allows an unprecedented subtyping resolution becoming the best epidemiological surveillance tool in outbreak investigations and monitoring programs of food processing plants, including the detection of *Lm* persistent strains and their characterization in terms of disinfectants resistance and stress survival genes.

Following a severe outbreak of listeriosis that occurred in Central Italy between 2015 and 2016 [21,22], the attention to this pathogen increased in this geographical area both with improved surveillance programs and characterization of *Lm* isolates. The present study was part of this purpose and reported a retrospective investigation on the persistence abilities of *Lm* strains isolated from 2013 to 2018 in a small-scale pork meat processing plant and in a dairy facility, in which positive samples (food and environment) for *Lm* had been recurrently found within the framework of the official food control plan and the own-check control system.

The main goal of the study was to improve knowledge about persistence and virulence characteristics of *Lm* strains associated with small-scale food processing companies of the studied area, in order to support Food Business Operators (FBOs) in contrasting *Lm* persistence in their establishments, to minimize the risk of food contamination and to avoid recurrence of severe outbreaks of listeriosis.

More in detail, the single objectives were to: (i) use WGS and bioinformatics analysis to assess the genetic relationships between the strains identifying persistent clones, (ii) characterize the isolates identifying in silico key genomic features contributing to stress response and persistence in FPEs, along with virulence potential and (iii) assess the biofilm forming-ability in vitro.

2. Materials and Methods

2.1. Bacterial Strains

The 66 *Lm* strains of the study were isolated by the Istituto Zooprofilattico Sperimentale of Umbria and Marche in a small-scale pork meat processing plant (Meat A) and in a dairy establishment (Dairy B) of Central Italy within the framework of the official food control plan and the own-check control system. The plants were located in different provinces of Marche Region and belonged to the two main traditional food-processing chains of Central Italy. Thirty-two strains were isolated from food and environmental samples collected in Meat A and analyzed between 2014 and 2018 (Table S1). Thirty-four strains were cultured during the period 2013–2016 from dairy products and surfaces, collected in Dairy B (Table S1). Multiple isolates from the same food sample were included in the study, to increase the representativeness of the *Lm* genetic diversity in each plant.

2.2. Molecular Serogrouping by PCR

Molecular serogrouping was performed for all the strains according to the EURL method, using a multiplex PCR assay based on the amplification of the same targets as described by Doumith et al. (2004) [23] and Kerouanton et al. (2010) [24]: *prs*, *lmo0737*, *ORF2110*, *lmo1118*, *ORF2819* and the *Lm*-specific gene *prfA*.

2.3. Whole Genome Sequencing

DNA of all the strains was extracted using the Maxwell 16 tissue DNA purification kit (Promega Italia Srl, Milan, Italy) according to the manufacturer's protocol and the purity of the extracts was evaluated by NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). Starting from 1 ng of input DNA, the Nextera XT DNA chemistry (Illumina, San Diego, CA, USA) was used for library preparation according to the manufacturer's protocols. Whole Genome Sequencing was performed on the NextSeq 500 platform (Illumina, San Diego, CA, USA) with the NextSeq 500/550 mid output reagent cartridge v2 (300 cycles, standard 150-bp paired-end reads).

For the analysis of WGS data, an in-house pipeline was used [25] which included steps for trimming (Trimmomatic v0.36) [26] and quality control check of the reads (FastQC v0.11.5). Genome de novo assembly of paired-end reads was performed using SPAdes [27]

v3.11.1 with default parameters for the Illumina platform 2 × 150 chemistry. Then, the genome assembly quality check was performed with QUAST v.4.3 [28].

The 66 *Lm* genome assemblies were deposited at DDBJ/ENA/GenBank under the BioProject PRJNA689809 (Table S2).

2.3.1. In Silico Multi Locus Sequence Typing (MLST)

The multi locus sequence typing (MLST) scheme used to characterize *Lm* strains is based on the sequence analysis of the following seven housekeeping genes: ABC transporter (*acbZ*), beta-glucosidase (*bglA*), catalase (*cat*), Succinyl diaminopimelate desuccinylase (*dapE*), D-amino acid aminotransferase (*dat*), lactate deshydrogenase (*ldh*) and histidine kinase (*lhkA*) [29]. The seven-gene of MLST scheme and the Clonal Complex (CC) were deducted in silico using the BIGSdb-*Lm* database (http://bigsdb.pasteur.fr/listeria; accessed on 26 August 2020).

2.3.2. Core Genome MLST

For the cluster analysis of the strains, the core genome MLST (cgMLST) according to the Institute Pasteur's scheme of 1748 target loci, was performed using the chewBBACA allele calling algorithm [30] available in the in-house pipeline. Agreeing to the guidelines for *Lm* cgMLST typing [20], only the genomes with at least 1660 called loci (95% of the full scheme) were considered. Using the software GrapeTree v.1.5.0 [31], a Minimum Spanning tree (MSTreeV2), showing the relationships among the strains in terms of allelic mismatches, was edited for each plant.

Strains presenting 7 or less allelic differences (similarity cut-off of 99.6%) [20] were considered as belonging to the same cgMLST cluster. The cgMLST allelic cut-off was used to identify persistent strains. In particular, cgMLST profiles repeatedly isolated in the same plant over the time were considered persistent.

2.3.3. Single Nucleotide Polymorphism (SNP) Analysis

A core-single nucleotide polymorphism (SNP)-based approach was used to perform Phylogenetic analysis and to deepen genetic relationships among the isolates. The reference-free tool KSNP3 [32] was used with a kmer size of 21 as indicated by Morganti et al. [33]. The core SNPs matrix was used as input to build a neighbor-joining (NJ) tree using MegaX [34].

Considering the possible evolution over time of a population of persistent strains in its environment, a relaxed 25-SNPs threshold was applied to define strains as belonging to the same cluster [35,36].

2.3.4. Detection of Genetic Determinants Involved in Persistence

The detection of genetic determinants involved in persistence was performed automatically using Prokka v.1.12 [37]. Furthermore, the genome assemblies were manually screened for the absence/presence of loci encoding for disinfectants and metal resistance using the "Metal & Detergent Resistance" function available on the BIGSdb-*Lm* platform (accessed on 10 September 2020). Stress Survival Islands (SSIs) and PMSC in the *inlA* gene were detected using the same online platform. Other genetic determinants in the field (*sugE*, *mdrl*, *lde*, *arsRDABC*, *cadAC*, *npr*) were also detected using the results of genome annotation for each tested genome.

The PlasmidFinder web Tool (https://cge.cbs.dtu.dk/services/PlasmidFinder/; version: 2.0.1 2020-02-07; accessed on 23 October 2020) [38] was used to detect the potential plasmids among the whole genome sequence. The "Listeria Stress Islands" function of the BIGSdb-*Lm* platform was interrogated to detect the LGI2 associated with one of the arsenic resistance cassettes of *Lm*.

2.3.5. Virulence-Associated Genes

All the assembled genomes were screened for virulence genes with ABRicate v.0.8 [39] using public databases as Virulence Factor Database (vfdb) (2597 sequences, [40], last

updated 9 July 2019), CARD [41] and Resfinder [42]. The results were visualized as a heatmap using the "ComplexHeatmap" package of R software v.3.6.1. [43].

Statistical Analysis

For statistical analysis the "stats" package of R software v.3.6.1 [43] was used. The number of virulence genes was compared both among the different cgMLST clusters and the CCs, using the Kruskal–Wallis rank sum test followed, when significant, by pairwise comparisons using Dunn's test with Bonferroni correction. An adjusted $p < 0.05$ was considered as significant.

2.4. Biofilm-Forming Ability In Vitro Assay

To assess the ability of the strains to form biofilm, a colorimetric assay staining biomass with crystal violet was performed as previously described by Di Bonaventura et al. (2008) [44], with minor modifications. Briefly, 150 µL of a Brain Heart Infusion (BHI) bacterial culture of each tested strain, approximately containing 10^8 CFU/mL, were transferred into each of the 10 central wells of a 96-well microtiter plate row. All the remaining wells, both in the central part and the edge, were filled with 150 µL of sterile BHI and 10 of them in a row, not in the edge, were selected to be used as negative controls. After incubation at 30 °C for 48 h, the growth media was removed from all the 96 wells. Removed media from "strains rows", was transferred in a sterile tube and plated on both sheep blood agar and ALOA to assess the microbial purity of inoculum after incubation. The same was done for the "negative controls' row" to assess its sterility. After being emptied, all the wells were gently washed three times with phosphate-buffered saline (PBS) to remove weakly or not adhering bacteria. The samples were fixed at 60 °C for 1 h and each well was stained with 150 µL of 2% crystal violet (Carlo Erba Reagents, MI, Italy) solution in 95% ethanol for 15 min at room temperature. After staining, plates were washed three times with distilled water and dried at 37 °C for 30 min. To de-stain the wells, 150 µL of 33% acetic acid were added and left to act at room temperature for 15 min. In order to measure the attached biomass, the absorbance at 540nm (OD_{540}) was determined with a microplate reader (Sunrise^{TM}, Tecan Trading AG, Männedorf, Switzerland). Three independent experiments for each strain were performed for a total of 30 results. For background correction, the absorbance mean of the negative controls' wells was calculated and used to adjust each result.

Statistical Analysis

ODs_{540} results for independent groups of strains were compared using Kruskall–Wallis test followed by a Dunn's test (with Bonferroni correction) to verify the differences between the possible pairs under comparison. In particular intra-cluster comparisons were performed to verify the presence of any absorbance differences within the individual clusters and inter-cluster comparisons were done to verify if there were differences between the clusters. The same statistical approach was applied to compare biofilm formation between different serogroups. Moreover, the non-parametric Mann–Whitney test was used to verify if there was a difference in biofilm formation between isolates from the two types of plants.

3. Results

3.1. Whole Genome Sequencing

For all the genomes, sequence data in agreement with the quality control thresholds recommended were obtained. Quality metrics for each genome are reported in Table S2. The average read quality after trimming and the number of read pairs returned 34 (min 32.05; max 35.08) and 1,984,489 (min 355,510; max 4,945,108), respectively. The average of the vertical coverage was 80.20 (min 16; max 229). The mean length of the 66 assemblies was 2,989,684 (min 2,879,332; max 3,095,373) with an average number of contigs of 88 (min

23; max 497). The mean values for N50 and L50 returned 362,335 (min 19,796; max 546,962) and 6.31 (min 2, max 47), respectively.

3.1.1. Meat A

Molecular Typing and Cluster Analysis

The 32 *Lm* strains from Meat A were typed as serogroups IIc (n = 16; 50%) and IIa (n = 15; 46.9%), with only one strain found to be IVb (3,1%). On the basis of MLST analysis, isolates were distributed among three CCs: CC9 (IIc), CC121 (IIa) and CC1 (IVb). According to the criteria given by Moura et al. [20], the cgMLST analysis grouped strains of serogroup IIc-CC9 into a larger cluster (cluster A) including 11 strains isolated from 2014 to 2017 and in a smaller one (cluster B) including two *Lm* strains isolated in 2017 and 2018, respectively (Figure 1). Three CC9 strains grouped outside clusters A and B, two of them (*Lm*_2270 and *Lm*_2272) formed the same node and one, *Lm*_2266, was a singleton. The 15 *Lm* serogroup IIa, CC121, instead, all isolated in 2018, grouped in the same cluster (cluster C) (Figure 1). The only IVb strain, CC1, isolated in 2018, was a singleton.

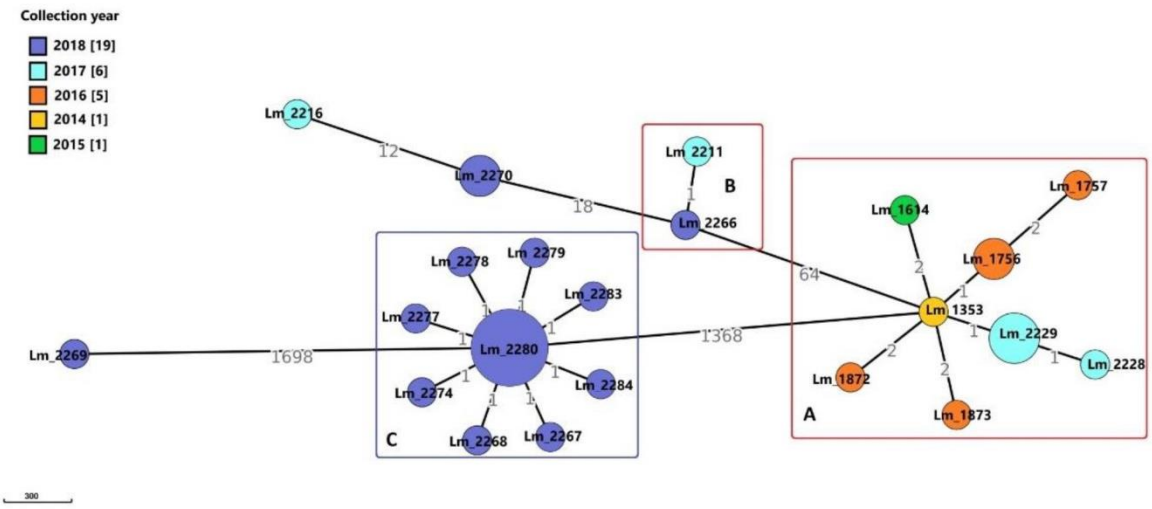

Figure 1. Cluster analysis of *Listeria monocytogenes* (*Lm*) strains isolated in a small-scale pork meat processing plant (Meat A): Minimum Spanning Tree (MSTv2) based on Institute Pasteur's core genome multi locus sequence typing (cgMLST) scheme. Number values between adjacent nodes indicate the number of allelic differences between nodes. In the legend, the numbers in the square brackets indicate the number of strains isolated during each year. Strains of clusters A and B (in red) belonged to serogroup IIc-CC9 together with the *Lm*_2270 and *Lm*_2216. Strains in cluster C (in blue) were IIa-CC121 and the singleton *Lm*_2269 was IVb-CC1. Note: the *Lm*_1756 node also included *Lm*_1791; the *Lm*_2229 node included *Lm*_2230 and *Lm*_2231; in the *Lm*_2270 node also grouped *Lm*_2272; the *Lm*_2280 node also included *Lm*_2271, *Lm*_2273, *Lm*_2275, *Lm*_2276, *Lm*_2282 and *Lm*_2285.

As reported in Table S1, some *Lm* were isolated from the same food sample.

Core SNPs analysis was performed to deepen genetic relationships between strains belonging to the IIc-CC9 clusters A and B, including the outlier strains. The obtained results, according to the 25-SNPs threshold, confirmed what observed with the cgMLST, identifying the same clusters with the same strains composition (Figure 2). In particular, according to the SNPs matrix, in cluster A, strains differed from a minimum of 0 to a maximum of 14 SNPs, in cluster B the two isolates differed by 4 SNPs. *Lm* strains belonging to the same node with the cgMLST differed from 0 to 6 SNPs. Strains of cluster A and those of cluster B differed for a range from 120 to 240 SNPs.

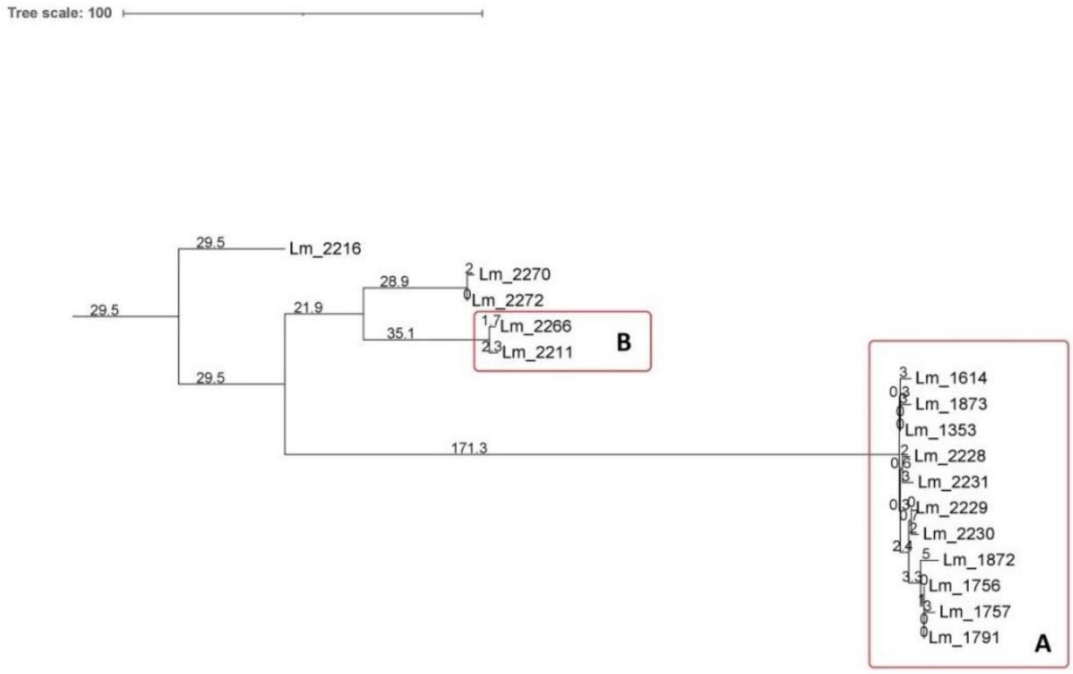

Figure 2. Cluster analysis of *Lm* strains isolated in Meat A: neighbor joining (NJ) tree obtained by core single nucleotide polymorphisms (SNPs) analysis of IIc-CC9 strains. Branch lengths are expressed in terms of changes per number of SNPs. The NJ tree showed the same clusters identified by cgMLST.

The outlier strains *Lm*_2270 and *Lm*_2272 differed by 2 SNPs from each other and by 65–69 SNPs from stains of cluster B. The *Lm*_2216 was more distant showing a SNPs difference ranging from 130 to 240 with cluster B, *Lm*_2270 and *Lm*_2272.

Core SNPs analysis was performed also for IIa-CC121 strains of cluster C, all isolated in 2018 (Figure 3). According to cgMLST results, all the isolates grouped in the same cluster, showing a SNPs difference ranging from 0 to 5.

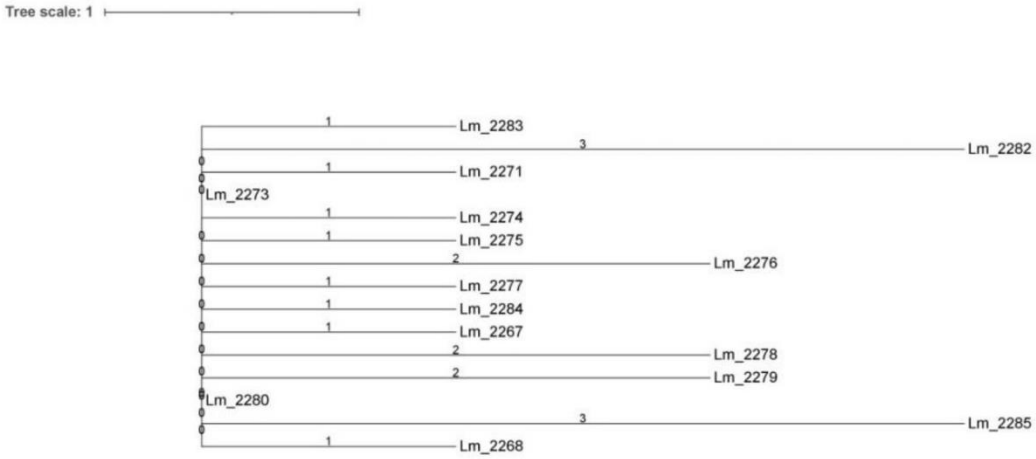

Figure 3. Cluster analysis of *Lm* strains isolated in Meat A: neighbor joining (NJ) tree unrooted obtained by core SNPs analysis of IIa-CC121 strains. Branch lengths are expressed in terms of changes per number of SNPs. The NJ tree showed the same cluster identified by cgMLST.

Genetic Determinants Involved in Persistence

Using the BIGSdb-*Lm* platform together with the annotation results, several disinfectants resistance genes were detected as well as genetic determinants for tolerance to environmental stresses and toxic compounds. In particular, all the strains (Clusters A, B, C and the outliers) carried determinants for different multidrug efflux-pumps: *sugE*, *mdrl*, *lde*, *norM* and *mepA* (Table 1). Strains from cluster C also presented the Tn*6188* conferring resistance to BC. Investigating tolerance to environmental stressors, we found that all the CC9 strains (cluster A and cluster B) carried SSI-1 while CC121 (cluster C) harbored SSI-2. In all the genomes the *gbuABC* cassette for osmotic stress resistance and the *npr* gene for oxidative stress resistance were also detected. Strains belonging to clusters A and C harbored *cadA* and *cadC*. In more detail, CC9 strains of cluster A carried the pLM33 (GenBank acc. no.: GU244485) containing the Tn*5422* with the *cadA1C1* cassette [45]. We used the nucleotide Basic Local Alignment Search Tool (BLASTn) to verify the alignment between the sequence of *cadA* as annotated by Prokka in CC9 strains and the one of *cadA1* harbored by the pLM33. Both coverage and identity were 100%. The same results were obtained for *cadC* and *cadC1* associated with pLM33 (Table 1). The CC121 strains of cluster C, harbored the pLM5578 (GenBank acc. no.: CP001603) plasmid known to carry the *cadA1C1* cassette [46]. We verified the alignment between the *cadA* sequence of the CC121 strains and the one present on the pLM5578 obtaining coverage and identity of 100%. The same results were obtained comparing the strains' *cadC* sequence and the one of *cadC1* on pLM5578. This plasmid also carried the *npr* gene (Table 1). CC9 strains of cluster B carried neither cadmium resistance genes nor plasmids.

The *csoR* gene, for copper-sensing transcriptional regulator, *copZ*, for copper chaperone and *copA*, for the copper-exporting ATPase, were detected in all the strains. Only strains belonging to cluster A carried *copY* for the copper repressor of the cop operon [13]. Genetic determinants for arsenic resistance were found in all the isolates from the Meat A plant. All of them harbored *arsB* and *arsC* with those belonging to clusters A and B carrying also *arsA*, *arsD* and *acr3* (Table 1).

All the CC9 and CC121 strains were found to carry a PMSC mutation in the *inlA* gene encoding for a truncated internalin A while none of the isolates carried the LGI2.

Virulence-Associated Genes

A total of 62 different virulence genes were detected in the 32 isolates of Meat A (Figure S1). A single isolate owned between 34 and 42 virulence genes. Virulence gene counts difference was not significant among clusters (Kruscall–Wallis test, $p = 0.14$) or among different CCs (Kruscall–Wallis test, $p = 0.05$). A set of 31 virulence genes was found in all isolates. All the CC9 and CC121 strains were also found to carry an *inlA* PMSC mutation. The CC1 strain (*Lm*_2269) carried the greatest number of virulence genes (42) and was the only one presenting the Listeria pathogenicity island LIPI-3 (llsY, llsX, llsP, llsH, llsG, llsD, llsB, llsA).

3.1.2. Dairy B

Molecular Typing and Cluster Analysis

All the 34 *Lm* strains isolated from Dairy B were serogroup IVb and CC2. Clustering analysis by cgMLST, according to the above definition, grouped all these strains in a single persistent cluster (cluster D) (Figure 4) lasting in the facility from 2013 to 2016 (Table S1). As reported in Table S1, some *Lm* were isolated from the same food sample.

Table 1. Stress response genes found in the *Lm* isolates.

Genetic Determinant Category	Gene or Islet	Specific Location	Cluster or Isolate							Predicted Resistance Functions
			Meat A						Dairy B	
			Cluster A	Cluster B	Cluster C	*Lm*_2270-2272	*Lm*_2216	*Lm*_2269	Cluster D	
SMR	*sugE*		+	+	+	+	+	+	+	Multidrug efflux-pumps
MFS	*Mdrl*		+	+	+	+	+	+	+	
	Lde		+	+	+	+	+	+	+	
MATE	*norM*		+	+	+	+	+	+	+	
	mepA		+	+	+	+	+	+	+	
QAC-specific resistance genes	*qacH*	Tn6188	-	-	+	-	-	-	-	QAC resistance
Heavy metals resistance genes	*arsA*		+	+	-	+	+	-	-	Arsenic resistance
	arsA1	LGI2	-	-	-	-	-	-	+	
	arsA2	LGI2	-	-	-	-	-	-	+	
	arsB		+	+	+	+	+	+	+	
	arsC		+	+	+	+	+	+	+	
	arsD		+	+	-	+	+	-	-	
	arsD1	LGI2	-	-	-	-	-	-	+	
	arsD2	LGI2	-	-	-	-	-	-	+	
	acr3								-	
	cadA1	pLM33	+	-	-	-	-	-	-	Cadmium resistance
		pLM5578	-	-	+	-	-	-	-	
	cadA4	LGI2	-	-	-	-	-	-	+	
	cadC1	pLM33	+	-	-	-	-	-	-	
		pLM5578	-	-	+	-	-	-	-	
	csoR		+	+	+	+	+	+	+	Copper resistance
	copA		+	+	+	+	+	+	+	
	copZ		+	+	+	+	+	+	+	
	copY		+	-	-	-	-	-	-	
	copB		+	-	-	-	-	-	-	
Stress survival determinants and Islet	*SSI-1*		+	+	-	+	+	+	-	Tolerance to low pH, high osmolarity, bile and nisin
	SSI-2		-	-	+	-	-	-	-	Alkaline and oxidative stress resistance
	gbuA		+	+	+	+	+	+	+	Osmotic stress resistance
	gbuB		+	+	+	+	+	+	+	
	gbuC		+	+	+	+	+	+	+	
	npr	pLM5578	-	-	+	-	-	-	-	Oxidative stress resistance
			+	+	-	+	+	+	-	

SMR—Small Multidrug Resistance Efflux Pumps; MFS—Major Facilitator Superfamily; MATE—Multidrug and Toxic Compounds Extrusion pumps.

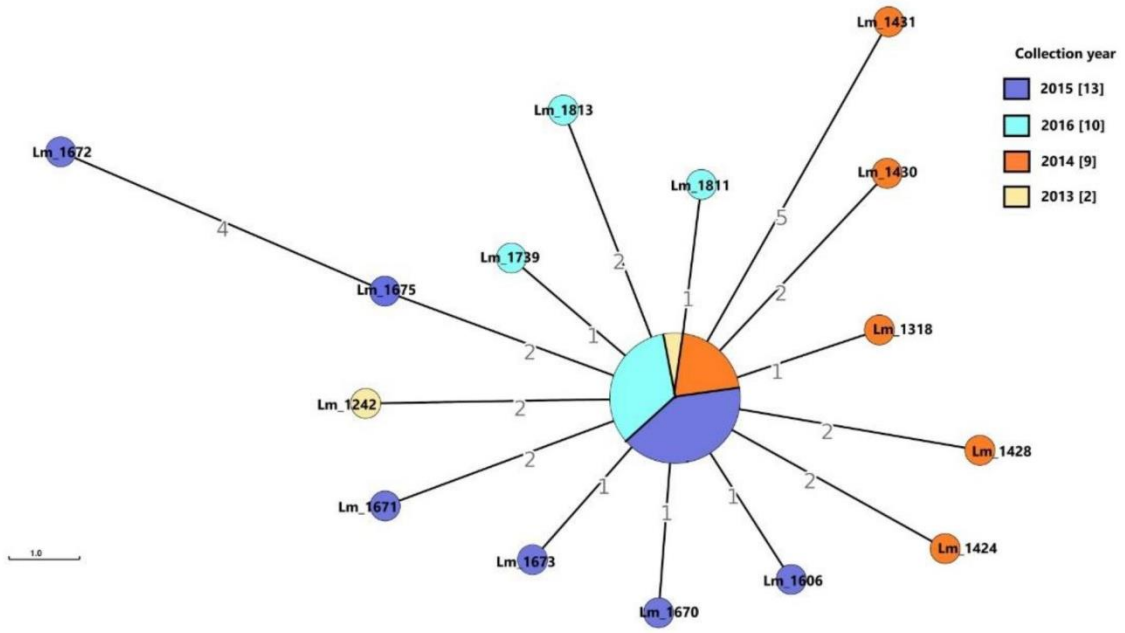

Figure 4. Cluster analysis of *Lm* strains isolated in the dairy establishment (Dairy B): MSTv2 based on Institute Pasteur's cgMLST scheme. Number values between adjacent nodes indicate the number of allelic differences between nodes. In the legend, the numbers in the square brackets indicate the number of strains isolated during each year. All the strains were serogroup IVb and grouped in the single persistent cluster D. Note: Central node included the following strains not shown in the figure: *Lm*_1306 (2013), *Lm*_1311 (2014), *Lm*_1425 (2014), *Lm*_1426 (2014), *Lm*_1429 (2014), *Lm*_1605 (2015), *Lm*_1607 (2015), *Lm*_1674 (2015), *Lm*_1676 (2015), *Lm*_1678 (2015), *Lm*_1679 (2015), *Lm*_1680 (2015), *Lm*_1741 (2016), *Lm*_1743 (2016), *Lm*_1744 (2016), *Lm*_1746 (2016), *Lm*_1747 (2016) and *Lm*_1812 (2016).

Core SNPs analysis was performed for all the IVb-CC2 strains of Dairy B, as they grouped together in the same cgMLST cluster. The obtained results confirmed the belonging to the same cluster for all the strains (Figure 5). The number of SNPs ranged from 0 to 38. Strains belonging to the cgMLST central node differed by a number of SNPs ranging from 0 to 6. *Lm*_1672, *Lm*_1671, *Lm*_1431 and *Lm*_1811 presented a SNPs distance greater than 25 between them but still grouped in the cluster D.

Genetic Determinants Involved in Persistence

According to the results obtained from the BIGSdb-*Lm* database interrogation and the Prokka annotation results, all the isolates of Dairy B carried determinants involved in multidrug efflux-related function, in particular *sugE*, *mdrl*, *lde*, *norM* and *mepA* (Table 1). None of the isolates presented the transposon *Tn6188* for tolerance to BC. All the strains lacked an SSI and carried the *gbuABC* cassette for osmotic stress resistance.

Determinants mediating cadmium and arsenic resistance were present in the genome of all these *Lm*. In particular, *cadA* was the only determinant shared for Cd resistance (Table 1). The PlasmidFinder web tool did not detect any plasmid in the genome of these strains. To assess the chromosomal location of this cadmium-resistance determinant and identify which *cadA* it was, the FASTA sequence on the .ffn file obtained from Prokka was entered in BLAST. The first result among the sequences producing a significant alignment, with a 100% of identity and query coverage, was the *Lm* strain Scott A (GenBank acc. no. CPO23862.1), known to harbor *cadA4*. Further, we performed a multiple alignment between the *cadA* sequence of the strains from Dairy B plant and the one of the *cadA4* carried by the Scott A strain (GenBank acc. no. KT946835.1). The percentage of identity

and the query cover were both 100% with an E value of 0.0. The arsenic resistance pattern included *arsA1*, *arsA2*, *arsB*, *arsC*, *arsD1* and *arsD2* and was fully present in all the isolates, except one that was missing *arsA1*. In all the strains *csoR*, *copZ* and *copA* were also detected.

All these strains harbored the LGI2 (Table 1) and a full length *inlA* gene.

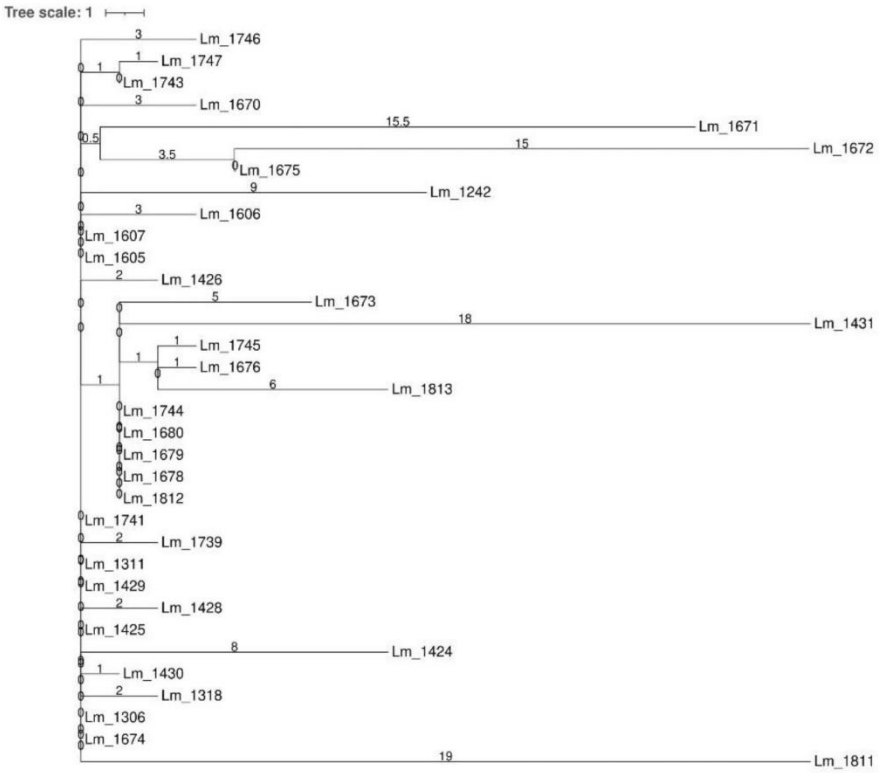

Figure 5. Cluster analysis of *Lm* strains isolated in Dairy B: neighbor joining (NJ) unrooted tree obtained by core SNPs analysis of all the IVb-CC2 strains. Branch lengths are expressed in terms of changes per number of SNPs. The NJ tree confirmed the belonging of all the strains to the same cluster (cluster D).

Virulence-Associated Genes

A total of 45 different virulence genes were identified in the 34 isolates of Dairy B (Figure S1). A single isolate could contain between 34 and 36 virulence genes. A set of 33 virulence genes and a full length *inlA* was found in all isolates. Virulence gene counts difference was not significant among CCs also including CC2 (Kruscall–Wallis test, $p = 0.05$).

3.2. Biofilm-Forming Ability

Among the *Lm* isolated in Meat A, nine strains were selected and tested for their biofilm-forming ability. As a selection criterion within clusters, where possible, strains isolated in different years or alternatively from different samples were selected. In particular, three strains from cluster A, both strains of B and two from C were tested. The CC9 singleton *Lm*_2216 and the only CC1 strain *Lm*_2269 were also tested.

All these *Lm* strains formed biofilm in vitro with OD_{540nm} median values ranging from 0.191 to 0.367 (Figure 6). Intra-cluster comparisons showed significant difference in biofilm formation within all the clusters (cluster A: Kruskall–Wallis k = 22.481, $p < 0.0001$; cluster B: Mann–Whitney U = 223, $p = 0.0008$; cluster C: Mann–Whitney U = 824, $p < 0.0001$). Dunn test with Bonferroni correction (adjusted p-value = 0.0167) was used for pair comparisons within the cluster A and showed significant differences (adjusted p-value < 0.0167)

only between *Lm*_1353 and *Lm*_1791 and between *Lm*_1791 and *Lm*_2228. Inter-cluster comparisons indicated a significant difference in biofilm formation between the different clusters (Kruskall–Wallis k = 84.992, $p < 0.0001$) and Dunn test results identified a statistical significance between clusters A and B and clusters A and C (Figure 7). *Lm*_2216 and *Lm*_2269 significantly produced less biofilm when compared with cluster B and C but no significant difference was found with cluster A.

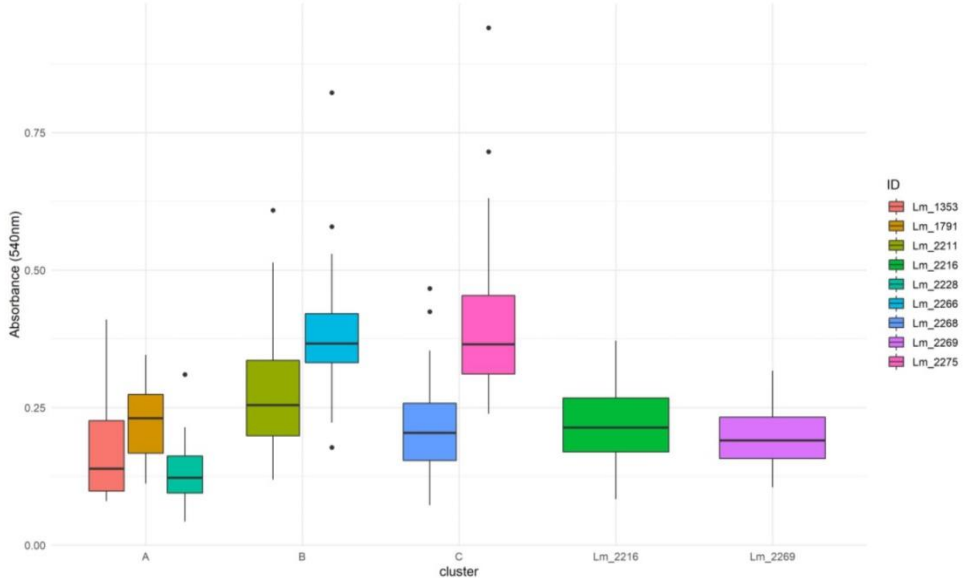

Figure 6. Box plots analysis of biofilm formation by *Lm* strains tested from Meat A. Boxplots represent the distribution of the thirty adjusted absorbance values obtained for each tested strain using the in vitro crystal violet assay. Single strains boxplots are grouped by cluster.

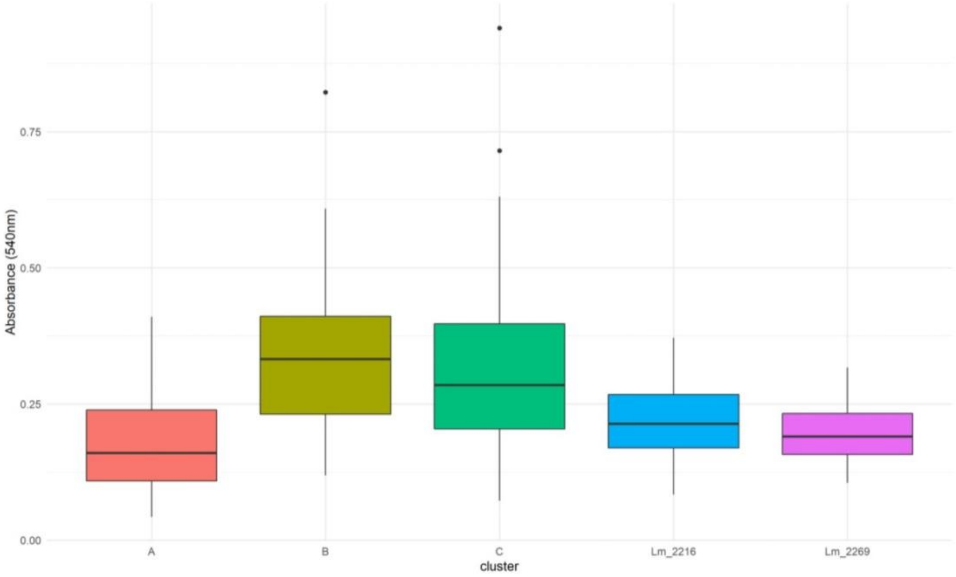

Figure 7. Box plots analysis of biofilm formation by *Lm* different clusters and the oulier strains from Meat A.

Among the Dairy B *Lm* collection, eight strains were selected to be tested for their biofilm-forming ability. As all the *Lm* isolated in this facility grouped in the same cluster, the selection was made considering the year of isolation and the origin (food or environments), in order to test at least one strain per year and/or matrix of isolation. Then one strain for 2013 (cheese), two for 2014 (one from cheese and the other from environment), three for 2015 (two from different cheeses and one from FPEs) and two for 2016 (one from cheese and the other from environment) were selected.

All the tested strains were able to form limited biofilm with OD_{540nm} median values ranging from 0.040 to 0.130 (Figure 8). Intra-cluster comparisons, involving all the strains of Dairy B composing together the cluster D, showed significant difference in biofilm formation (Kruskall–Wallis k = 53.099; $p < 0.0001$). Pairwise comparisons showing significant differences by the Dunn test with Bonferroni correction (Bonferroni adjusted value = 0.0018) are reported in Table 2.

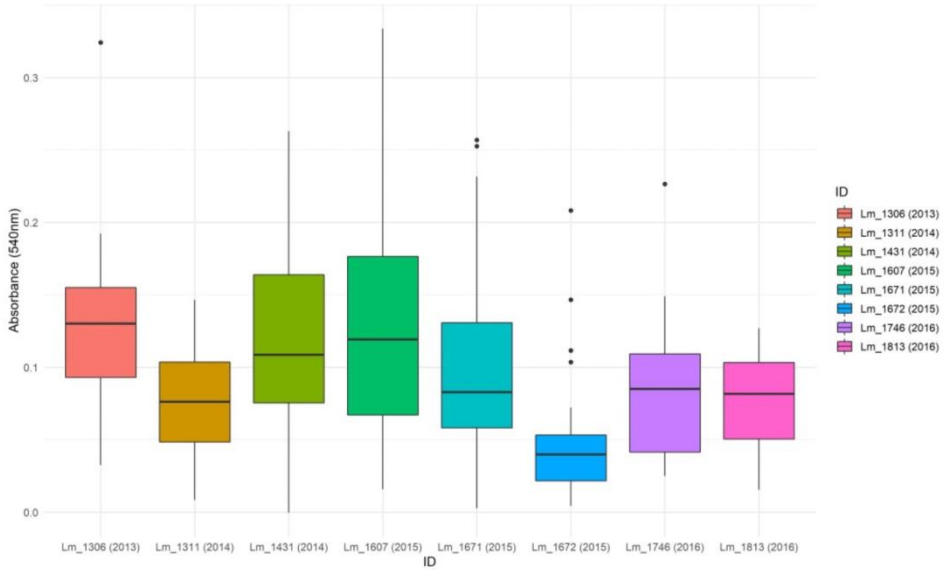

Figure 8. Box plots analysis of biofilm formation by *Lm* strains tested from Dairy B. Boxplots represent the distribution of the thirty adjusted absorbance values obtained for each tested strain using the in vitro crystal violet assay.

Table 2. Pairwise comparisons of biofilm production between strains of Dairy B. The Dunn test was used as statistical method followed by Bonferroni correction. The Bonferroni adjusted *p*-value was 0.0018. Significant differences are reported in bold.

	***Lm*_1306**	***Lm*_1311**	***Lm*_1431**	***Lm*_1607**	***Lm*_1671**	***Lm*_1672**	***Lm*_1746**	***Lm*_1813**
*Lm*_1306	1	**0.001**	0.338	0.586	0.039	**<0.0001**	0.002	**0.001**
*Lm*_1311	**0.001**	1	0.016	0.005	0.193	0.009	0.800	0.956
*Lm*_1431	0.338	0.016	1	0.679	0.270	**<0.0001**	0.031	0.019
*Lm*_1607	0.586	0.005	0.679	1	0.129	**<0.0001**	0.010	0.006
*Lm*_1671	0.039	0.193	0.270	0.129	1	**<0.0001**	0.294	0.212
*Lm*_1672	**<0.0001**	0.009	**<0.0001**	**<0.0001**	**<0.0001**	1	0.004	0.007
*Lm*_1746	0.002	0.800	0.031	0.010	0.294	0.004	1	0.844
*Lm*_1813	**0.001**	0.956	0.019	0.006	0.212	0.007	0.844	1

Making a comparison of the biofilm absorbance values between the different serogroups (IIa, IIb and IVb), we found a significant difference (Kruskall–Wallis test k = 203,197, $p < 0.0001$). According to the results obtained with the Dunn test (Bonferroni adjusted

p-value < 0.0167), all the pair comparisons showed statistically significant differences, with IVb presenting the lowest values.

The biofilm production by *Lm* isolated in Meat A was significantly different from that of Dairy B strains (Mann–Whitney U = 7372, $p < 0.0001$) with the latter presenting lower values.

4. Discussion

This work was focused on *L. monocytogenes* persistence in small-scale food processing facilities repeatedly tested positive in food and environmental surfaces within the framework of official control and the own-check control system. A cluster analysis was performed to define the genetic relationships between *Lm* strains isolated in the same plant in order to identify persistent clusters. Persistence features and virulence profiles of all the strains, belonging or not to persistent groups, were also investigated.

Strains isolated from the same sample very often grouped in the same cluster and probably consisted exactly of the same strain. This did not affect our considerations as the interest was in identifying genetic clusters containing isolates of different years and in defining persistence and virulence abilities of all *Lm* strains associated with each studied plant.

The microbial population detected in Meat A was more heterogeneous than those from Dairy B. Indeed, in the Meat A plant we found strains belonging to CC9 (serogroup IIc), CC121 (IIa) and CC1 (IVb) and both cgMLST and SNPs analysis identified three different clusters and four strains clustering outside. All the strains from Dairy B, instead, belonged to CC2 (IVb) and grouped into the same cluster through the cgMLST and SNPs analysis (cluster D).

According to previous studies [47,48], the CC9 and CC121 are hypo-virulent clones infecting mostly highly immune-compromised individuals, are more frequently isolated from food and seem to be better adapted to FPEs. Although they have been found in all production sectors, there is a strong association with meat products and it is consistent with our results. In contrast, CC1 and CC2 are considered hyper-virulent MLST clones as they have high clinical frequency. Even though different authors report their association with certain food types [4,49,50], including dairy products, they have been isolated from a huge diversity of foods, and have been implicated in outbreaks involving different food types [51].

In the Meat A plant, cluster A (IIc, CC9) was recurrently isolated from 2014 to 2017, while cluster B (IIc, CC9) consisted of only two strains, one isolated in 2017 and the other in 2018. These results suggested the persistence of these clusters in the plant. The 15 *Lm* strains of cluster C (IIa, CC121) were detected for the first time in 2018 and the same was for the outlier strains *Lm*_2270/*Lm*_2272 (IIc, CC9) and *Lm*_2269 (IVb, CC1). The *Lm*_2216 (IIc, CC9) was isolated in 2017 and since it was a singleton, related strains were found neither in 2018 nor in the previous years. This strain was probably sporadic in the plant. In the Dairy B plant, cluster D was isolated from 2013 to 2016, indicating a worrying persistence of a clone frequently involved in human listeriosis outbreaks. The long-lasting colonization of these FPEs by *Lm* could explain the recurrent contamination of food in both the plants.

Investigating the main characteristics known to be involved in persistence, we found that all the studied strains carried various efflux-pumps genetic determinants. Although these transport mechanisms have many efflux-related functions, they have been previously associated with BC tolerance. In particular, Jiang et al. [52] reported the association between the presence of *sugE* and a higher values of minimum inhibitory concentration of BC, while Tamburro et al. [53] and Jiang et al. [54] observed a significant overexpression of *mdrl* in *Lm* strains exposed to BC stress. Similarly, Rakic-Martinez et al. [55] reported an increased expression of *lde* in BC-selected *Lm*. NorM and MepA, belonging to the MATE family, are also known to be associated with extrusion of QACs and in particular of BC [17,56]. In addition, in all CC121 strains from the Meat A plant, we found the presence of Tn6188, carrying the *qacH* gene, a QAC-specific efflux determinant associated with the export of

BC. This result was consistent with previous reports [4]. Although the resistant phenotype should be verified by in vitro assays [54,57], all these findings suggested that QACs may have been ineffective at controlling *Lm* in these food processing plants. Efflux-mediated QAC resistance received significant interest because it has a genetic origin, confers co-resistance to antibiotics and it is transferable among species through horizontal gene transfer [17,58].

In the Meat A plant, all the strains belonging to clusters A, B and C and the CC9 outlier strains carried an SSI. In particular, consistently with previous studies [4,59], all the CC9 strains (cluster A and cluster B) carried the SSI-1 while the CC121 strains (cluster C) harbored the SSI-2. The SSI-1 contributes to the growth of *Lm* at low pH, high salt concentrations and at both refrigeration temperature (4 °C) and at 15 °C [60] while the SSI-2 supports survival under alkaline and oxidative stresses [59]. *Lm* faces this last stress during cleaning and sanitation procedures in the FPEs, as oxidizing agents (hydrogen peroxide, chlorine dioxide, peracetic acid and sodium hypochlorite) are frequently applied as antimicrobials in food industry. This finding suggested that other disinfectants besides QACs may also have been ineffective at controlling *Lm* in Meat A. Both the SSIs may have enhanced listerial adaptation to FPEs conferring to the bacterial population associated with the plant an adaptive advantage. This may have contributed to the long-lasting colonization of clusters A and B and suggested a potential persistence also for cluster C and the outlier *Lm*_2270/*Lm*_2272. The CC1 singleton detected in Meat A as well as all the CC2 strains from Dairy B lacked an SSI, consistently with their belonging to serogroup IVb [61].

All the strains both from Meat A and Dairy B carried the *gbuABC* cassette for osmotic stress resistance but only strains from Meat A presented the *npr* gene for oxidative stress resistance [62]. In CC121 strains of cluster C, this gene was carried on the pLM5578.

Another important adaptive mechanism we investigated was associated with detoxification of heavy metals, existing in natural environments in a variety of chemical forms and typically at low levels, although their concentrations can increase due to various anthropogenic interventions. All the *Lm* strains studied in both the facilities carried genetic determinants for tolerance to arsenic, cadmium and copper. It is currently not clear how such resistance may contribute to overall fitness of *Lm* in the FPEs and in foods. However, several studies provide suggestive evidence that cadmium resistance, in particular, may promote *Lm* persistence and fitness in food or FPEs. In the Meat A plant, the CC9 strains belonging to cluster A, carried *cadA1C1* on the plasmid-borne Tn5422 contained in the pLM33. The presence of these determinants associated with high-level resistance to cadmium [9], may have contributed to the long-term persistence of this cluster. The *cadA1C1* cassette was also found in all the CC121 strains of cluster C but in them, it was carried by the pLM5578.

The spread of hypo-virulent *Lm* strains carrying genetic determinants of persistence on mobile elements as plasmids or transposons represented a risk of horizontal gene transfer conferring enhanced survival to FPE-associated stressors even to hyper-virulent *Lm* clones and to other species of foodborne pathogens circulating in the same plant.

All the strains from Dairy B instead, presented *cadA4*, harbored by the LGI2 and associated with lower-level cadmium resistance. Consistently with our results, the majority of reported *cadA4*-harboring strains belonged to CC2 [8,63]. The presence of this low-level cadmium resistance gene was not considered relevant for the long-term persistence of the *Lm* strains in Dairy B.

Coming to arsenic resistance, all the strains from Meat A harbored *arsB* and *arsC* with those belonging to clusters A and B also carrying *arsA*, *arsD* and *acr3*. In agreement with Parsons et al. [11], these CC9 and CC121 strains lacked the LGI2, predominantly responsible for arsenic and cadmium resistance in serotype 4b isolates and in particular in those belonging to CC2. Consistently, CC2 strains from Dairy B presented LGI2 and carried the arsenic pattern associated with this genomic island (*arsA1*, *arsA2*, *arsB*, *arsC*,

arsD1 and *arsD2*). This cassette is known to confer tolerance to higher concentrations of arsenic and may have contributed to the persistence of the CC2-cluster in Dairy B.

Investigating in vitro the biofilm-forming ability, considered one of the most influent mechanisms of persistence in *Lm*, we found statistically significant differences in biofilm formation among strains belonging to the same genetic clusters. Therefore, our results showed that strains with the same genotype may exhibit a different biofilm-forming ability. In the Meat A plant, cluster A produced significantly less biofilm than clusters B and C, indicating that the long-term persistence of this cluster was not determined by the level of biofilm production. Statistically significant differences were found in the biofilm production by different *Lm* serogroups (IIa, IIb and IVb), with serogroups IIa and IVb presenting the highest and lowest values respectively. These findings were consistent with previous studies reporting that serogroup IVb strains demonstrated a lower capacity for biofilm formation and that CC9 and CC121 were able to make more biofilm than CC1 and CC2 [4,61]. Moreover, Keeney et al. [61] reported that the presence of SSI-1 was strongly correlated with biofilm formation and according to Franciosa et al. [15], the PMSC mutation in the *inlA* gene was associated with enhanced biofilm levels when compared to the wild type *inlA*. Further studies investigated at the same time the influence of SSI-1 and a truncated inlA protein, finding that they were both significantly associated with increased levels of biofilm [14]. According with these previous works, all CC9 and CC121 tested strains, harbored an SSI and a PMSC mutation in the *inlA* gene, and produced significantly more biofilm in vitro when compared with the singleton CC1 and the CC2 strains from Dairy B, lacking an SSI and presenting a full length *inlA*.

We also compared the biofilm producing ability by plant and we obtained a statically significant difference indicating that *Lm* isolated from Dairy B, produced less biofilm if compared with strains from Meat A. Although this result was easily predictable considering that all the strains from Dairy B were serogroup IVb, it indicated that even for cluster D, the amount of produced biofilm was not the determining factor of its prolonged persistence.

Anyway, we considered the ability to produce biofilm rather than the effective amount of biofilm, as an influent advantage in persisting. First, because as reported by Azeredo et al. [64], the microtiter plate dye-staining method, although the most commonly used, thanks to its versatility and high-throughput, may sometimes lack reproducibility with results laboratory dependent. This is mostly due to the lack of reference strains certified to be good biofilm producers and the over or underestimation of biofilm biomass depending on the washing steps. Based on these limitations, we only used the method to assess whether a strain was able of adhering and forming biomass on an inert substrate. Moreover, even just a thin layer of biofilm, if formed in niches that are difficult to reach during sanitation procedures, represents a persistent source of contamination.

Overall, as previously reported [65,66], our results indicated clones CC9 and CC121 as more adapted to FPEs with a higher prevalence of stress resistance, the presence of BC-specific tolerance genes and higher biofilm production capability. On the other hand, the CC2 population associated with the Dairy B plant, despite the lack of these genetic determinants and the lower biofilm production, persisted over the years remaining extremely stable and homogenous, probably as the result of a strong long-term selective pressure. All these findings reflected how different FPEs might present very different selective conditions influencing the associated bacterial population.

Finally, investigating the virulence profiles of the studied strains, we did not observe statistically significant differences for the number of genes between different CCs. However, it was evident that the only CC1 strain of Meat A (*Lm*_2269) presented many more virulence factors if compared with the other CCs but the fact that it was the only one CC1 in the study may affected the statistics. Only in this singleton we found the LIPI-3, encoding a biosynthetic cluster involved in the production of Listeriolysin S (LLS), a hemolytic and cytotoxic factor conferring a greater virulence to *Lm* [67]. LLS is expressed only under oxidative stress conditions and this confers a better ability in terms of phagosome escape. Therefore, the presence of LIPI-3 is considered responsible for the increased virulence in

some strains and is the best candidate to date to explain the greater association of lineage I with human listeriosis [68,69]. Another factor playing a fundamental role in host cells invasion and, in particular, in crossing human intestinal barrier during infection is InlA, encoded by the *inlA* gene. The PMSC mutations in *inlA* correlated with the inability of the *Lm* isolates to invade Caco-2 cells and so with a less virulence [70]. Both the CC1 strain and all the CC2 carried a full length *inlA* while all CC9 and CC121 strains presented a PMSC mutation. All these findings supported the definition of CC1 and CC2 as hyper-virulent clones and CC9 and CC121 as hypo-virulent.

5. Conclusions

Many mechanisms may contribute to *Lm* persistence in FPEs, with complex interactions of changing factors from case to case. A multidisciplinary approach based on both genotypic and phenotypic investigation is required to better understand this phenomenon. WGS currently provides the highest possible microbial typing resolution and is considered the most practical and relevant laboratory technique to study the full genomes of bacteria. The combination of different bioinformatics solutions evidenced intra-plant *Lm* clones persisting over years in food products and environment of two different facilities of Central Italy. In addition, it provided insights into the dynamics of stress tolerance-related genetic markers promoting the persistence of *Lm* CCs in FPEs and gave information about their virulence potential. On the other hand, despite its known limits, the in vitro assessment of biofilm-forming ability added important information about the main known strategy used by *Lm* to colonize and persist in FPEs. In particular, we found that strains belonging to the same genetic cluster may exhibit a different biofilm-forming phenotype and that the amount of produced biofilm did not seem to be decisive for long-term persistence in FPEs.

It is known that hypo-virulent clones, in particular CC9 and CC121, more efficiently persist in food-production environments. Nevertheless, our results showed that even hyper-virulent clones could warningly persist for long time. In the Dairy B plant, in fact, we found the same CC2 cluster persisting over four years. These findings demonstrated that persistence of *Lm* is not necessarily or exclusively the result of a contamination by strains having specific and unique genetic traits or phenotypic abilities. The fitness of a strain is relative to the environment with which it is interacting. Strains having such persistence abilities could be, at the same time, more adapted to one environment and less in another. In addition, besides the specific characteristic of the FPE (presence of ecological niches, non-compliant structures and equipment) and the survival abilities of the strains, other factors can influence *Lm* persistence such as reintroduction of contaminated raw materials, inappropriate processing and ineffective cleaning and sanitizing protocols.

The small number of food-producing plants involved in this study obviously, does not allow us to consider our results as representative of the *Lm* persistence situation in all the FPEs of Central Italy, but that was not our goal. This study precisely aimed to investigate the persistence dynamics influencing bacterial populations associated with individual plants. With this in mind, reporting the long-term (years) persistence of two different CC9 and one CC2 clusters, we contributed to deepen the current knowledge on *Lm* persistence in the main traditional food production chains of Central Italy, while providing new data on the persistence abilities of *Lm* clones in Italy.

One of our future perspectives will be the in vitro assessment of disinfectants resistance in the studied strains, with particular regard to BC (QAC), to demonstrate the phenotypical expression of the carried tolerance genes. It would also be very useful to test sanitizing agents specifically used in the plants in order to assess their effectiveness on the circulating *Lm* strains. The next goal for the future is also to extend the study to other food producing plants located in Central Italy.

Concluding, the identification of the main mechanisms promoting *Lm* persistence in a specific food processing plant by investigating survival biomarkers is the major goal to provide recommendations to FBOs in order to remove or reduce resident *Lm*. Those measures should be adapted to individual plants and could involve, for example, use of

different sanitizer agents in a rational combination or turning them, or increase attention to environmental niches or harborage points, to improve the management of the pathogen in the food industry minimizing risk of food contamination and recurrence of severe outbreak of listeriosis as that which occurred in Central Italy between 2015 and 2016.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/2/376/s1, Figure S1: Heat map showing the in silico detected virulence-associated genes, Table S1: *L. monocytogenes* isolates used in this study by food processing plants. Table S2: Quality control check of sequence data.

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Supplementary Materials

The following are available online at https://www.mdpi.com/2076-2607/9/2/376/s1

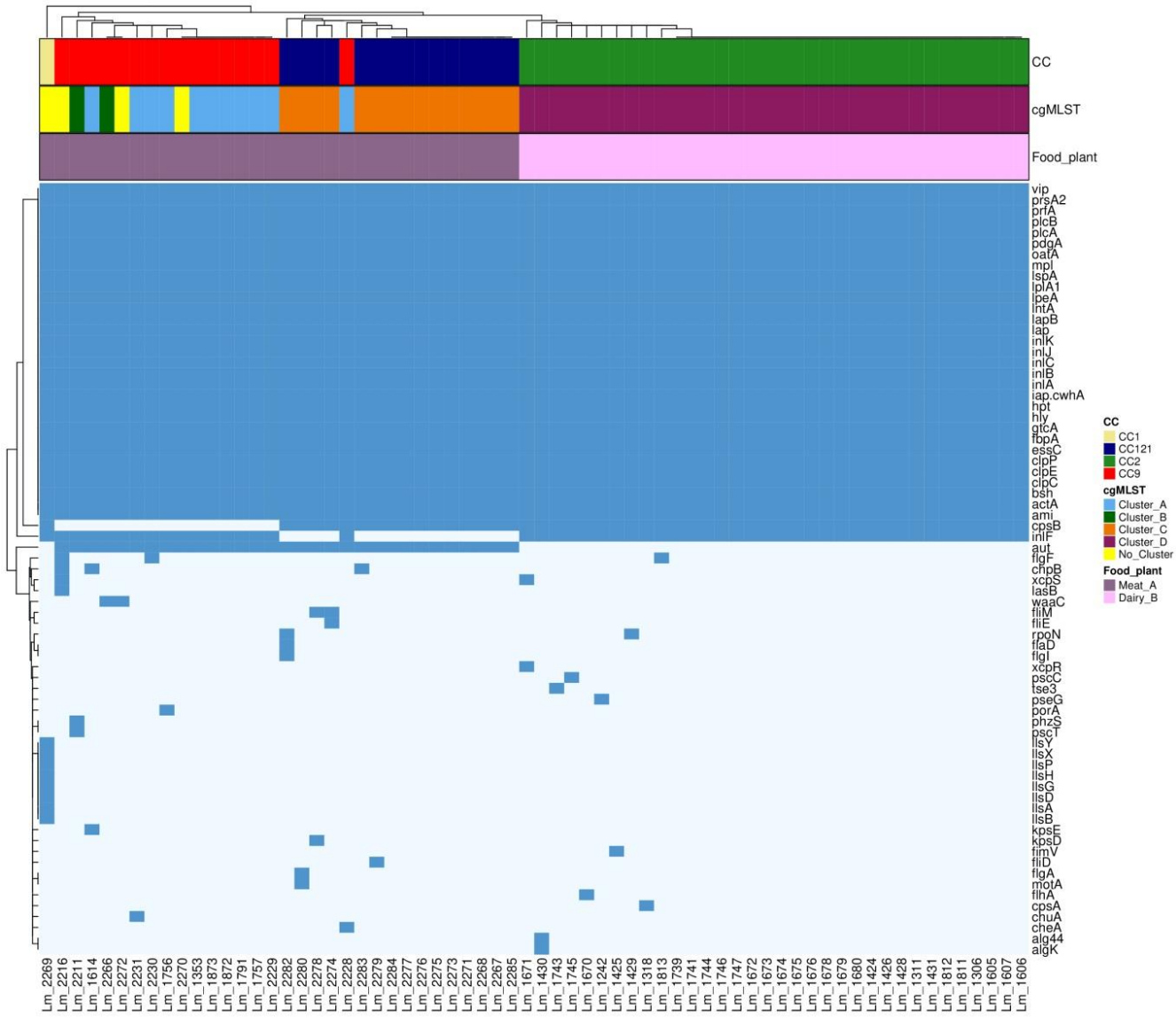

Figure S1. Heat map showing the *in silico* detected virulence-associated genes. In blue the present genes and in light blue absent genes are represented. The CC, cgMLST cluster and food plant for each isolate are also reported.

Table S1: *L. monocytogenes* isolates used in this study by food processing plants. Matrices and years of isolation are also reported. Isolates within the same source-box in the Table were from the same sample.

Food processing plant	ID	Year	Source
Meat processing plant (Meat A)	*Lm*_1353	2014	Sausage (pork)
	*Lm*_1614	2015	Salami (pork)
	*Lm*_1756	2016	Salami (pork)
	*Lm*_1757	2016	Salami (pork)
	*Lm*_1791	2016	Environment
	*Lm*_1872	2016	Environment
	*Lm*_1873	2016	Environment
	*Lm*_2211	2017	Salami (pork)
	*Lm*_2216	2017	Salami (pork)
	*Lm*_2228	2017	Salami (pork)
	*Lm*_2229	2017	
	*Lm*_2230	2017	
	*Lm*_2231	2017	
	*Lm*_2266	2018	Salami (pork)
	*Lm*_2267	2018	
	*Lm*_2278	2018	
	*Lm*_2279	2018	
	*Lm*_2280	2018	
	*Lm*_2285	2018	
	*Lm*_2268	2018	Salami (pork)
	*Lm*_2269	2018	
	*Lm*_2270	2018	
	*Lm*_2271	2018	
	*Lm*_2272	2018	
	*Lm*_2273	2018	
	*Lm*_2282	2018	
	*Lm*_2283	2018	
	*Lm*_2274	2018	Sausage (pork)
	*Lm*_2275	2018	
	*Lm*_2276	2018	
	*Lm*_2277	2018	
	*Lm*_2284	2018	
Dairy plant (Dairy B)	*Lm*_1306	2013	Dairy product
	*Lm*_1242	2013	Dairy product
	*Lm*_1431	2014	Environment
	*Lm*_1430	2014	Environment
	*Lm*_1318	2014	"Pasta filata" cheese
	*Lm*_1311	2014	"Pasta filata" cheese
	*Lm*_1429	2014	"Pasta filata" cheese
	*Lm*_1428	2014	
	*Lm*_1426	2014	
	*Lm*_1425	2014	
	*Lm*_1424	2014	
	*Lm*_1607	2015	Mozzarella cheese
	*Lm*_1606	2015	Mozzarella cheese
	*Lm*_1605	2015	Mozzarella cheese
	*Lm*_1680	2015	"Pasta filata" cheese
	*Lm*_1679	2015	
	*Lm*_1678	2015	
	*Lm*_1676	2015	"Pasta filata" cheese
	*Lm*_1675	2015	
	*Lm*_1674	2015	
	*Lm*_1673	2015	
	*Lm*_1672	2015	
	*Lm*_1671	2015	Environment
	*Lm*_1670	2015	Environment

	*Lm*_1813	2016	Mozzarella cheese
	*Lm*_1811	2016	Environment
	*Lm*_1812	2016	Environment
	*Lm*_1747	2016	"Pasta filata" cheese
	*Lm*_1746	2016	Environment
	*Lm*_1745	2016	Environment
	*Lm*_1744	2016	Environment
	*Lm*_1743	2016	"Pasta filata" cheese
	*Lm*_1741	2016	"Pasta filata" cheese
	*Lm*_1739	2016	

Table S2. Quality control check of sequence data. Reads' quality control metrics reported are after trimming.

ID	Acc. Number	Average read quality score	N° read pairs	Vertical coverage	N° contigs	Total length (bp)	N50	L50
*Lm*_1353	JAEUCJ000000000	34.94	2,776,150	106	55	3,017,736	476,295	3
*Lm*_1614	JAENUT000000000	34.52	2,402,274	92	50	3,016,349	449,477	3
*Lm*_1756	JAEUCI000000000	34.61	2,447,608	103	49	3,018,396	479,972	3
*Lm*_1757	JAENUS000000000	34.79	2,440,666	94	52	3,017,039	396,821	4
*Lm*_1791	JAEUCH000000000	33.60	2,968,674	119	38	3,014,976	479,972	3
*Lm*_1872	JAENUR000000000	34.97	2,835,032	109	45	3,016,048	480,409	3
*Lm*_1873	JAENUQ000000000	34.87	1,957,116	77	65	2,997,322	344,036	4
*Lm*_2211	JAEUCG000000000	34.67	1,427,476	58	114	3,017,786	445,404	4
*Lm*_2216	JAFDUX000000000	34.80	2,147,888	84	147	3,035,554	477,697	3
*Lm*_2228	JAENUP000000000	34.83	2,551,018	95	80	3,019,036	222,045	6
*Lm*_2229	JAEUCF000000000	34.49	2,457,534	112	59	3,019,090	449,371	3
*Lm*_2230	JAEUCE000000000	34.79	3,360,098	134	73	3,023,370	477,699	3
*Lm*_2231	JAEUCD000000000	34.92	2,498,750	93	60	3,017,718	448,988	3
*Lm*_2266	JAENUO000000000	34.87	2,498,894	96	63	3,005,992	477,700	3
*Lm*_2267	JAEUCC000000000	34.52	2,488,638	114	70	3,007,165	527,818	2
*Lm*_2278	JAENUN000000000	34.99	3,774,680	137	41	2,999,601	524,870	2
*Lm*_2279	JAENUM000000000	34.93	2,512,944	96	45	3,000,573	524,621	2
*Lm*_2280	JAENUL000000000	34.79	3,143,864	130	45	3,000,409	524,661	2
*Lm*_2285	JAENUK000000000	34.75	2,486,370	108	47	3,001,532	524,821	3
*Lm*_2268	JAENUJ000000000	34.88	2,068,200	81	47	3,000,872	358,803	3
*Lm*_2269	JAENUI000000000	34.90	2,741,128	106	39	2,879,332	517,194	2
*Lm*_2270	JAENUH000000000	34.95	3,418,932	128	58	3,009,742	509,887	3
*Lm*_2271	JAENUG000000000	34.92	3,219,152	122	39	2,998,570	524,644	2
*Lm*_2272	JAENUF000000000	34.99	3,098,148	112	45	3,005,583	480,080	3
*Lm*_2273	JAEUCB000000000	34.58	2382568	108	51	3,002,223	524,661	2
*Lm*_2282	JAENUE000000000	34.84	2,543,230	97	37	2,997,531	524,674	2
*Lm*_2283	JAEUCA000000000	34.36	2,162,954	99	48	3,001,343	524,621	2
*Lm*_2274	JAEUBZ000000000	34.97	3,075,776	113	61	3,004,736	524,657	2

*Lm*_2275	JAEUBY000000000	34.78	1,879,428	78	55	3,000,008	150,641	6
*Lm*_2276	JAENUD000000000	34.83	2,704,886	105	36	2,998,365	524,797	2
*Lm*_2277	JAEUBX000000000	34.75	2,368,796	99	141	3,025,713	541,650	2
*Lm*_2284	JAENUC000000000	34.79	2,518,230	96	38	2,998,847	543,126	2
*Lm*_1306	JAENUB000000000	32.24	830,972	34	60	2,972,528	301,962	4
*Lm*_1242	JAENUA000000000	34.86	402,616	17	262	2,951,907	21,959	40
*Lm*_1431	JAENTZ000000000	32.70	429,068	16	113	2,963,009	60,224	18
*Lm*_1430	JAENTY000000000	32.41	1,452,132	59	92	2,970,623	115,722	10
*Lm*_1318	JAENTX000000000	32.74	1,772,528	70	37	2,967,337	321,423	4
*Lm*_1311	JAENTW000000000	32.52	1,001,012	39	34	2,964,508	321,341	4
*Lm*_1429	JAENTV000000000	32.70	824,678	32	41	2,962,561	272,532	5
*Lm*_1428	JAENTU000000000	32.33	1,163,634	46	36	2,965,562	321,101	4
*Lm*_1426	JAENTT000000000	32.49	685,136	25	45	2,964,059	244,259	4
*Lm*_1425	JAENTS000000000	32.58	1,403,212	55	226	3,009,267	163,962	7
*Lm*_1424	JAEUBW000000000	32.05	642,580	26	197	3,001,085	206,535	6
*Lm*_1607	JAEUBV000000000	33.07	2,708,136	125	63	2,975,191	546,857	3
*Lm*_1606	JAEUBU000000000	32.86	2,305,234	106	65	2,975,270	546,760	3
*Lm*_1605	JAEUBT000000000	33.15	2,856,586	132	61	2,974,749	546,481	3
*Lm*_1680	JAENTR000000000	32.05	2,220,912	93	54	2,972,192	546,857	3
*Lm*_1679	JAENTQ000000000	32.17	2,279,188	93	45	2,970,054	546,608	3
*Lm*_1678	JAENTP000000000	32.36	2,297,500	91	29	2,965,454	359,489	3
*Lm*_1676	JAENTO000000000	32.39	1,242,190	49	23	2,963,873	546,318	3
*Lm*_1675	JAENTN000000000	32.35	1,632,908	65	30	2,965,523	321,341	4
*Lm*_1674	JAENTM000000000	32.63	1,346,274	52	33	2,965,382	321,337	4
*Lm*_1673	JAENTL000000000	32.84	525,594	20	80	2,966,700	122,660	9
*Lm*_1672	JAENTK000000000	34.89	394,394	16	337	2,955,269	19,796	47
*Lm*_1671	JAENTJ000000000	34.55	355,510	16	216	2,957,165	35,574	30
*Lm*_1670	JAEUBS000000000	34.81	3,481,330	151	175	3,002,777	371,328	3
*Lm*_1813	JAENTI000000000	33.20	506,418	18	161	2,960,422	35,397	23
*Lm*_1811	JAEUBR000000000	32.91	458,900	17	147	2,961,707	44,986	21
*Lm*_1812	JAENTH000000000	32.75	631,548	24	48	2,962,223	184,282	7
*Lm*_1747	JAENTG000000000	35.08	1,346,064	52	122	2,982,344	181,143	6
*Lm*_1746	JAEUBQ000000000	34.65	639,028	30	119	2,971,472	95,525	11
*Lm*_1745	JAEUBP000000000	34.74	4,945,108	229	497	3,095,373	546,962	3
*Lm*_1744	JAEUBO000000000	34.86	1,243,342	53	78	2,975,505	220,395	6
*Lm*_1743	JAEUBN000000000	35.03	1,412,468	56	144	2,991,686	218,541	5
*Lm*_1741	JAEUBM000000000	34.84	1,549,700	67	95	2,977,157	145,478	7
*Lm*_1739	JAEUBL000000000	35.05	633,276	25	151	2,970,646	76,892	12

Original Research Paper II

This work consists of a retrospective WGS-based study conducted on *Lm* strains isolated during an intensive environmental monitoring plan involving 86 FPEs put in place by the Competent Authorities following a severe listeriosis outbreak occurred in Marche region (Central Italy) between 2015 and 2016 and caused by a CC7 cluster of infection associated with a pork-meat product. The sampling plan was performed both at FPP and retail grocery stores (RS) level and longitudinal follow-up sampling was conducted where positive samples were found after cleaning and sanitation to verify their effectiveness and to give evidence of the elimination of the contamination by *Lm*.

The main objectives of the study were to: (i) provide a snapshot on *Lm* circulation in different FPPs and RSs, (ii) use WGS data to study the genetic diversity of the *Lm* isolates, identifying the most frequent and widespread clones and their virulence and stress resistance profiles, (iii) evaluate the genetic relationships between the isolates, identifying strains detected in more than one FBO or persisting despite sanitation.

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Article

Intensive Environmental Surveillance Plan for *Listeria monocytogenes* in Food Producing Plants and Retail Stores of Central Italy: Prevalence and Genetic Diversity

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Abstract: *Listeria monocytogenes* (*Lm*) can persist in food processing environments (FPEs), surviving environmental stresses and disinfectants. We described an intensive environmental monitoring plan performed in Central Italy and involving food producing plants (FPPs) and retail grocery stores (RSs). The aim of the study was to provide a snapshot of the *Lm* circulation in different FPEs during a severe listeriosis outbreak, using whole genome sequencing (WGS) to investigate the genetic diversity of the *Lm* isolated, evaluating their virulence and stress resistance profiles. A total of 1217 samples were collected in 86 FPEs with 12.0% of positive surfaces at FPPs level and 7.5% at RSs level; 133 *Lm* isolates were typed by multilocus sequencing typing (MLST) and core genome MLST (cgMLST). Clonal complex (CC) 121 (25.6%), CC9 (22.6%), CC1 (11.3%), CC3 (10.5%), CC191 (4.5%), CC7 (4.5%) and CC31 (3.8%) were the most frequent MLST clones. Among the 26 cgMLST clusters obtained, 5 of them persisted after sanitization and were re-isolated during the follow-up sampling. All the CC121 harboured the *Tn6188_qac* gene for tolerance to benzalkonium chloride and the stress survival islet SSI-2. The CC3, CC7, CC9, CC31 and CC191 carried the SSI-1. All the CC9 and CC121 strains presented a premature stop codon in the *inlA* gene. In addition to the *Lm* Pathogenicity Island 1 (LIPI-1), CC1, CC3 and CC191 harboured the LIPI-3. The application of intensive environmental sampling plans for the detection and WGS analysis of *Lm* isolates could improve surveillance and early detection of outbreaks.

Keywords: foodborne pathogen; food processing environments; monitoring plan; WGS typing; environmental stress resistance; QAC-resistance; persistence; virulence

1. Introduction

Listeria monocytogenes (*Lm*) is a major foodborne pathogen causing human listeriosis, a severe zoonoses with high mortality. Invasive forms of the disease are particularly dangerous for the elderly, immuno-compromised people, newborns and pregnant women, leading to sepsis, meningitis, encephalitis, abortion and stillbirth [1]. Although listeriosis is

relatively rare if compared with other foodborne disease, hospitalization (between 90% and 97%, for invasive forms) and fatality rates (approximately 20% to 30%, despite effective antibiotic treatment) make it a significant public health concern [2,3].

Lm is widespread in the natural environment, animals and food. Ready-to-eat products (RTE) (Table S1), such as salads or deli meat, are of special concern due to the lack of a heating step prior to consumption. Due to its ubiquity, the probability of introducing *Lm* into food producing plants (FPP), either with raw materials, through equipment or via employees is very high [4]. Therefore, it can be assumed that no FPP is *Lm* free [5]. Moreover, once introduced into a food processing environment (FPE), several factors increase the probability of a strain to establish long-lasting colonization of niches (harbourage sites) and to persist. Among them, there are the abilities of *Lm* to survive and grow under a wide range of environmental conditions (low pH, high salt concentration, alkaline and oxidative stress and refrigeration temperatures), to form biofilm and to show tolerance to common disinfectants used in the FPEs [4,6].

The European Commission Regulation 2073/2005 (Article 5) states that food business operators (FBO) manufacturing RTE, which may pose a *Lm* risk for public health, shall sample the processing areas and equipment for *Lm* as part of their Hazard Analysis and Critical Control Points plans. Such sampling activity aims at detecting *Lm*, including persistent strains, and at implementing corrective actions to eliminate the contamination. In Italy, except for the specific monitoring plan involving FPPs that are authorized to the export of meat products and other food of animal origin to the USA (DGISAN 42841-25 June 2019), a systematic and extensive monitoring of food surfaces is not included in the food safety surveillance programs performed by the FBO and competent authorities.

Advanced molecular typing methods enable source attribution and investigation of *Lm* strains introduction and persistence in FPEs [7,8]. In particular, whole genome sequencing (WGS) allows an unprecedented subtyping resolution and is now considered the best typing tool in routine epidemiological surveillance of contamination. This method improves the detection of outbreaks, the understanding of distribution of virulent *Lm* strains in food and enables source attribution [9].

There is ample evidence of high variability regarding the virulence potential and pathogenicity of *Lm* isolates. Epidemiological data combined with results of WGS and from animal models, indicated that different levels of virulence may be associated with different clonal complexes (CCs). Maury et al. (2016) [10] distinguished CCs in 'infection-associated', 'food-associated' or 'intermediate' depending on the relative proportion of isolates from clinical cases, food or both. Clones CC1, CC2, CC4 and CC6 were strongly associated with human infection, whereas CC121 and CC9 were frequently isolated from food. Moreover, using a humanised mouse model, the authors observed that infection-associated clones were hyper-virulent, while the food-associated CCs were less invasive and hypo-virulent [10]. However, despite the observed variability in their virulence potential, almost every *Lm* strain has the ability to result in human listeriosis because of the complex interaction between the pathogen, food and the host [11].

Following a severe listeriosis outbreak occurred in Central Italy between 2015 and 2016 [12], with 24 confirmed clinical cases and associated with a pork-meat product, the Regional Competent Authority put in place an intensive environmental monitoring plan involving 86 FPEs in the affected area. The sampling plan was here described and was performed both at FPP and retail grocery stores (RS) level. Longitudinal follow-up sampling was conducted where positive samples were found, after cleaning and sanitation to verify their effectiveness and to give evidence of the elimination of the contamination by *Lm*.

The main objectives of the study were to: (i) provide a snapshot on *Lm* circulation in different FPP and RS, (ii) use WGS data to study the genetic diversity of the *Lm* isolates, identifying the most frequent and widespread clones and their virulence and stress resistance profiles, (iii) evaluate the genetic relationships between the isolates, identifying strains detected in more than one FBO or persisting despite sanitation.

2. Materials and Methods

2.1. Sampling and L. monocytogenes Detection

From January to August 2016, 1217 FPE surfaces were sampled from 41 FPP producing RTE and 45 RS located in Marche region, the samples were tested according to ISO 11290-1:1996/Adm1:2004 for *Lm* detection. In each FPE, a first sampling session was performed during processing and usually included 10 food-contact surfaces (FCS), as working tables, slicers, cutters, mixing and stuffing machines, containers, utensils, gloves, and 5 non-food-contact surfaces (NFCS), as floors, drains, sinks, walls, equipment framework, table legs, doors, boots, cleaning tools. If positive samples were found, the surfaces were tested again after extraordinary cleaning and sanitation (follow-up sampling). In accordance with the European Union Reference Laboratory for *Lm* (EURL) guidelines [13], the total sampled area varied depending on the sampling site, but was as large as possible to improve the probability of detecting *Lm*.

2.2. Statistical Analysis

All comparisons were made using a Bayesian approach with beta distribution by calculating 95% confidence intervals (CI 95%) (BETAINV function, Microsoft Excel, Redmond, WA, USA) for the percentages of positive sample. Differences between percentages were considered statistically significant when their CI 95% did not overlap.

2.3. Strains Collection

Up to five *Lm* colonies from one sample were randomly selected and screened for their belonging to one of the five major serogroups (IIa, IIb, IIc, IVa and IVb), using a multiplex PCR assay according to the EURL method [14,15]. One isolate for each serogroup found in each sample was submitted to WGS.

2.4. Whole Genome Sequencing and Bioinformatics Analysis

DNA extraction was performed using the Maxwell 16 tissue DNA purification kit (Promega Italia Srl, Milan, Italy) according to the manufacturer's protocol and the purity of the extracts was evaluated by NanoDrop2000 (ThermoFisher Scientific, Wältham, MA, USA). Starting from 1 ng of input DNA, the Nextera XT DNA chemistry (Illumina, San Diego, CA, USA) for library preparation was used according to the manufacturer's protocols. WGS was performed on the NextSeq 500 platform (Illumina, San Diego, CA, USA) with the NextSeq 500/550 mid output reagent cartridge v2 (300 cycles, standard 150-bp paired-end reads).

For the analysis of WGS data, an in-house pipeline [16] was used which included steps for trimming (Trimmomatic v0.36) (base quality parameters—Leading: 25; Trailing: 25; Slidingwindow: 20:25) [17] and quality control check of the reads (FastQC v0.11.5). Genome de novo assembly of paired-end reads was performed using SPAdes v3.11.1 [18] with the parameters suggested by the manual for the Illumina platform 2 × 150 chemistry (–only-assembler –careful -k 21, 33, 55, 77). Then, the genome assembly quality check was performed with QUAST v.4.3 [19].

The genome assemblies were deposited at DDBJ/ENA/GenBank under the BioProject PRJNA737760.

The MLST scheme used to characterize *Lm* strains is based on the sequence analysis of seven housekeeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh and lhlA*) [20].

The seven-gene of MLST scheme and the clonal complexes (CCs) were deducted in silico using the BIGSdb-*Lm* database (http://bigsdb.pasteur.fr/listeria; accessed on 14 April 2021).

For the cluster analysis of the strains, the core genome MLST (cgMLST), according to the Institut Pasteur's scheme of 1748 target loci, was performed using the chewBBACA allele calling algorithm [21], available in the in-house pipeline. Agreeing to the guidelines for *Lm* cgMLST typing [22], only the genomes with at least 1660 called loci (95% of the full scheme) were considered. Using the software GrapeTree [23] a Minimum Spanning

tree (MSTreeV2 method), showing the relationships among the strains in terms of allelic mismatches was generated.

The genomes of the strains belonging to the most frequently isolated CCs, for which at least five isolates were detected, were further characterized using "*Metal and detergent resistance genes*", "*Stress Islands*" and "*Virulence*" tools of the BIGSdb-*Lm* database (accessed on 18 May 2021). The gene presence/absence matrices according to their MSTree were visualized using Phandango v 1.3.0 [24] (accessed on 30 July 2021).

3. Results

3.1. Sampling and L. monocytogenes Detection

A total of 1217 samples were collected in the first sampling session. Forty-six out of the 86 different establishments showed at least one positive sample and were considered contaminated by *Lm*. The percentage of positive facilities was 60.9 % (CI 95%: 45.6–74.4%) at FPP and 46.7% (CI 95%: 32.9–61.0%) at RS.

A total of 118 samples (9.7%; CI 95%: 8.2%–11.5%) were positive for *Lm*, of which there were 72 (12.0%; CI 95%: 9.6–14.8%) at FPP and 46 (7.5%; CI 95%: 5.7–9.8%) at RS. No statistically significant difference was found in the amount of *Lm* positive samples between these two types of FBO. At single establishment level, the percentage of positive samples ranged between 7% and 80% at FPP and from 7% to 40% at RS.

Overall, *Lm* was detected in 76 (9.6%; CI 95%: 7.8–11.9%) FCS and 42 (9.8%; CI 95%: 7.3–13.0%) NFCS. The difference between positive FCS at FPP (12.9%; CI 95 %: 9.9–16.7%) and at RS (6.6%; CI 95%: 4.6–9.4%) was found to be significant (Table 1).

Table 1. Results of tested surfaces based on the food environment typology.

Surfaces	FCS				NFCS			
Food Producing Environments	***n***	***Lm+***	**%**	**CI 95%**	***n***	***Lm+***	**%**	**CI 95%**
FPP	379	49	12.9	9.9–16.7	222	23	10.4	7.0–15.1
RS	409	27	6.6	4.6–9.4	207	19	9.2	6.0–13.9
Total	788	76	9.6	7.8–11.9	429	42	9.8	7.3–13.0

FPP: food processing plant; RS: retail store; FCS: food contact surfaces; NFCS: non-food contact surfaces; *Lm+*: *Listeria monocytogenes* positive samples.

In order to identify differences in prevalence, both FCS and NFCS were grouped into five categories after sampling: equipment (e.g., working tables, containers, hooking bars, cutting boards), industrial systems (e.g., floors, walls, drains, sinks, door handles, freezers), machines (e.g., slicers, blenders, mincers, hoppers, labellers), clothing (e.g., shoes and gloves) and tools (e.g., knives, ladles and tongs) (Table S2).

In Table 2 the results of FCS and NFCS are showed according to the category they belonged to.

Table 2. Results of the tested FPEs based on the category.

	FCS				NFCS			
Surface Category	***n***	***Lm+***	**%**	**CI 95%**	***n***	***Lm+***	**%**	**CI 95%**
Equipment	346	39	11.3	8.4–15.0	83	6	7.2	3.4–14.9
Industrial systems	64	5	7.8	3.4–17.0	267	29	10.9	7.7–15.2
Machines	259	20	7.7	5.0–11.6	64	4	6.3	2.5–15.0
Clothing	5	1	20.0	3.6–62.5	9	3	33.3	12.1–64.6
Cleaning tools	0	0	0	0	2	0	0	0
Tools	114	11	9.6	5.5–16.5	3	0	0	0
Not classifiable surface	0	0	0	0	1	0	0	0
Total	788	76	9.6	7.8–11.9	429	42	9.8	7.3–12.9

FCS: food contact surfaces; NFCS: non-food contact surfaces; *Lm+*: *Listeria monocytogenes* positive samples.

Not including the clothing category, for which the samples number was small when compared with the others, the FCS of the equipment appeared the most contaminated. The NFCS presented the highest number of positive samples in the industrial system category (Table 2). These differences were not statistically significant.

At RS, the percentage of positive surfaces belonging to industrial systems, machines and tools was lower than what was observed in FPP (Table 3).

Table 3. Results of the samples tested reported based on the FPP or RS and category of surfaces (n = 1214 *).

	FPP				RS			
Surface Category	***n***	***Lm+***	**%**	**CI 95%**	***n***	***Lm+***	**%**	**CI 95%**
Equipment	221	24	10.9	7.4–15.7	208	21	10.1	6.7–14.9
Industrial systems	156	22	14.1	9.5–20.4	175	12	6.9	4.0–11.6
Machines	162	16	9.9	6.2–15.4	161	8	5.0	2.5–9.5
Clothing	7	1	14.2	2.6–51.3	7	3	42.9	15.8–75.0
Tools	52	9	17.3	9.4–29.7	65	2	3.1	0.9–10.5
Total	598	72	12.0	9.7–14.9	616	46	7.5	5.7–9.8

* The table does not consider the three samples: the two negative cleaning tools and the not classifiable surface. FPP: food processing plant; RS: retail store; *Lm+*: *Listeria monocytogenes* positive samples.

A follow-up sampling session was carried out in 33 out of the 46 FPP that tested positive, after cleaning and sanitation. Positive surfaces were still found in 2 FPP and 2 RS. They were re-sampled and re-analysed until they resulted as negative for the presence of *Lm*. During these follow-up activities, a total of 279 samples were collected and 11 of them tested positive.

3.2. Strains Collection

A total of 133 *Lm* strains, 121 isolated within the first sampling session and 12 during the follow-up activities, were selected and collected to be typed (Tables S3 and S4). Eighty isolates were from FPP and 53 from RS. In Table S5, all the *Lm* strains of the study were reported. Among all the *Lm* strains analysed, four serogroups (IIa, IIb, IIc, and IVb) were identified. The main was the serogroup IIa revealed for 55 strains (28 RSs, 27 FPPs), followed by IIc for 30 strains (26 FPPs, 4 RSs), IIb for 27 strains (20 FPPs, 7 RSs) and finally, serogroup IVb revealed for 21 strains (15 FRs, 6 FPPs). In 11 plants, both from FPP and RS, at least two different serogroups were detected.

3.3. WGS and Bioinformatics Analysis

For all the 133 genomes, sequence data were obtained in agreement with the quality control thresholds recommended. Quality metrics of sequence data obtained for each genome are reported in Table S5.

3.3.1. Distribution of CCs and cg-MLST Clusters

MLST analysis grouped the strains in 19 CCs (Table S3, Figure 1). More in detail, the *Lm* strains belonged to the following CCs: CC121 (25.6%), CC9 (22.6%), CC1 (11.3%), CC3 (10.5%), CC191 (4.5%), CC7 (4.5%), CC31 (3.8%), CC2 (3.0%), CC517 (2.3%), CC8 (2.3%), CC14 (2.3%), CC363 (1.5%), CC6 (1.5%), CC155 (0.8%), CC224 (0.8%), CC429 (0.8%), CC475 (0.8%), CC101 (0.8%). In one strain, isolated in the retail plant RS9, exact allele matches were found only for five of the seven genes of the MLST scheme. The genome of this strain was submitted to the BIGSdb-*Lm* database to be typed and its MLST profile resulted new in the database (submitted on 13 August 2021). New alleles and profiles were defined with the assignation of CC2764 (Figure 1 and Table S3).

Among the CCs isolated in both the FBO types, CC121, CC9 and CC3 presented most of the isolates from FPP, while CC1, CC7 and CC14 from RS. *Lm* strains belonging to CC191, CC17, CC6 and CC429 were found only in FPP, while CC31, CC2, CC8, CC36, CC101, CC155, CC224 and CC475 were exclusively isolated in RS.

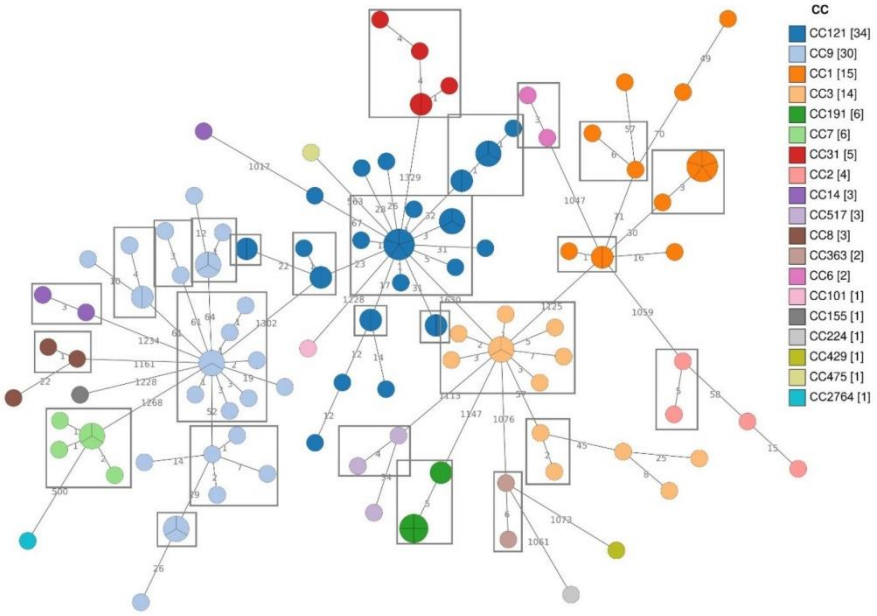

Figure 1. Minimum Spanning Tree (MST) based on the cgMLST profiles of 133 *Lm* strains, coloured according to CCs.

The cgMLST cluster analysis was performed to deepen the relationships among the *Lm* isolates (Figure 1). According to the cgMLST allelic threshold (≤7) for cluster definition [22], 26 cgMLST clusters were identified among all the isolates. Strains belonging to CC121, CC9, CC1 and CC3 grouped into more than one cluster (Table 4).

Table 4. cgMLST analysis: number of isolates within cgMLST clusters observed in each clonal complex (CCs) presenting more than one cluster.

CC	cgMLST Clusters	No. of Isolates	No. of Singleton Strains
CC121	6	12	7
		6	
		3	
		2	
		2	
		2	
CC9	6	9	5
		4	
		4	
		3	
		3	
		2	
CC1	3	6	4
		3	
		2	
CC3	2	9	3
		2	

All the CC2, CC6, CC7, CC8, CC14, CC31, CC191 and CC363 strains presented a single cgMLST cluster with CC2, CC8 and CC14 also including some singletons. The remaining CCs presented only singleton strains (Figure 1).

Several CCs and cgMLST clusters were isolated from different sampling points, both in FPP and RS (Table S3). Five cgMLST clusters belonging to CC7 (1), CC9 (2) and CC121 (2), were detected at different time points, both during the first sampling and the follow up control, in the same FPE (Figures S1–S5). The FPEs in which a specific cgMLST cluster was re-isolated after sanitation were 4: two FPPs and two RSs.

More in detail, CC121 isolates (Figure S1) were from six FPPs and six RSs. For this CC, none of the cgMLST clusters found was shared by more than one FPE (Figure S1).

Lm strains belonging to CC9 were isolated in 10 FPPs and 2 RSs. Two CC9 cgMLST clusters were detected in more than one FBO (Figure S2).

CC1 strains were isolated in five FPPs and four RSs. (Figure S3).

All CC3 strains were isolated during the first sampling session from FPP2, FPP22, FPP25, RS4, RS14 and RS17 (Table S3). Strains belonging to the same cgMLST cluster were detected in RS4, FPP2 and RS17 (Figure S4).

The CC191 clone was represented by six strains, all isolated from FPP1 and belonging to the same cgMLST cluster.

All the CC7 strains grouped in the same cluster isolated in RS10, RS13, RS14 and FPP4 (Tables S3 and S4, Figure S5).

CC31 was exclusively isolated from RSs and consisted of five strains, isolated in five different RSs (RS12, RS18, RS19, RS20 and RS21) (Table S3) and belonging to the same cgMLST cluster.

Strains belonging to CC2 were collected from RS5, RS8 and RS13 (Table S3). Only two of them, both isolated from RS8 during the first sampling session, belonged to the same cluster.

Three CC8 were isolated from RS4 and RS7 during the first sampling session (Table S3). Allelic differences $\leq$7 were found only in two strains belonging to this CC, both isolated in the RS4 exercise.

Three CC14 *Lm* were found in this study and they were collected from FPP18 and RS6 (Tables S3 and S4). Both the strains from the RS6 isolated during the first and the follow-up sampling session, respectively, belonged to the same cluster.

For the remaining CCs, only one strain was isolated during the study.

3.3.2. Detection of Stress Resistance and Virulence Genes

CC1, CC3, CC7, CC9, CC31, CC121 and CC191 were considered the most frequently detected CC as for each of them at least five *Lm* were isolated. Strains belonging to these CCs, 110 in all, were further characterized.

The in silico results on presence/absence of disinfectants resistance genes, SSIs and virulence genes (Figure 2) showed that all the CC121 strains harboured the *Tn6188*_qac for tolerance to benzalkonium chloride (BC). This gene was also detected in 10 CC1 (66.7%) and in three CC9 (10.0%) strains.

All the strains belonging to CC9 CC3, CC191, CC7 and CC31 carried out the five genes of SSI-1 (*lmo*0444, *lmo*0445, *lmo*0446, *lmo*0447 and *lmo*0448), while in CC121 and CC1, only *lmo*0447 gene was found. Moreover, the two genes of SSI-2 (*lin*0464 and *lin*0465) were only detected in CC121 strains.

Regarding the virulence genes, all CC1, CC3, CC191, CC7 and CC31 strains carried a full length *inl*A for Internalin A and *inl*B for Internalin B. A Premature Stop Codon Mutation (PMSC) in the *inl*A gene was detected in all the CC121 and the CC9 strains.

All the studied CCs presented *prf*A, *plc*A, *hly*, *mpl*, *act*A and *plc*B, forming together the *Lm* Pathogenicity Island 1 (LIPI-1). Moreover, CC1, CC3 and CC191 strains also harboured a complete LIPI-3 composed of the eight *lls* genes (*lls*A, *lls*G, *lls*H, *lls*X, *lls*B, *lls*Y, *lls*D, *lls*P).

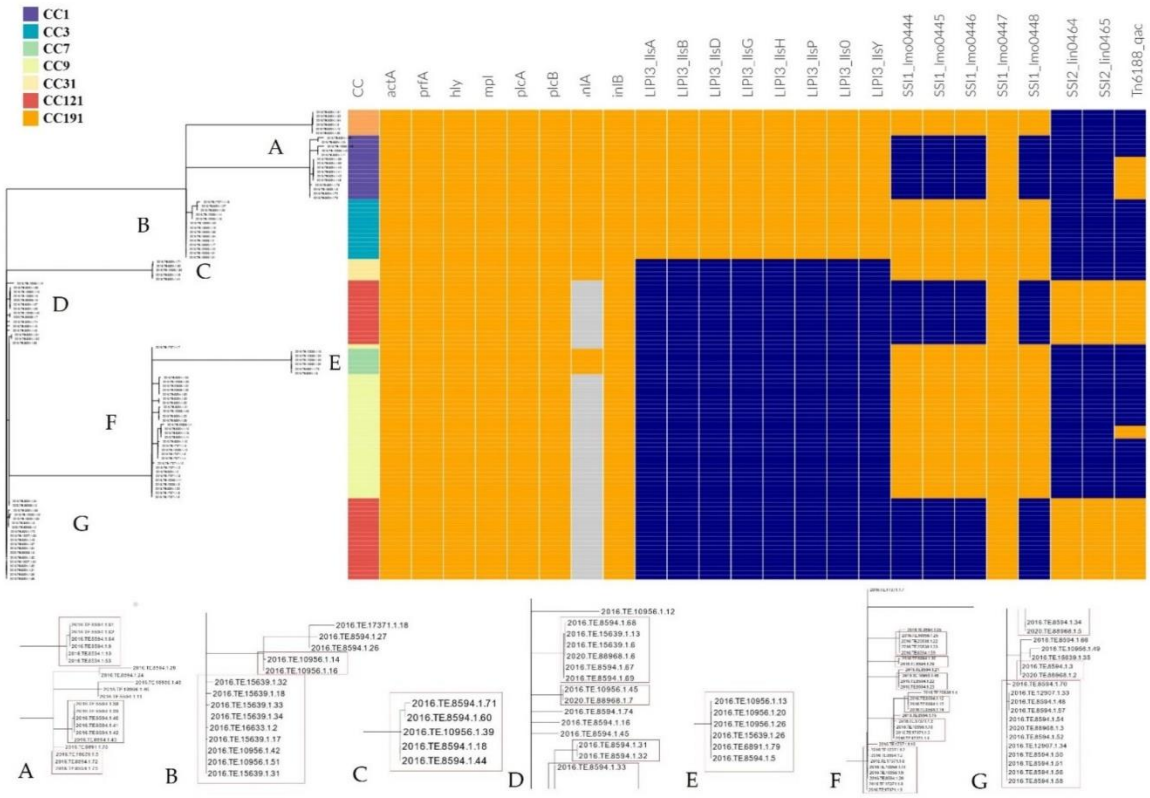

Figure 2. Stress resistance and virulence patterns according to CCs and cgMLST clustering. Orange: presence of the gene; light blue: presence of premature stop codon; blue: absence of the gene. Enlarged detail images of different nodes are shown at the bottom of the figure and are indicated with the letters from (**A**–**G**). The red boxes enclose the isolates belonging to the same cgMLST cluster. The incomplete cluster of image D is completed by the one in the image (**G**).

4. Discussion

This retrospective study reported the results of an intensive environmental monitoring plan carried out during the investigation tracing steps of a severe listeriosis outbreak that occurred in Central Italy between 2015 and 2016 [12]. The results provided information about the environmental contamination of *Lm* circulating in pork-meat FPP and RS of Marche Region.

4.1. Sampling and L. monocytogenes Detection

The importance of the study was to give evidence of the distribution and diversity of *Lm* strains, as in previously studies carried out on FPP and in RS [25–28]. We collected surfaces samples, including both FCS and NFCS, from 41 ready-to-eat pork-meat FPPs and 45 RSs of the studied area and tested them for *Lm* detection.

We found that 60.9% of FPPs and 46.7% of RSs were contaminated by *Lm*. A total of 72 (12%) samples were positive for *Lm* at FPP and 46 (7.5%) at RS levels. Results showed no significant differences for FPP and RS, surfaces were widely positive for *Lm* and possible sources of food contamination.

Antoci et al. (2021) reported in their study that 50% of the FPPs were contaminated by *Lm*, with at least one sample positive for the pathogen [29]. They also reported that 9.8% of FCS and 6.1% of NFCS were contaminated by *Lm*. We found a higher percentage of contaminated FPPs, all located in the same Region of Italy. Even the percentage of positive

FCS found at the FPPs was higher in our study (12.9%) and it was probably due to the high levels of *Lm* contamination existing in raw pork [30,31].

Antoci et al. (2021), in fact, included in their monitoring plan also FPPs for meat, fishery and dairy products, while in the present study all the FPPs were in the pork chain [29]. All these findings suggested a massive spread of *Lm* in the pork production chain of Marche Region, emphasizing the need for more assiduous monitoring and more effective risk containment measures to prevent food contamination.

As reported by Forauer et al. (2021), many studies in this field were carried out in the U.S.A [32]. Hoelzer et al. (2011) and Sauders et al. (2009), in their non-longitudinal deli-focused studies conducted in RS of the New York State, reported that approximately 60% of them were positive for *Lm* [26,33]. Etter et al. (2017) [34], in a recent longitudinal study in 30 U.S.A. delis, found that about 97% of them tested positive for *Lm* at least once, while Burnett et al. (2020) [27] monitored 30 grocery stores across seven U.S.A states, finding that *Lm* was isolated at least once from 83% of them. All these results showed higher percentages of RSs contaminated by *Lm* than the level reported in our study, probably linked to the larger geographical area involved in the U.S.A studies.

The obtained results showed how FCS and NFCS could equally harbour *Lm*, representing sources of food contamination or possible persistence niches. These findings emphasized the importance of sanitation procedures including effective strategies to clean and sanitize NFCS. More in detail 12.9% FCS and 10.4% NFCS tested positive at FPP, while 6.6% FCS and 9.2% NFCS at RS.

The difference between FCS and NFCS was not significant, even within each type of FPE tested. This result was not in line with previous studies reporting that *Lm* prevalence was significantly lower on FCS than on NFCS and indicated a widespread of *Lm* in the studied FPEs [26,34,35].

The number of positive FCS at FPPs level was significantly higher than at RS level. This result could be explained with the large amount of raw material handled in FPPs also considering that high levels of *Lm* contamination in raw pork have been regularly reported [30,31].

All the surfaces' categories, including both FCS and NFCS sampled (equipment, industrial systems, machines, etc.), reported positive results for *Lm* indicating the need to include them all in the monitoring plans. The lack of statistical significance resulting from comparisons between these categories (FCS vs. NFCS; FPP vs. RS) was in part due to the different sample size as we grouped surfaces only after sampling to further investigate the prevalence of *Lm* contamination.

During the follow-up sampling session in two FPPs and two RSs, FCS previously resulted positive for *Lm*, were found contaminated again despite sanitation. The persistence of *Lm* contamination on these surfaces could be explained by the ineffectiveness of cleaning and sanitation procedures used, the incorrect application or specific stress-resistance abilities of the contaminating *Lm* strains.

A limitation of this work was that, being a retrospective study performed years after the emergency when the monitoring plan was finished, it cannot give information about sources of *Lm* contamination and transmission routes.

4.2. Distribution of CCs and cg-MLST Clusters and Their Virulence and Stress Resistance Profiles

Lm serogrouping is considered a first typing step useful to evaluate the microbial population diversity. The serogroup IIa was reported as the most frequently isolated [36,37], in agreement with our results (Table S3) and other studies conducted in meat products and environmental surfaces [35,38–40].

To have more insight regarding the spread of *Lm* in different FPE, WGS was performed to analyse the diversity of the *Lm* strains detected during the study, identifying genetic relationships between the strains and detecting virulence and stress resistance associated determinants. The MLST and the cgMLST analysis showed a great heterogeneity of the *Lm* population circulating in the studied area, identifying 19 different CCs and 26 cgMLST

clusters. The MLST clones most frequently isolated in this study (Figure 1; Table S3), were already defined as the most frequent clones in many countries [39,41,42].

CC1, CC3, CC9 and CC121 presented the greatest genetic diversity, in terms of cgMLST clusters.

With the exception of CC31 and CC191, isolated only at RS and FPP, respectively, all the CCs were found in both type of FPE. In some cases, it was just the same cgMLST cluster to be isolated in different FPP and RS FPEs.

According to previous studies, the CC9 and CC121 were considered hypo-virulent clones, able to cause disease in highly immune-compromised individuals and seeming to be better adapted to FPE and presenting strong association with the meat processing environment [6,39,41]. Several authors also reported that CC9 and CC121 presented a higher prevalence of stress resistance and BC tolerance genes, a higher survival and biofilm formation ability and were able to persist in FPE even for years [6,41]. Moreover, in a recently published study, Guidi et al. (2021) reported two different CC9 clusters persisting, for four and two years, respectively, in a pork-meat processing plant of the same studied area of central Italy [6]. All these findings were consistent with our results and in particular with the isolation and persistence of these CCs after sanitation. Indeed, most of the strains isolated both during the first sampling and the follow-up in the same FPE belonged to CC121 and CC9, harbouring the *Tn6188*_qac transposon for tolerance to BC, a quaternary ammonium compound widely used in food industry. The SSIs are known to confer resistance to stresses, in particular the SSI-1, linked to environmental stress, such as low pH, high osmolarity, bile and nisin, and the SSI-2, linked to tolerance to alkaline and oxidative stresses. According to our results, SSI-1 were frequently observed in *Lm* strains belonging to different clones, whereas the SSI-2 genes were mainly found in CC121 isolates [43], suggesting a possible contribution to strains adaptation and persistence in FPPs [44].

In previous studies the CC1 clone was isolated in the pork-meat production and in other production sectors although it was more abundant and strongly associated with milk and the dairy sector [45]. Moreover, CC1 was previously defined hyper-virulent with a high clinical frequency [10,41]. For this reason, its spread in the FPE of the studied area should be taken into consideration, for the risk of cross-contamination between surfaces and food.

The clone CC3 was one of the most prevalent in cooked products according to Wang et al. (2018) [46], while a recent study reported it was over-represented in the RTE of poultry origin and in meat FPPs [47].

CC7 isolates were previously globally recovered (North and South America, Europe, Oceania, Africa and Asia) from a variety of sources, such as wild animals, ruminants, poultry, silage, fish, slaughterhouse floors, compost and human infections [48]. A high prevalence of CC7 at the dairy farm level in the USA was also reported [49]. Among the CC7 strains isolated during this monitoring plan, four belonged to the same genetic cluster (analysis results not shown) causing the severe invasive listeriosis outbreak reported by Duranti et al. (2018) and occurred between 2015 and 2016 in the studied area [12]. This cluster, never detected before in any of the studied FPEs, were recovered in one FPP and two different RS, showing how the *Lm* outbreak strain was widely circulating in the FPE of central Italy. Moreover, this clone re-emerged in the same area during 2018, when it was isolated from a child affected by listeriosis, presenting only 13 single-nucleotide polymorphisms (SNPs) of difference from the original outbreak strain [50]. All these findings emphasized the need for continuous monitoring in order to avoid the recurrence of new listeriosis outbreaks.

The CC191 clone was poorly reported in the literature. Recently, Kurpas et al. (2020) included in their study a CC191 strain isolated from a slaughterhouse [51].

Maury et al. (2016) [10] observed a strong association of CC31 with meat and meat products, as reported also by the European Food Safety Authority [7]. The spread of CC31 in FPE of FPP of the meat chain, in agreement with our results, was also confirmed by a

recent published study, reporting CC31 isolates from environmental samples collected in meat FPP and farms [47].

The other CCs presenting less than five isolates were mostly linked to the RS and included both hypo- and hyper-virulent clones. Among them, CC2 was previously defined as a hyper-virulent infection-associated clone as the CC1, described above, and the CC6. Maury et al. (2019) evaluating the CC proportion in different food categories, found CC2 isolates from different food groups without a strong association with anyone in particular [41]. Recently, Guidi et al. (2021) reported CC2 strains persisting over four years in a dairy facility both at food and environmental levels [6].

According to several authors, the other remaining CCs detected in this study were previously isolated from different sources, such as food and environments [10,45,46,48,52–57].

Within each FPE we found different degree of diversity both at CCs and cgMLST clusters level. More in detail within the FPPs studied, a maximum of two different CCs and three cgMLST clusters were detected, while at the RS level, up to five different CCs were isolated in the same FPE, although a less strain variability within each CC was observed. The greater genetic diversity observed at RS level was most likely related to the high variability of food products and food categories, from different suppliers, handled at RS level.

Only five cgMLST clusters were detected both at the first sampling and at the follow up control in four different FPEs (Figures S1–S5). Four of five clusters, two belonging to CC121, one to CC7 and one to CC9, included strains with 0–1 allelic differences, therefore their re-isolation after cleaning and sanitation could be considered as due to persistence of the same strain. The last cgMLST cluster (the second cluster of CC9), instead, was composed by two isolates with five alleles difference, in this case the hypothesis of a reintroduction could not be excluded.

Very interestingly, the CC7 and the two CC9 cgMLST recurrent clusters detected after sanitation in the same FPE (RS13) were not carriers of the Tn6188_*qacH* gene, specific for BC resistance. The lack of specific determinants for tolerance to sanitizers and the re-isolation of three different cgMLST types after sanitation in the same FPE, suggested that cleaning and sanitation protocols used were ineffective. In contrast, all the CC121 strains grouping in the same cgMLST clusters were carriers of the Tn6188_*qacH* gene and their isolation at different time points could be due to their resistance to sanitation. Although we do not have detailed information regarding the specific disinfectants used in each FPE, it is known that QAC and specifically BC, are the most commonly used in the food industry.

From all these findings several recommendations were provided to FBOs in order to remove or reduce resident *Lm*, such as the use of different sanitizers combining or turning them and the application of procedures needed to clean and disinfect niches or harbourage points.

Virulence factor analysis was performed on seven CCs (CC1, CC3, CC7, CC9, CC31, CC121 and CC191) presenting at least five isolates and considered widespread in the studied area (Figure 2).

As previously reported, among the major virulence factors crucial for the intracellular lifestyle of *Lm* there is the LIPI-1, highly conserved among *Lm* strains and containing the *prfA*, *plcA*, *hly*, *mpl*, *ActA* and *plcB* genes [36,58]. As expected, LIPI-1 was detected in all the strains.

All CC1, CC3, CC7 and CC191 isolates carried a full-length *inl*A and *inl*B, considered one of the most influent factors on the *Lm* invasiveness [58]. Internalins are surface proteins used by *Lm* to invade and cross the human intestinal barriers invading epithelial cells during the infection process and among them Internalin A (InlA) and B (InlB) are considered the most relevant [58–60]. These findings confirmed CC1 as hyper-virulent clone, as reported before [10,41] and suggested the same for CC3, CC191 and also for CC7, to which belonged the outbreak strain described by Duranti et al. (2018) [12]. On the contrary, a PMSC in the *inl*A gene, mainly detected in all the CC121 and the CC9 strains, confirmed these clones as hypo-virulent.

Moreover, according to previous reports [36], all the CC1, CC3 and CC191 also harboured a complete LIPI-3, encoding a biosynthetic cluster involved in the production of Listeriolysin S (LLS) [61]. LLS (hemolytic and cytotoxic factor conferring a greater virulence to *Lm*) is expressed only under oxidative stress conditions and this confers a better ability in terms of phagosome escape. Therefore, the presence of LIPI-3 is considered responsible for the increased virulence in some strains [61,62].

5. Conclusions

The present study represented the first intensive *Lm* FPE monitoring plan performed in central Italy, both at FPP and RS level, within the pork-meat chain. Results highlighted that FPEs widely harboured *Lm*, both on FCS and NFCS, representing potential sources of food cross contamination. A systematic *Lm* monitoring of FPEs in Italian food safety surveillance plans performed by the competent authority should be included, designing an effective, risk-based environmental monitoring program, and defining the guidelines for key design elements, such as the number, location, timing and frequency of sampling as well as standard criteria for classifying surfaces into specific categories. Moreover, there are no common standard criteria to classify surfaces into a specific category. Therefore, it should be very important to define a standard categorisation of food surfaces to be used in monitoring plans in order to obtain comparable results. However, these recommendations should take into account that each FPE has specific characteristics and different critical points and so provide for flexible and adaptable criteria to each food associated reality. Moreover, sinks and drains (NFCS) should not be excluded as they were very often contaminated with *Lm*. The *Lm* circulation in FPEs, with a common presence of strains at FPP and RS level after the follow-up sampling, should focus the attentions at the efficacy of cleaning and disinfection procedures.

Thanks to the highly discriminatory power, WGS is now routinely used for the surveillance of human listeriosis and for food-safety monitoring. The great benefit and the potential of WGS analysis emerged from this study, emphasizing how this advanced molecular typing method should also be considered an essential tool in the environmental monitoring plans. Moreover, WGS could also easily detect the possible presence of different *Lm* clones and clusters within the same *Lm* positive sample, showing how is extremely important trying to isolate more than one strain from each positive sample analysed.

Through the cgMLST cluster analysis, the genetic relationships between isolates were investigated allowing us to identify strains persisting after sanitization in the same FPE as well as strains contaminating different FPEs. The spread, both at FPP and RS, of hypo-virulent CCs, more adapted to FPEs and able to persist after cleaning and sanitation represents a significant risk of food cross contamination. On the other hand, the detection of hyper-virulent clones, including an outbreak strain, even without evidence of persistence, posed an even more warring risk for the public health.

The provided information contributed to increasing knowledge on the environmental spread of *Lm* in meat FPP and RS of Marche Region, following a severe listeriosis outbreak occurred between 2015 and 2016. The lack of European environmental monitoring studies including both FPP and RS and in particular the paucity of data on *Lm* FPE contamination at RS, emphasize the need to add FPEs to the sampling plan and collect data on the topic in this continent. From the results obtained in this study arose several recommendations to be provided to FBOs and aimed at improving the management of the pathogen minimizing risk of food contamination and recurrence of severe outbreak of listeriosis.

In conclusion, the application of intensive environmental sampling plans, considering several different surfaces, for the *Lm* detection and the isolation, when possible, of more than one *Lm* strain from each positive sample might be extremely important, in order to have improved surveillance, better clusters detection and early foodborne outbreak detection.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10081944/s1, Figure S1: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC121 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red. Figure S2: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC9 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red. Figure S3: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC1 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red. Figure S4: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC3 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red. Figure S5: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC7 *Lm* strains coloured according to sampling session; All the strains belonged to the same cgMLST cluster. Table S1: Abbreviation list; Table S2: List of sampled surfaces grouped into five categories; Table S3: Positive samples and *Lm* strains' molecular typing results for each production FPP and RS. Table S4: Positive samples and molecular typing results for *Lm* strains isolated during the follow-up sampling; Table S5: Quality control check of sequence data. Reads' quality control metrics reported are after trimming.

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Supplementary Materials

The following are available online at
https://www.mdpi.com/article/10.3390/foods10081944/s1

Figure S1: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC121 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red.

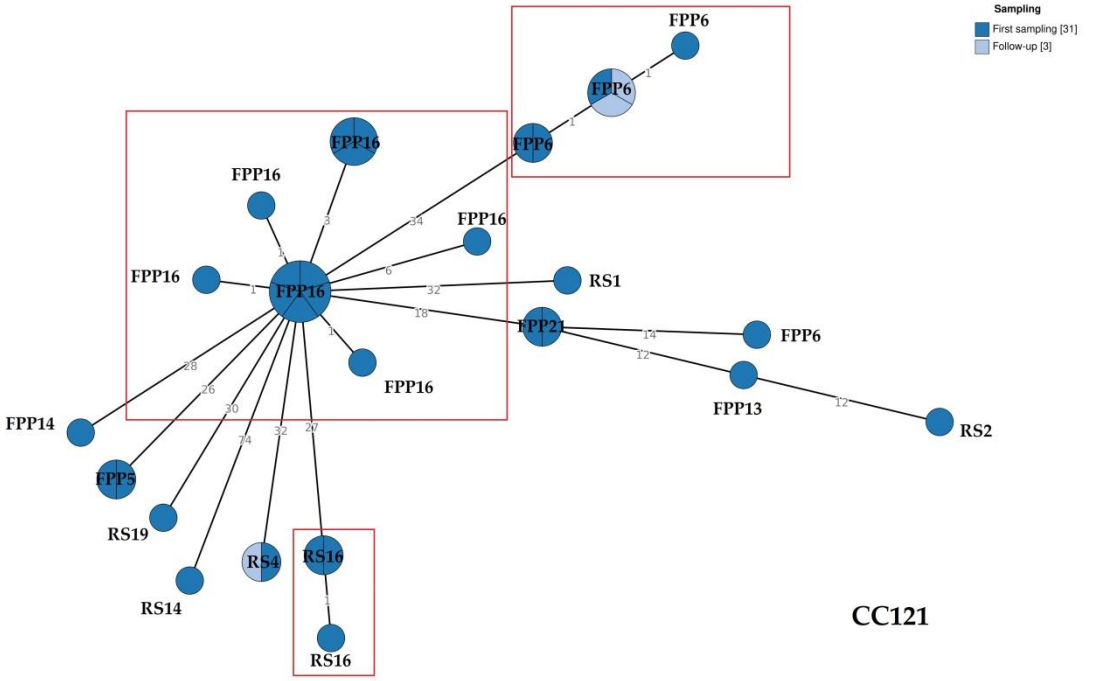

Figure S2: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC9 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red.

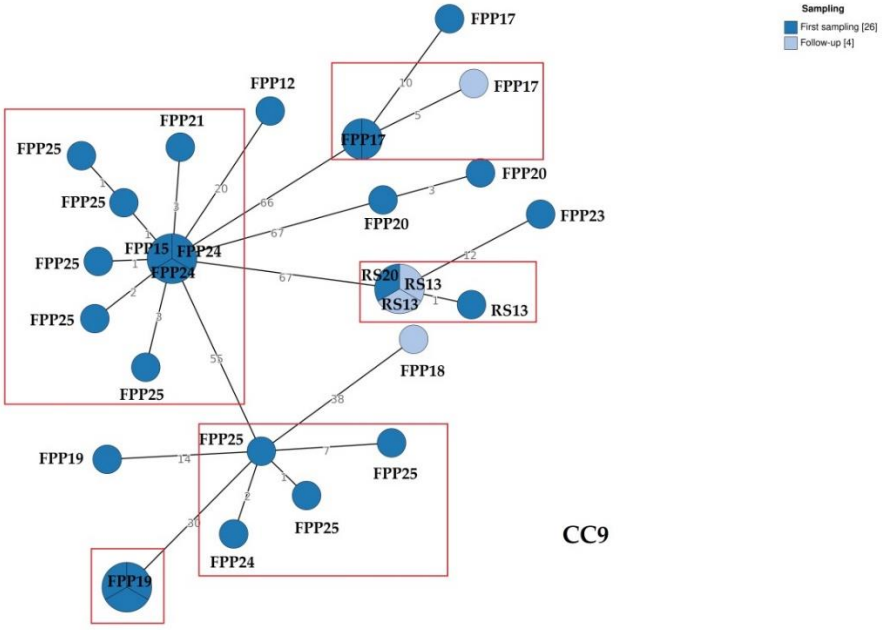

Figure S3: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC1 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red.

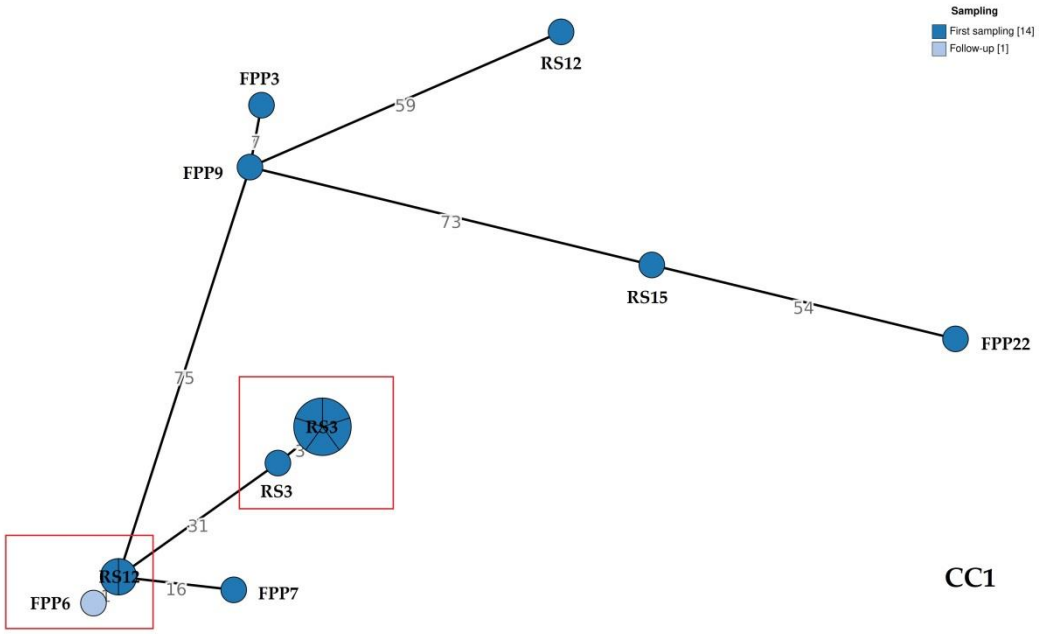

Figure S4: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC3 *Lm* strains coloured according to sampling session; the cgMLST clusters containing more than two strains are highlighted in red.

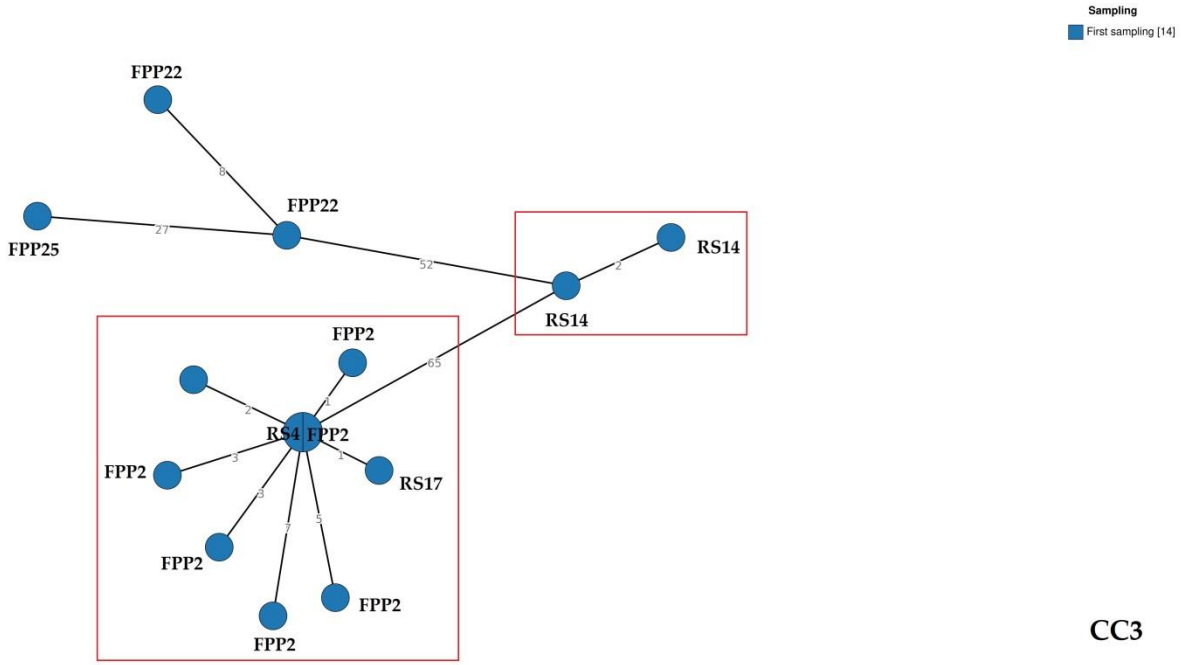

Figure S5: Minimum Spanning Tree (MST) based on the cgMLST profiles of CC7 *Lm* strains coloured according to sampling session; All the strains belonged to the same cgMLST cluster.

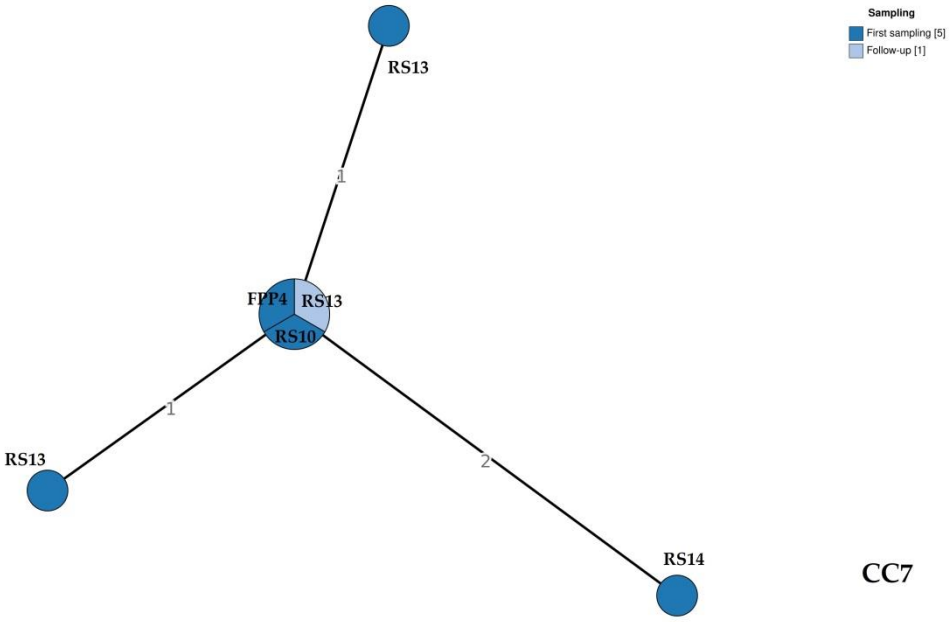

Table S1. Abbreviation list.

Abbreviation	**Explenation**
FPE	Food processing environment
FPP	Food producing plants
RS	Retail store
FBO	Food business operators
RTE	Ready-to-eat
WGS	Whole genome sequencing
MLST	Multilocus sequencing typing
cgMLST	Core genome multilocus sequencing typing
MST	Minimum spanning tree

Table S2: List of sampled surfaces grouped into five categories.

Surface category	sampled surfaces
Equipment	industrial cupboards/shelves/drawers
	basins/baskets
	carts
	baldresca/containers
	waste/processing waste containers
	meat cart containers
	meat hanger trolleys/racks for hanging sausages
	hooking bars
	hooks
	cookware
	hams holders
	pushbuttons/keyboards/control panels/switches
	cutting boards
	working/packing tables
	trays/dishes
Industrial systems	air intake systems/cooker hoods
	air conditioning systems
	cooling systems for cold rooms/blast chillers/refrigerators
	cooling systems for refrigerated exhibitors/exhibitor mural refrigerators
	sinks
	drain channels
	drain wells
	cold rooms
	drying rooms
	seasoning rooms
	goods lifts
	walls
	floors
	door/door handles
	food wormers
	pipes
Machines	slicers
	scales
	packaging machines
	meat-bone separators
	labeling machines
	ovens/cooking boilers
	grater machines
	pressure washers
	kneaders
	sausage stuffers
	meat tenderizers
	meat tying machines
	centrifugal fans
	conveyors belts
	bone saws/band saws
	meat injector machines

	knives sterilizers
	meat grinders
Clothing	cut resistant gloves stainless steel
	apron
	gloves
	boots/shoes
Cleaning Tools	water tube
Tools	knife sharpeners
	Can openers
	knives
	spoons/ladles/scoops/strainers
	forks
	tongs
	salami prickers

Table S3. Positive samples and *Lm* strains' molecular typing results for each production FPP and RS.

Food processing plant/retail store ID	Positive samples (%)[a]	Typed strains	Serogroup	ST	CC
FPP1	6/15 (40%)	6	IIb	191	191
FPP2	7/15 (47%)	7	IIb	3	3
FPP3	1/11 (9%)	1	IVb	1	1
FPP4	**1/15 (7%)**	**1**	**IIa**	**7**	**7**
FPP5	2/15 (13%)	2	IIa	121	121
FPP6	5/15 (33%)	5	IIa	121	121
		1	IIb	517	517
FPP7	1/13 (7%)	1	IVb	1	1
FPP8	1/15 /7%)	1	SNT	SNT	SNT
FPP9	1/15 (7%)	1	IVb	1	1
FPP10	2/15 (13%)	2	IIb	517	517
FPP11	1/15 (7%)	1	IVb	6	6
FPP12	1/15 (7%)	1	IIc	9	9
FPP13	1/15 (7%)	1	IIa	121	121
FPP14	1/15 (7%)	1	IIa	121	121
FPP15	1/15 (7%)	1	IIc	9	9
FPP16	12/15 (80%)	12	IIa	121	121
FPP17	3/15 (20%)	3	IIc	9	9
FPP18	2/15 (13%)	1	IIa	14	14
		1	IIb	429	429
FPP19	4/15 (27%)	4	IIc	9	9
FPP20	2/15 (13%)	2	IIc	9	9
FPP21	2/15 (13%)	1	IIc	9	9
		2	IIa	121	121
FPP22	3/15 (20%)	1	IVb	1	1
		2	IIb	3	3
FPP23	1/15 (7%)	1	IIc	9	9
FPP24	3/15 (20%)	3	IIc	9	9

FPP25	8/15 (53%)	1	IIb	3	3
		8	IIc	9	9
RS1	1/15 (7%)	1	IIa	121	121
RS2	2/15 (13%)	1	IVb	1	1
		1	IIa	121	121
RS3	6/15 (40%)	6	IVb	1	1
RS4	5/15 (33%)	1	IIb	3	3
		1	IIa	121	121
		2	IIa	8	8
		1	IIa	101	101
RS5	1/15 (7%)	1	IVb	2	2
RS6	1/15 (7%)	1	IIa	91	14
RS7	1/15 (7%)	1	IIa	8	8
RS8	2/15 (13%)	2	IVb	2	2
RS9	1/7 (14%)	1	IIa	2764	2764
RS10	**1/15 (7%)**	**1**	**IIa**	**7**	**7**
RS11	3/12 (25%)	2	IIb	363	363
		1	IIb	224	224
RS12	3/15 (20%)	1	IIa	325	31
		2	IVb	1	1
RS13	**3/16 (19%)**	1	IIc	9	9
		2	**IIa**	**7**	**7**
RS14	5/15 (33%)	2	IIb	3	3
		1	IIa	7	7
		1	IIa	504	475
		1	IIa	121	121
RS15	1/15 (7%)	1	IVb	1	1
RS16	2/15 (13%)	3	IIa	121	121
RS17	1/15 (7%)	1	IIb	3	3
RS18	1/15 (7%)	1	IIa	325	31
RS19	3/15 (20%)	1	IIa	325	31
		1	IIa	155	155

		1	IIa	121	121
RS20	2/15 (13%)	1	IIa	325	31
		1	IIc	9	9
RS21	1/15 (7%)	1	IIa	325	31

ST-sequence type. CC-clonal complex. a: environmental samples positive for *Lm* compared to the total samples tested. Strains belonging to the outbreak clone are in bold font. SNT- strain not typed: the bacterial strain was not available for further genomic analysis other than serogroup.

Table S4: Positive samples and molecular typing results for *Lm* isolated during the follow-up sampling.

Food processing plant/retail store ID	**Typed isolates**	**Serogroup**	**ST**	**CC**
FPP6	2	IIa	121	121
	1	IVb	1	1
FPP11	1	IVb	6	6
FPP17	1	IIc	9	9
FPP18	1	IIc	9	9
RS4	1	IIa	121	121
RS6	1	IIa	14	91
RS13	1	IVb	2	2
	1	**IIa**	**7**	**7**
	2	IIc	9	9

ST-sequence type. CC-clonal complex. Strains belonging to the outbreak clone are in bold font.

Table S5. Quality control check of sequence data. Reads' quality control metrics reported are after trimming.

Strain ID	Sequence ID	Average read quality score	N° read pairs	Vertical coverage	N° contigs	Total length (bp)	N50	L50
Lm_1958	2016.TE.10956.1.10.fsa	32.1	4,013,638	118	65	2,967,331	489,281	3
Lm_1959	2016.TE.10956.1.11.fsa	31.79	2,872,928	92	59	3,017,215	510,825	3
Lm_1960	2016.TE.10956.1.12.fsa	32.7	4,052,512	169	167	2,977,541	524,626	2
Lm_1961	2016.TE.10956.1.13.fsa	31.86	2,182,764	67	52	2,983,122	508,735	2
Lm_1962	2016.TE.10956.1.14.fsa	32.4	2,715,462	115	119	3,025,935	516,315	2
Lm_1963	2016.TE.10956.1.15.fsa	31.97	2,229,538	37	176	3,007,080	489,058	2
Lm_1964	2016.TE.10956.1.16.fsa	32.18	1,945,568	81	82	3,019,088	516,602	2
Lm_1967	2016.TE.10956.1.19.fsa	32.27	3,115,196	133	168	3,028,288	579,539	3
Lm_1968	2016.TE.10956.1.20.fsa	31.79	1,952,728	60	214	2,943,512	48,265	17
Lm_1969	2016.TE.10956.1.25.fsa	32.48	3,931,966	167	159	3,038,424	489,317	3
Lm_1970	2016.TE.10956.1.26.fsa	32.11	4,806,552	137	49	2,935,177	449,383	2
Lm_1974	2016.TE.10956.1.36.fsa	31.61	2,259,980	72	63	2,974,292	268,528	3
Lm_1975	2016.TE.10956.1.37.fsa	32.11	2,255,916	94	95	2,895,712	510,943	2
Lm_1976	2016.TE.10956.1.38.fsa	32.26	2,034,124	85	504	3,096,610	483,057	3
Lm_1977	2016.TE.10956.1.39.fsa	32.18	2,338,262	96	131	3,093,756	431,753	3
Lm_1954	2016.TE.10956.1.40.fsa	32.1	2,270,532	96	81	2,931,423	1,507,844	1
Lm_1948	2016.TE.10956.1.41.fsa	32.25	2,787,522	117	129	3,068,386	526,076	2
Lm_1949	2016.TE.10956.1.42.fsa	32.29	2,498,646	106	145	3,038,436	517,91	2
Lm_1950	2016.TE.10956.1.43.fsa	32.24	2,190,292	91	197	3,109,352	438,891	4
Lm_1951	2016.TE.10956.1.44.fsa	33.46	949,4	31	114	3,065,689	64,672	15
Lm_1952	2016.TE.10956.1.45.fsa	32.39	3,005,852	128	153	3,124,487	481,686	3
Lm_1971	2016.TE.10956.1.46.fsa	32.31	2,519,256	95	75	3,049,803	449,54	4
Lm_1953	2016.TE.10956.1.47.fsa	32.32	2,685,800	114	110	2,924,335	580,305	2
Lm_1978	2016.TE.10956.1.48.fsa	32.23	2,467,484	104	94	2,968,632	479,534	3
Lm_1980	2016.TE.10956.1.49.fsa	32.23	2,307,802	97	112	3,112,802	474,812	3
Lm_1979	2016.TE.10956.1.51.fsa	32.29	2,463,636	105	107	3,025,717	257,394	3
Lm_1957	2016.TE.10956.1.9.fsa	32.09	3,161,934	89	64	3,011,702	507,416	3
2016.TE.12907.1.33	2016.TE.12907.1.33.fsa	34.4	5,305,850	196	53	3,060,351	465,251	4
2016.TE.12907.1.34	2016.TE.12907.1.34.fsa	33.7	2,626,610	117	57	3,061,472	481,622	3
Lm_2001	2016.TE.15639.1.13.fsa	33.06	3,363,790	145	97	3,109,823	358,803	4
Lm_2029	2016.TE.15639.1.17.fsa	33.66	1,440,170	51	67	3,092,990	236,795	5
Lm_2028	2016.TE.15639.1.18.fsa	32.95	3,250,534	142	72	3,098,833	302,516	3
2016.TE.15639.1.26	2016.TE.15639.1.26.fsa	33.48	3,938,672	157	95	2,948,386	449,383	2
Lm_2017	2016.TE.15639.1.28.fsa	33.71	5,612,492	206	116	2,993,205	546,52	3
Lm_2030	2016.TE.15639.1.31.fsa	33.7	2,269,562	81	38	3,008,710	517,925	2
Lm_2032	2016.TE.15639.1.32.fsa	33.73	2,012,494	73	56	3,092,090	302,516	4
Lm_2031	2016.TE.15639.1.33.fsa	33.71	3,892,974	139	58	3,094,072	302,516	4
Lm_2034	2016.TE.15639.1.34.fsa	33.78	3,776,486	132	43	3,053,607	518,041	2
Lm_2042	2016.TE.15639.1.35.fsa	32.94	2,896,316	129	69	3,063,850	510,936	2

Lm_2002	2016.TE.15639.1.5.fsa	33.03	3,842,786	159	85	3,070,489	581,263	2
Lm_2003	2016.TE.15639.1.6.fsa	33.13	2,423,078	105	75	3,103,422	358,803	4
Lm_2033	2016.TE.16633.1.2.fsa	33.83	4,901,730	159	70	3,060,119	518,062	2
Lm_2058	2016.TE.17371.1.10.fsa	34.01	1,392,426	54	61	3,016,064	509,792	3
Lm_2060	2016.TE.17371.1.11.fsa	34.15	1,377,960	47	71	3,012,509	260,686	4
2016.TE.17371.1.18	2016.TE.17371.1.18.fsa	34.17	4,834,256	185	59	3,023,196	518,148	2
Lm_2050	2016.TE.17371.1.2.fsa	34.11	1,218,070	47	66	2,961,586	511,505	3
Lm_2051	2016.TE.17371.1.3.fsa	34.27	4,162,334	146	60	2,928,885	604,997	2
Lm_2052	2016.TE.17371.1.4.fsa	34.08	1,365,764	52	48	2,965,550	489,274	3
Lm_2053	2016.TE.17371.1.5.fsa	34.14	1,378,382	51	46	2,964,998	564,627	3
Lm_2054	2016.TE.17371.1.6.fsa	34.3	4,067,056	142	66	2,976,457	565,717	3
Lm_2055	2016.TE.17371.1.7.fsa	34.17	2,240,044	83	37	3,013,890	511,857	3
Lm_2056	2016.TE.17371.1.8.fsa	34.3	2,647,480	93	41	3,005,661	477,264	3
Lm_2057	2016.TE.17371.1.9.fsa	33.88	3,070,536	131	104	3,023,172	476,54	3
Lm_2091	2016.TE.20838.1.22.fsa	34.03	1,234,212	49	34	3,006,776	476,139	3
Lm_2092	2016.TE.20838.1.23.fsa	33.74	2,416,166	108	75	3,017,832	605,869	2
Lm_2070	2016.TE.20838.1.3.fsa	33.6	3,982,536	167	75	3,029,069	361,235	3
Lm_2069	2016.TE.20838.1.4.fsa	34.23	4,080,212	150	59	3,045,587	435,205	4
Lm_1854	2016.TE.6891.1.78.fsa	33.45	2,067,704	69	53	3,018,896	356,99	2
Lm_1868	2016.TE.6891.1.79.fsa	33.53	1,952,826	68	51	2,935,422	449,383	2
Lm_1936	2016.TE.8594.1.10.fsa	33.32	3,507,926	139	102	3,010,565	294,126	2
Lm_1943	2016.TE.8594.1.11.fsa	33.16	2,222,298	95	60	2,889,692	540,602	2
Lm_1876	2016.TE.8594.1.12.fsa	33.82	3,717,144	137	60	2,985,538	482,817	3
Lm_1877	2016.TE.8594.1.13.fsa	33.26	2,429,142	105	225	3,028,756	482,817	3
Lm_1878	2016.TE.8594.1.14.fsa	33.96	4,800,432	161	121	2,999,846	481,413	3
Lm_1879	2016.TE.8594.1.15.fsa	33.87	3,877,522	142	68	2,972,547	566,621	3
Lm_1880	2016.TE.8594.1.16.fsa	33.89	3,933,478	136	99	3,077,371	479,525	3
Lm_1881	2016.TE.8594.1.17.fsa	33.96	4,363,138	151	47	2,894,234	434,414	3
Lm_1882	2016.TE.8594.1.18.fsa	34.11	3,247,418	99	100	3,086,621	431,753	3
Lm_1883	2016.TE.8594.1.19.fsa	33.6	3,936,462	153	205	3,053,904	505,536	3
Lm_1955	2016.TE.8594.1.2.fsa	34.05	5,651,654	180	77	3,017,824	509,117	3
Lm_1884	2016.TE.8594.1.20.fsa	33.55	3,826,672	154	59	3,014,033	509,119	3
Lm_1885	2016.TE.8594.1.21.fsa	33.19	1,971,426	85	63	3,045,866	429,54	4
Lm_1886	2016.TE.8594.1.22.fsa	33.14	2,419,094	101	59	3,044,510	429,594	4
Lm_1887	2016.TE.8594.1.23.fsa	33.11	3,059,626	128	100	3,055,720	429,594	4
Lm_1888	2016.TE.8594.1.24.fsa	33.82	4,869,248	177	306	3,062,594	357,44	2
Lm_1889	2016.TE.8594.1.25.fsa	33.17	1,909,250	82	52	3,011,401	605,869	2
Lm_1890	2016.TE.8594.1.26.fsa	33.38	2,677,658	111	74	3,036,749	484,925	3
Lm_1891	2016.TE.8594.1.27.fsa	34.08	4,379,378	137	68	3,063,947	484,133	3
Lm_1892	2016.TE.8594.1.28.fsa	33.6	3,735,040	136	57	2,961,573	357,44	2
Lm_1872	2016.TE.8594.1.29.fsa	33.55	3,125,588	101	62	3,018,366	449,477	3
Lm_1956	2016.TE.8594.1.3.fsa	33.62	3,511,986	141	95	3,109,434	358,803	4
Lm_1873	2016.TE.8594.1.30.fsa	34.04	3,041,014	93	64	2,998,313	476,295	3
Lm_1874	2016.TE.8594.1.31.fsa	33.5	3,273,412	128	127	3,116,013	358,802	4
Lm_1875	2016.TE.8594.1.32.fsa	33.94	4,157,440	136	84	3,104,808	482,291	3

Lm_1900	2016.TE.8594.1.33.fsa	33.66	3,168,604	121	89	3,100,462	358,802	4
Lm_1901	2016.TE.8594.1.34.fsa	33.6	3,540,108	132	620	3,244,089	358,802	4
Lm_1902	2016.TE.8594.1.35.fsa	33.25	2,813,316	119	49	2,908,723	580,306	2
Lm_1903	2016.TE.8594.1.36.fsa	33.81	4,710,798	168	84	2,990,627	540,999	2
Lm_1904	2016.TE.8594.1.37.fsa	33.12	1,991,218	84	67	2,967,112	299,173	2
Lm_1905	2016.TE.8594.1.38.fsa	33.49	2,650,140	103	167	3,044,341	356,99	2
Lm_1906	2016.TE.8594.1.39.fsa	33.92	3,456,078	118	72	3,019,502	356,99	2
Lm_1907	2016.TE.8594.1.40.fsa	33.93	3,246,432	103	70	3,018,019	356,99	2
Lm_1908	2016.TE.8594.1.41.fsa	33.86	1,917,054	63	124	3,031,225	356,99	3
Lm_1909	2016.TE.8594.1.42.fsa	33.91	2,627,260	91	113	3,068,187	376,529	2
Lm_1910	2016.TE.8594.1.43.fsa	33.37	2,939,254	117	71	3,020,560	356,989	3
Lm_1911	2016.TE.8594.1.44.fsa	34.09	5,225,150	158	101	3,086,604	431,753	3
Lm_1912	2016.TE.8594.1.45.fsa	33.41	1,823,574	77	454	3,198,918	358,803	4
Lm_1913	2016.TE.8594.1.46.fsa	33.91	5,523,864	191	96	2,983,671	295,975	4
Lm_1914	2016.TE.8594.1.47.fsa	33.39	2,928,668	123	76	2,979,956	308,368	4
Lm_1918	2016.TE.8594.1.48.fsa	33.68	4,600,160	181	116	3,076,932	482,727	3
2016.TE.8594.1.5	2016.TE.8594.1.5.fsa	34.11	3,919,570	126	40	2,932,597	437,349	2
Lm_1920	2016.TE.8594.1.50.fsa	33.39	2,292,424	96	723	3,244,126	344,532	4
Lm_1921	2016.TE.8594.1.51.fsa	34.12	3,824,204	124	55	3,061,470	482,464	3
Lm_1922	2016.TE.8594.1.52.fsa	34.03	4,307,648	142	81	3,067,703	481,06	3
Lm_1924	2016.TE.8594.1.54.fsa	33.98	2,980,650	103	57	3,061,073	465,251	3
Lm_1926	2016.TE.8594.1.56.fsa	34.1	5,039,372	160	127	3,078,656	344,262	4
Lm_1927	2016.TE.8594.1.57.fsa	33.36	1,906,334	82	64	3,062,541	358,322	4
Lm_1928	2016.TE.8594.1.58.fsa	34.03	5,004,246	165	45	3,059,159	482,727	3
Lm_1930	2016.TE.8594.1.59.fsa	33.38	2,973,294	126	141	3,034,201	564,718	3
Lm_1931	2016.TE.8594.1.60.fsa	33.43	3,335,494	136	119	3,113,267	431,753	3
Lm_1933	2016.TE.8594.1.61.fsa	33.34	1,862,716	77	64	3,042,551	294,126	2
Lm_1934	2016.TE.8594.1.62.fsa	32.89	2,101,804	83	154	3,067,070	294,126	3
Lm_1935	2016.TE.8594.1.63.fsa	34.05	4,107,590	125	61	3,000,428	294,126	2
Lm_1937	2016.TE.8594.1.64.fsa	34.04	3,740,640	112	63	3,042,414	294,126	2
Lm_1938	2016.TE.8594.1.65.fsa	34.01	3,242,648	103	77	2,974,254	262,424	2
Lm_1939	2016.TE.8594.1.66.fsa	34.15	3,032,410	95	82	3,074,215	481,38	3
Lm_1940	2016.TE.8594.1.67.fsa	33.18	2,350,758	97	92	3,107,778	307,644	4
Lm_1941	2016.TE.8594.1.68.fsa	33.98	4,343,402	142	61	3,100,318	482,801	3
Lm_1942	2016.TE.8594.1.69.fsa	33.6	2,499,532	96	64	3,100,133	482,429	3
2016.TE.8594.1.70	2016.TE.8594.1.70.fsa	33.69	3,898,756	147	207	3,161,174	358,181	4
Lm_1944	2016.TE.8594.1.71.fsa	33.45	3,234,282	130	157	3,117,503	431,752	3
Lm_1945	2016.TE.8594.1.72.fsa	33.25	2,101,106	83	113	3,075,044	356,99	2
Lm_1946	2016.TE.8594.1.73.fsa	33.18	2,057,446	83	134	3,080,721	357,182	2
Lm_1917	2016.TE.8594.1.74.fsa	33.25	2,284,278	98	105	3,109,227	482,505	3
Lm_1947	2016.TE.8594.1.9.fsa	33.2	1,621,580	65	53	3,039,033	261,325	2
Lm_1981	2016.TE.9198.1.24.fsa	33.15	1,272,262	44	28	2,992,761	262,448	3
Lm_1982	2016.TE.9198.1.25.fsa	32.97	1,500,928	52	62	3,001,267	262,447	2
2020.TE.88968.1.2	2020.TE.88968.1.2.fsa	36.8	1,513,740	75	42	3,100,088	482,067	3
Lm_1923	2020.TE.88968.1.3.fsa	36.77	1,268,386	63	29	3,056,186	465,338	3

Lm_1973	2020.TE.88968.1.4.fsa	36.78	1,452,784	72	21	2,846,401	1,464,595	1
2020.TE.88968.1.5	2020.TE.88968.1.5.fsa	36.78	1,250,626	62	47	3,098,667	480,32	3
2020.TE.88968.1.6	2020.TE.88968.1.6.fsa	36.78	1,736,240	86	46	3,099,491	482,504	3
Lm_1999	2020.TE.88968.1.7.fsa	36.78	1,238,390	61	48	3,098,775	480,726	3

Original Research Paper III

In this study an *in vitro* model to evaluate adhesion and invasiveness of *Lm* towards intestinal cells was applied to nine strains isolated from food and human cases of listeriosis.

The main purposes of the study were to (i) use WGS to analyze *Lm* genomes identifying Clonal Complexes (CC) and key virulence-associated determinants and (ii) characterize the ability of the *Lm* strains to adhere and invade human colon carcinoma cell line Caco-2 evaluating the possible correspondence with their genomic virulence profile.

Whole Genome Sequencing analysis of *Listeria monocytogenes* virulence profiles and cell adhesion/invasion assessment *in vitro*

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Abstract

Listeria monocytogenes (*Lm*) is the causative agent of human listeriosis. *Lm* strains have different virulence potential. We used WGS to type the virulence profile of nine *Lm* strains on a wide panel of markers. The adhesion and invasion abilities of the strains were also assessed *in vitro*.

The clinical strains belonged to Clonal Complex (CC) 1, CC31 and CC101 and showed low invasiveness. The *Lm* strains isolated from food were assigned to CC1, CC7, CC9 and CC121 with the CC7 and a CC1 showing high invasiveness.

All CC1 carried the Listeria Pathogenicity Island (LIPI) 3. Premature Stop Codons in the inlA gene were found in *Lm* food strains belonging to CC9 and CC121. The highly invasive CC7 strain, belonging to an epidemic cluster, carried the additional internalins' genes *inlG* and *inlL*. The human CC31 strain, lowely invasive, lacked *lapB* and *vip*.

The genetic determinants of hypo- or hypervirulence not necessarily predicted the cell adhesion and/or invasion ability *in vitro* indicating that the presence of specific virulence-associated genes not necessarily indicates the expression of the relative virulence factors Anyway, listeriosis results from the interplay between the host and the virulence features of *Lm* and even hypovirulent clones are able to cause infection in immunocompromised people.

1. Introduction

Listeria monocytogenes (*Lm*) is a major foodborne pathogen causing human listeriosis, a severe disease with the highest fatality rates among all others foodborne diseases [1, 2]. Invasive infections mainly occur in immunocompromised people, the elderly, pregnant women and neonates [3–6] and are caused by the ability of *Lm* to invade human cells crossing multiple host barriers [7].

Transmission to humans occurs primarily via consumption of food, mainly ready-to-eat (RTE) foods including meat and dairy. Once ingested, *Lm* can invade intestinal epithelial cells, gaining access to the lymphatic system and blood stream, resulting in the dissemination of cells to the liver, spleen, central nervous system, and, in pregnant women, to the placenta [8].

The ability of *Lm* to adhere and invade phagocytic and non-phagocytic cells is an important aspect of disease pathogenesis that includes multiple stages such as cell adhesion, internalization, vacuole escape, intracellular replication, movement by actin mobilization and cell-to-cell spread [9]. Over the last decade, major advances have been made in understanding the role of virulence factors involved in the pathogenesis of *Lm*.

The pathogenicity of *Lm* is mediated by a wide range of virulence factors which allow it to infect, survive, and replicate in a variety of host cell types [10, 11]. Thanks to the numerous studies conducted to investigate the adhesion, invasion and/or virulence regulation of this pathogen, the roles of different virulence factors have been well characterized in different cell types or animal models together with the relative encoding genes [11, 12]. More in detail, four Listeria pathogenicity islands (LIPI-1, LIPI-2, LIPI-3 and LIPI-4) have been identified so far [13–16]. LIPI-1, necessary for intracellular survival and spread, is present in all *Lm* strains and is composed by six genes including *prfA*, *actA*, *hly*, *mpl*, *iap*, *plcA* and *plcB*. LIPI-2 is a 22 kb gene cluster involved in phagosome disruption [16–18]. LIPI-3 is composed by eight genes (*llsAGHXBYDP*) and encodes a biosynthetic cluster involved in the production of Listeriolysin S (LLS), an haemolytic and cytotoxic factor that is known to be required for *Lm* virulence in vivo [15,19]. LIPI-4 is a cluster of six genes and is involved in neural and placental infection [20, 21].

Internalin A (InlA) and B (InlB), encoded by the *inlAB* operon, bind the eukaryotic cell membrane receptors, E-cadherin and Met, and the receptor of the hepatocyte growth factor (HGF), inducing the bacterial uptake through receptor-mediated endocytosis [12, 18, 22]. Many studies have previously reported multiple distinct mutations leading to a premature stop codon (PMSC) in the *inlA* gene that cause a dysregulated expression of the internalin protein

[23,24] with a significant decrease in the invasiveness of *Lm* towards human epithelial cells [25].

Other proteins such as fibronectin binding protein (FbpA), Auto and Vip are suggested to have a role in mediating *Lm* entry into the host cell [9]. In addition, *Lm* utilizes Listeria adhesion proteins (Lap and LapB) to exploit epithelial defences and cross the intestinal epithelial barriers [9, 26].

To date, *Lm* is classified into four major evolutionary lineages (I, II, III, IV), 13 agglutination serotypes, five molecular serogroups and several Multi Locus Sequence Typing (MLST) clonal complexes (CCs) [13, 20, 27, 28].

Serotypes 1/2b and 4b, along with serotype 1/2a, are the main serotypes that cause human disease and represents 90–95% of cases [29]. Recent advances in *Lm* infection biology have reported the existence of hypo- and hypervirulent CCs [20, 30]. In particular, certain CCs such as CC1, CC2, CC4, and CC6 are more frequently associated with clinical cases and are hypervirulent in a humanized mouse model, whereas others like CC9 and CC121 are mainly of food-borne origin and show hypovirulence in vivo [20,30].

Methods for determining strains virulence include in vivo bioassays (animal models), *in vitro* cell assays and molecular methods to detect virulence genes [31].

Several mammalian cell lines have been used in *in vitro* studies aimed at evaluating the pathogenic potential of Listeria species. Among these, the Caco-2 human colon adenocarcinoma cell line, whose characteristics simulate structural and functional features of mature enterocytes *in vitro*, has been most widely used to investigate intestinal adherence and invasion as well as intracellular replication of *Lm* [32, 33].

The analysis of genetic virulence determinants, previously undertaken mainly through PCR detection, takes advantage of Whole Genome Sequencing (WGS) technology and bioinformatic analysis with appropriate virulence analytic tools capable to detect a wide panel of genes responsible for the pathogenicity of the strains. Indeed, WGS provides the most comprehensive overview of a bacterial strain with the highest possible microbial subtyping resolution compared to the other typing methods. For this reason, WGS has become a new typing standard in public health and food microbiology replacing former gold standard typing tools like PFGE and serotyping. This approach outperforms traditional methods with respect to robustness, discriminatory power, comparability and ease of data exchange and costs [34].

In this study, we selected nine *Lm* strains isolated between 2013 and 2016 from sporadic cases of human listeriosis and foods and we characterized them both identifying genetic virulence-associated markers and assessing their virulence abilities *in vitro*.

The main purposes of the study were to (i) use WGS to analyze *Lm* genomes identifying Clonal Complexes (CCs) and key virulence-associated determinants and (ii) characterize the ability of the *Lm* strains to adhere and invade human colon carcinoma cell line Caco-2 evaluating the possible correspondence with their genomic virulence profile.

2. Materials and methods

2.1 Bacterial strains

The nine strains of *Lm* tested in this study included isolates of food origin (n = 6) and from human cases of listeriosis (n = 3). The main characteristics and the isolation source of the *Lm* strains used in this study are reported in Table 1.

	Strain ID	**Source**	**Serotype**
H**uman**	490	Blood	1/2a
	566	Blood	1/2a
	1498	Cerebrospinal fluid	4b
RTE-food	1484	"Coppa di testa" head cheese	1/2b
	1608	"Coppa di testa" head cheese	1/2a
	1487	Fresh salami	4b
	1643	Salami	4b
	2018	Spit roasted pork	1/2a
	1715	"Coppa di testa" head cheese	1/2a

Table 1: *Listeria monocytogenes* strains typed in this study.

The food-derived strains were collected within the framework of the official control plan activity (Reg EC 2073/2005) [35] carried out in Marche region between 2015 and 2016, when a severe outbreak of human listeriosis [36] led to the intensification of the surveillance activities. One of these strains, the 1715, belonged to the same epidemic cluster causing the outbreak.

The clinical strains were isolated between 2013 and 2015 within the Italian surveillance network of human listeriosis coordinated by the Italian National Institute of Health (Istituto Superiore di Sanità, ISS).

The selection was made in such a way as to have clinical and food isolates belonging to the serotypes of major epidemiological concern: 1/2a, 1/2b and 4b [29].

The non-pathogenic *Listeria innocua* (ATCC 33090) was used as a negative control in the adhesion and invasion assays.

2.2 Whole Genome Sequencing (WGS)

DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen Hilden, Germany) following the manufacturer's protocol with minor modifications according to Portmann et al., 2018.

The purity of the extracts was evaluated using a NanoDrop 2000 (ThermoFisher Scientific, Wältam, MA, USA). Starting from 1 ng of input DNA, the Nextera XT DNA chemistry (Illumina, San Diego, CA) was used for library preparation according to manufacturer's protocols. WGS was performed on the NextSeq 500 platform (Illumina, San Diego, CA) with the NextSeq 500/550 mid output reagent cartridge v2 (300 cycles, standard 150-bp paired-end reads).

For the analysis of WGS data, an in-house pipeline [37] was used which included steps for trimming (Trimmomatic v0.36) [38] and quality control check of the reads (FastQC v0.11.5). Genome de novo assembly of paired-end reads was performed using SPAdes v3.11.1 [39] with default parameters for the Illumina platform 2 × 150 chemistry. Then, the genome assembly quality check was performed with QUAST v.4.3 [40].

2.2.1 Multi Locus Sequence Typing (MLST)

The MLST scheme used to characterize *Lm* strains is based on the sequence analysis of the following seven housekeeping genes: *acbZ* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (Succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (lactate deshydrogenase), and *lhkA* (histidine kinase) [41]. The seven-gene of MLST scheme and the CCs were deducted in silico using the BIGSdb-Lm database (http://bigsdb.pasteur.fr/listeria ; accessed on 29 April 2021).

2.2.2 Virulence-associated genes detection

The "Virulence" tool of the BIGSdb-Lm database (http://bigsdb.pasteur.fr/listeria ; accessed on 3 September 2021) was used to detect virulence genes in the genomes of the selected strains. Based on the output of gene presence/absence, a heatmap was generated using Morpheus matrix visualization and analysis software from the Broad Institute (https://software.broadinstitute.org/morpheus/ ; accessed on 3 September 2021).

The presence of Premature Stop Codons (PMSC) in the *inlA* gene was also investigated. When the BIGSdb-Lm database reported that a PMSC mutation was present, the mutation position and the length of the resulting truncated InlA protein were specified [42].

2.3 In vitro assays

2.3.1 Epithelial cell line

Human colon carcinoma epithelial cell line (Caco-2) (ECACC 86010202) cells were obtained from the European Collection of Authenticated Cell Culture (Saint Louis, MO).

Caco-2 cells were cultured as monolayers in 75-cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 1% L-glutamine, 1% sodium pyruvate. Once the flasks reached 90% confluence, the cells were digested using trypsin and seeded at desirable density onto 6-well plates (Corning, USA). Cells were used at least 24 h to fully confluence after seeding. The cell line used in this study was grown at 37°C and 5% CO_2. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3.2 Adhesion assay

Two days prior to the assay, Caco-2 cells were seeded in 6 well plates to obtain semi-confluent monolayers (1.5 x 10^5 cells/ml). On the day of assay, cells were washed with Phosphate Buffered Saline (PBS, pH 7.4), and fresh prewarmed media without FBS was added to the wells. Overnight cultures of clinical and food *Lm* strains were grown in TSYEB with shaking at 200 rpm and used in the experiment adjusted to an OD600 = 1.0. The bacterial concentration of the contaminating culture was determined by colony forming units (CFU) confirmed by plating 10-fold dilutions onto TSYEA and incubating at 37°C for 24 h.

The Caco-2 cells grown in 6-well tissue culture plates were infected with c. 10^7 bacteria to yield a multiplicity of infection (MOI) of about 100 CFU per cell. The precise number of inoculated bacterial CFU added at T_0 was subsequently calculated according to plate count on TSYEA. To synchronize adhesion without forcing adhesion, bacteria were spun down on the cell layer for 1 min at 200 g. After incubation at 37° C, 5% CO_2 for 1 h with bacteria to allow adherence, monolayers were thoroughly washed five times in cold PBS to remove bacteria that had not adhered. Serial dilutions were plated on TSYEA and incubated at 37°C for 24 h; then *Lm* colonies were enumerated to determine the number of adhered bacteria.

The adhesion efficiency (%) for each strain was expressed as the percentage of the number of bacteria attached to the cells of the total number of CFU provided in the inoculation, multiplied by 100. Not infected wells were used as negative controls, and each assay was performed in triplicate. *L.innocua* ATCC33090 was included as negative control.

2.3.3 Invasion assay

Caco-2 cells were infected as described in the adhesion assay and incubated at 37°C and 5% CO_2. After 3 h post infection, cells were washed five times with cold PBS, and fresh media containing 50 µg/ml gentamycin (Sigma Aldrich, St. Louis, MO, USA) was added with an additional 90 min of incubation under the same conditions to kill extracellular bacteria. After incubation, cells were extensively washed with cold PBS to remove gentamycin and then intracellular bacteria were recovered by lysis of the monolayers using 500 µl of cold 0.1% Triton X-100 and sonication (Fisher Scientific Sonic Dismembrator Model 100, setting 3, 3 pulses, 6 s each).

The resulting suspension was ten-fold diluted, spread on TSYEA, and grown at 37°C for 24 h. The number of CFUs was considered as the number of bacteria that had invaded the Caco-2 cells. It was considered that counts obtained 3 h after the onset of infection represented the number of bacteria that had been internalized. Uninoculated wells were used as negative controls, and each assay was performed in triplicate wells and was repeated at least two times. *L.innocua* ATCC33090 was included as negative control.

The invasion level (%) for each strain was calculated by dividing the number of CFU that invaded the cells (with gentamycin) by the total number of CFU obtained without gentamycin treatment and was expressed as a percentage.

2.3.4 Hoechst staining

The adhesion and invasion capacity of *Lm* in Caco-2 cells were qualitatively analyzed by fluorescent microscopy. Caco-2 cells were plated and infected as described above (adhesion and invasion assays). Following the process of washing, infected cells were fixed with 4 % paraformaldehyde in PBS, permeabilized with cold methanol, and stained using 10 µM with Hoechst 33342 Staining Dye Solution (Sigma-Aldrich, St. Louis, MO, USA). After further PBS washes, cells were observed by fluorescent microscopy using a “DAPI” filter.

2.3.5 Statistical analysis

The unpaired, two-tailed t-test was applied to evaluate the statistical differences between adherent bacteria or intracellular bacteria and the reference negative control (*L. innocua* ATCC 33090). Differences were considered significant at $p < 0.05$. The analyses were conducted using GraphPad Prism5 Software.

3. Results

3.1 Whole Genome Sequencing (WGS) and Bioinformatics analysis

Sequences in agreement with the quality control thresholds recommended [43] were obtained for all the strains. The quality metrics for each genome are reported in Table 2.

ID	Vertical coverage	N° contigs	Total legth (bp)	N50	L50
***Lm*_490**	105.7	46	3023546	308142	3
***Lm*_566**	515.12	59	3082646	417896	3
***Lm*_1498**	219.6	130	2945468	556758	2
***Lm*_1484**	74	50	2927103	147035	1
***Lm*_1487**	104	111	3079929	524763	3
***Lm*_1608**	123	52	3024307	563871	2
***Lm*_1643**	92.4	61	3023637	580655	2
***Lm*_1715**	133	40	2934721	437349	2
***Lm*_2018**	51.1	71	3123917	531830	2

Table 2: quality metrics of genome assembly.

For each strain, exact matches were found for all the seven genes of the MLST scheme and the relative CC was identified. Three strains belonged to CC1, two were CC121 and the remaining ones were CC7, CC9, CC31 and CC101 respectively (Table 3).

On a scheme of 92 targets, a total of 71 different virulence genes were detected in the nine analyzed isolates. A single isolate owned between 57 and 66 virulence genes. The presence/absence of virulence genes for each strain is detailed in the heatmap reported in Figure 1.

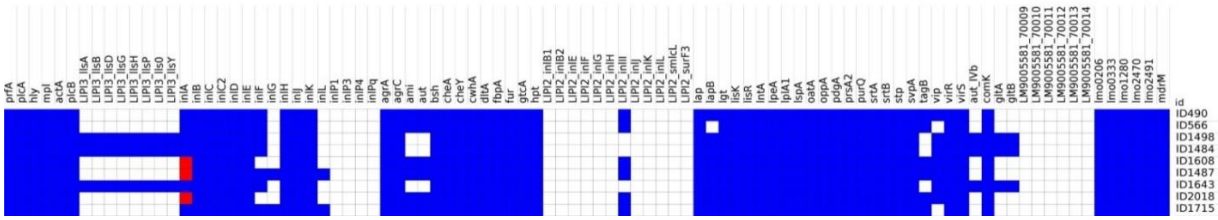

Figure 1. Heatmap of virulence genes detected in silico using the BIGSdb-Lm scheme. Blue: presence of the gene; red: presence of a premature stop codon; white: absence of the gene.

As expected, all the strains carried the LIPI-1 including *prfA*, *actA*, *hly*, *mpl*, *plcA*, *plcB*, and *iap* (recently renamed *cwhA*, as reported in Fig. 1). The CC1 strains also carried LIPI-3 (*llsA*, *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, *llsD*, *llsP*), the teichoic acid biosynthesis genes *gltA* and *gltB* and

the invasion gene *aut_IVb*. None of the studied *Lm* carried a complete LIPI-2 or LIPI-4 (protein sequences LM9005581_70009 to LM9005581_70014). However, the presence of LIPI2_*inlII* (LIV_RS06070) was observed in all the strains except those belonging to CC1. *Lm* 1487 and 1715 also carried the internalins' genes *inlG* and *inlL*. Only the human strain 566 lacked the *lapB* and *vip* genes.

Lm 1487, 1608 and 2018 showed a PMSC in the *inlA* gene predicting the translation of a truncated InlA protein instead of the full-length InlA of 800 aa (Table 3). In particular 1608 and 2018 carried a mutation firstly observed by Olier et al. (2003) and described as PMSC of type 6 by Moura et al., 2016. This mutation is known to produce a truncated form of InlA of 491aa. Strain 1487, instead, presented a type 29 PMSC resulting in a 576 aa length inlA (Moura et al., 2016). All the other strains presented a full-length sequence of the inlA gene.

ID strain	**Isolation source**	**CC**	***inlA* allele**	**PMSC**	**PMSC type**	**InlA type**	**Mutation position**	**InlA length**
490	human	CC101	21	-	-	Full length		800 aa
566	human	CC31	153	-	-	Full length		800 aa
1498	human	CC1	3	-	-	Full length		800 aa
1484	food	CC1	3	-	-	Full length		800 aa
1487	food	CC9	**47**	+	**29**	**Truncated**	1635 (Deletion A)	576 aa
1608	food	CC121	**49**	+	**6**	**Truncated**	1474 (C -->T)	491 aa
1643	food	CC1	3	-	-	Full length		800 aa
1715	food	CC7	2	-	-	Full length		800 aa
2018	food	CC121	**49**	+	**6**	**Truncated**	1474 (C -->T)	491 aa

Table 3. MLST and *inlA* typing results: Clonal Complex (CC), *inlA* allele (BIGSdb-Lm), PMSC type, InlA protein sequence type, PMSC position and predicting InlA length.

3.2 Adhesion and invasion

All the nine *Lm* strains were able to adhere to and to invade Caco-2 cells; the results are detailed in Fig. 2.

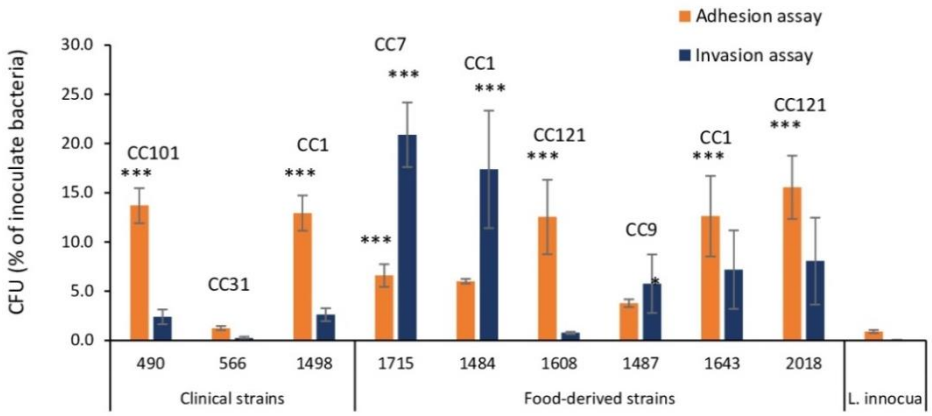

Figure 2. Ability of *Lm* isolates to adhere to and invade Caco2 human intestinal epithelial cells. Data were plotted as percentages of starting viable inoculum. For each strain the CC is reported.

The levels of adhesion of clinical strains ranged from 1.25% to 13.70 %. Clinical strains 490 and 1498 showed adhesion efficiencies of 13.70% (± 3.10%) and 12.94% (± 3.11%), respectively, which were significantly ($p<0.001$) higher than that of *L. innocua*. Clinical strain 566 showed lower adhesion efficiency than the others (1.25%; ± 0.35%) without any significant difference with *L. innocua*.

The levels of invasion for the clinical strains ranged from 0.24% to 2.61%.

More in detail, strains 490, 1498 and particularly 566, showed low invasion levels of 2.40% (± 1.69%), 2.61% (± 1.47%) and 0.24% (±0.23%) respectively but without any significant difference with *L. innocua*.

Results obtained for food strains indicated a wide variability of adhesion levels, with higher values for strains 2018 (15.55% ± 5.55%), 1643 (12.63% ± 7.08%), and 1608 (12.54% ± 6.57%), and lower levels for strain 1715 (6.59% ± 1.99%) and 1484 (6.00% ± 0.40%) respectively. Strain 1487 showed the lowest adhesion level (3.78% ± 0.68%).

When compared with the *L. innocua* ATCC33090, a high level of significance was found for strains 1715, 1608, 1643 and 2018 ($p <0.001$), while for strains 1484 and 1487 no significant difference was found. Adhesion levels were not necessary associated with an increase in the number of bacteria that penetrate the epithelial cells.

Food strains 2018, 1487 and 1643 showed similar invasiveness percentages of 8.06% (± 7.64%), 5.75% (± 5.15%) and 7.19% (± 6.92%), respectively. Strain 1608 showed the lowest invasiveness 0.77% (± 0.19%), while the strains 1715 and 1484 presented the highest percentage of invasion of 20.90% (± 5.70%) and 17.40% (±1.03 %) respectively.

When compared with *L.innocua* ATCC33090, significant differences ($p < 0.001$) were found for strains 1715 and 1484.

The adhesion and invasion of *Lm* isolates were also analyzed by fluorescence microscopy staining cells with Hoechst dye (Figure 3).

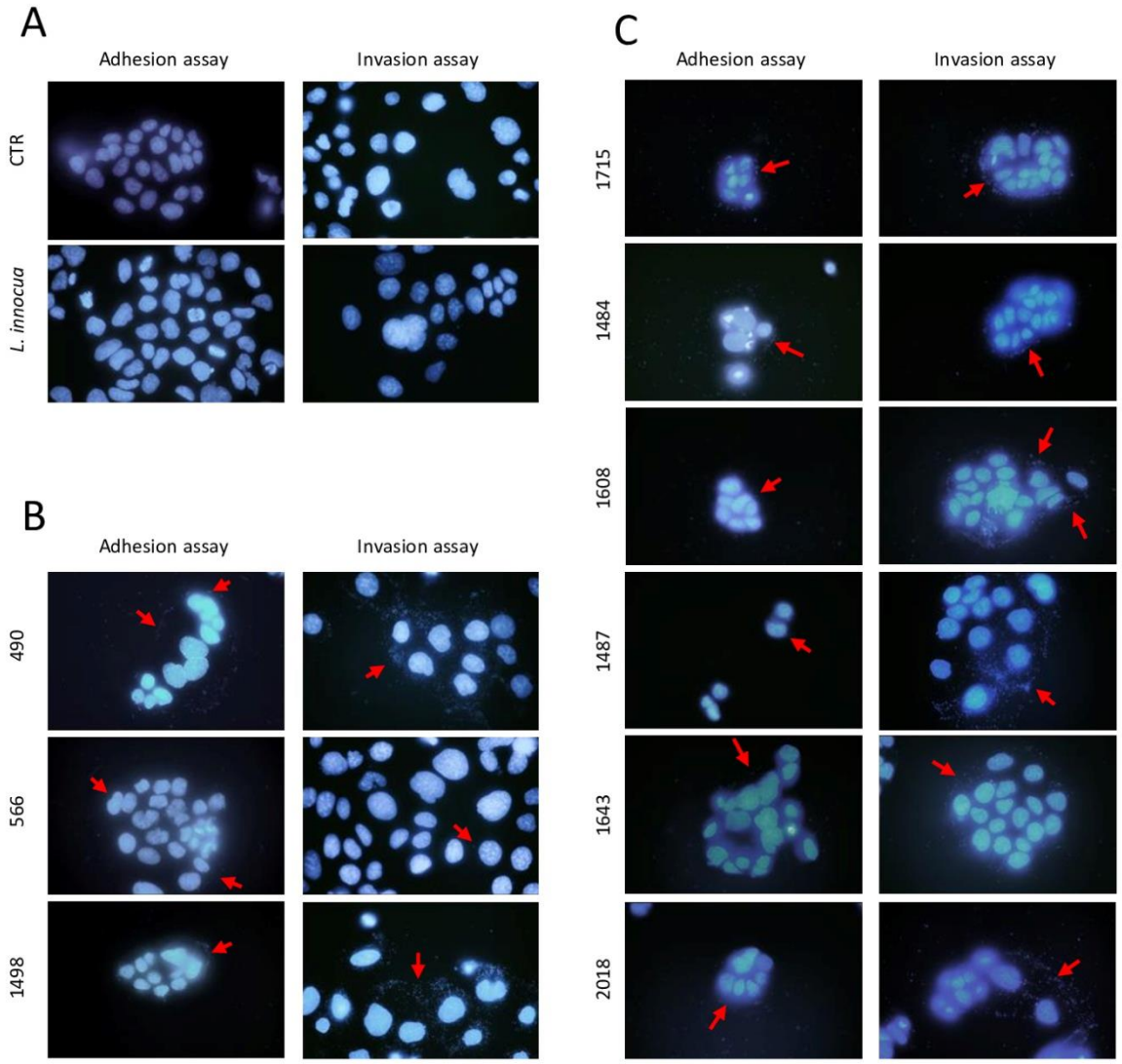

Figure 3. Hoechst staining. Panl A: CTR, uninfected Caco-2 monolayer, and *L. innocua* ATCC33090 infected monolayer; Panel B: Caco-2 monolayer infected with clinical strains; Panel C: Caco-2 monolayer infected with food strains.

As expected, neither adhesion nor invasion were visible in Caco-2 cells exposed to *L. innocua* ATCC 33090 (panel A). *Lm* strains of human and food origin, were detectable after both adhesion and invasion assays (panel B and C), with *Lm* 566 showing a not relevant adhesive

or invasive capacity. Due to the difficulty of distinguishing if the fluorescent bacteria were inside or over the Caco-2 cells, these data should be interpreted as qualitative.

3.2.1 Correlation between adhesion and invasion properties of all strains

As reported in Fig. 4A, in the clinical strains we found a correlation between the adhesion and invasion levels (R^2 = 0,9868), while no correlation was found between the two indexes in the strains isolated from foods (Fig. 4B).

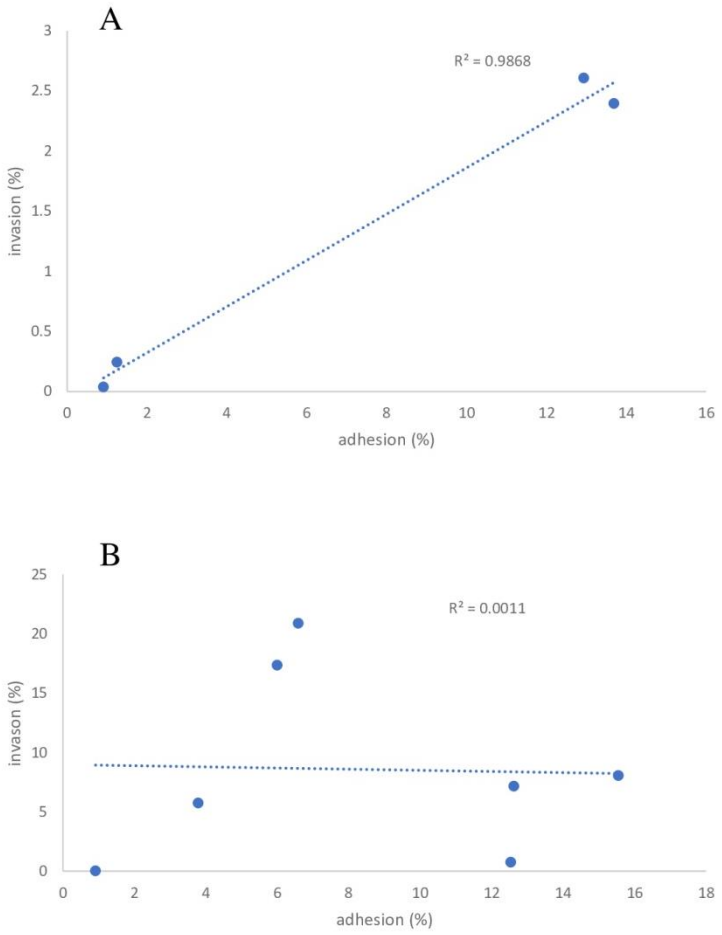

Figure 4. Correlation plot of the adhesion and invasion levels of 3 clinical (490, 566, 1498) (A) and 6 food (B) (1484, 1487, 1608, 1643, 1715, 2018) *L. monocytogenes* strains.

4. Discussion

Lm is an important foodborne pathogen with a significant public health concern worldwide. *Lm* presents a great genetic diversity and a wide variability in virulence potential. Several studies focused on *Lm* virulence potential distinguishing hypo- and hypervirulent clones on

the basis of the observed clinical frequency, virulence genes' profile and in vivo and *in vitro* assays [20, 30, 44].

The presence/absence of specific virulence associated determinants is considered a marker of increased or attenuated pathogenicity [11, 30]. In particular, virulence factors promoting adhesion and invasion of phagocytic and non-phagocytic cells, as well as the escaping from the vacuoles, are considered the most relevant in the prediction of virulence potential [12–16, 18, 22]. Previous authors aimed to evaluate the association between the presence/absence of the major virulence determinants and the ability of *Lm* to adhere and invade host cells obtaining different results [11, 14, 32].

The strains tested in this work were previously analyzed by Amagliani et al. (2021) [45] using a rt-PCR method targeting five virulence genes. We applied WGS to deepen the study of virulence extending it to a wider panel of genetic markers and evaluating the belonging to hypo- or hypervirulent CCs. The adhesion and invasion abilities of the strains were also assessed *in vitro* on the Caco-2 cells line. The obtained results confirmed those reported by Amagliani et al. (2021) [45] for the targeted genes *inlC*, *inlJ*, *inlF*, *lapB* and *lntA*, except for the absence of *inlF* in *Lm* 1498. In this strain the WGS analysis identify the *inlF* gene, showing higher sensitivity.

The human strain 566 was assigned to CC31, a clone sporadically isolated from humans and most frequently found in food [42, 46]. The belonging of this strain to a clone not defined hypervirulent was consistent with its low invasiveness; its ability to cause disease may have been due to the host's immunosuppression. The other clinical isolates belonged to CC1 and CC101, previously reported as clinical-source associated CCs with CC1 being considered one of the most hypervirulent [20, 47]. Despite that, these strains showed a low invasiveness during our experiments.

The *Lm* strains isolated from food were assigned to CC1, CC7, CC9, and CC121. As previously reported, CC1 and CC7 were frequently associated with human listeriosis but they were also detected in food products [20]. In particular, CC1 is considered the most prevalent clinical CC in several countries and it is strongly associated with cattle and dairy products [48]. CC7 instead, was previously defined an intermediate MLST clone between those mainly associated with infection and those strongly associated with food and was able to cause severe listeriosis outbreaks in the past [36, 49]. CC9 and CC121, instead, were previously defined hypovirulent clones with low clinical frequency but particularly adapted to food processing environments due to their high resistance to stresses [20, 30].

Consistent with the above, 1484 and 1715, belonging to CC1 and CC7 respectively, showed the highest level of invasiveness if compared with the other food isolates. The CC1 strain 1498 instead, unexpectedly presented a low invasiveness percentage despite having a good level of adhesiveness. In particular, results obtained for *Lm* 1715 were interesting considering that this strain belonged to the epidemic cluster causing the severe listeriosis outbreak occurred in Central Italy between 2015-2016. *Lm* belonging to CC9 and CC121 showed lower levels of invasiveness.

Investigating the virulence profiles, we observed that virulence gene count substantially differed only between CC1 strains and all others. Among the typed strains in fact, those belonging to CC1 were the only ones carrying the LIPI-3 in addition to the widely distributed LIPI-1. Consistently with these results, LIPI-3 was mainly described in lineage I and was previously reported in CC1 and CC4. It encodes a biosynthetic cluster involved in the production of Listeriolysin S (LLS), a hemolytic and cytotoxic factor conferring a greater virulence to *Lm* [15, 20, 21]. *LLS* is expressed only under oxidative stress conditions and this confers a better ability in terms of phagosome escape. Moreover, pathogenicity studies on murine models demonstrated that LIPI-3 was responsible for the increased virulence of some strains [14].

Among the typed strains presenting LIPI-3, 1484 and 1643, presented a good level of invasiveness while 1498 showed unexpectedly a low level.

PMSCs in the *inlA* gene were found only in *Lm* of food origin, in accordance with Van Stelten et al. (2010), who reported that a significantly greater proportion of RTE food isolates carried such mutation than human clinical isolates, which carried a full-length *inlA*. Moreover, consistently with several studies all the typed strains presenting a PMSC mutation belonged to CC9 or CC121 and two of them presented a low Caco-2 cells invasion ability *in vitro* [20,30,44].

The highest percentage of invasiveness showed by the strain 1715 could be explained to the presence in its genome of additional internalins' genes (*inlG* and *inlL*). Although the same genes were also carried by the *Lm* strain 1487 which did not show the same result *in vitro,* in this strain, the presence of a PMSC in the *inlA* gene may have reduced the invasion ability.

The teichoic acid biosynthesis genes *gltA* and *gltB* and the invasion gene *aut_IVb*, significantly more frequent among CC1, CC2, and CC6 clones than strains of the other CCs, were consistently detected only in 1484, 1498 and 1643 strains, all belonging to CC1 [50].

The remaining CC31 (566) and CC101 (490) did not present particular genetic features of hypo- or hypervirulence previously described. These strains presented low levels of

invasiveness and it was particularly noteworthy for 566. The extremely low level of invasiveness of this strain could be due, at least partly, to the lack of some virulence genes, such as *lapB* and *vip* or to the observed lower ability to adhere to Caco-2 cells. However, not for all strains there was a direct correlation between the level of adhesiveness and the one of invasiveness.

Despite the low number of tested strains, we observed the presence of MLST clones having a different virulence potential on a genetic basis with some of them carrying specific genetic determinants of hypo- or hypervirulence. These features were not necessarily predictive of the cell adhesion and/or invasion ability *in vitro*. This could be explained considering that the presence of specific virulence-associated genes not necessarily indicates the expression of the relative virulence factors but it can be used to evaluate the virulence potential of *Lm*.

Anyway, listeriosis results from the interplay between host and virulence features of the pathogen: the less immunocompromised host is, the more virulent *Lm* strain needs to be to cause disease [16].

The great limitation in performing studies evaluating *in vitro* virulence on a large number of strains is the use of cell culture models very laborious and expensive. A future perspective could be to extend the study to a larger number of strains using innovative biological models such us use of larvae that do not require any specific caging, are easy to handle [51].

5. Conclusions

In this study we observed that clinical strains responsable for cases of human listeriosis belonged to both hypo- and hypervirulent CCs and exhibited very low levels of invasiveness, reflecting how the occurrence of the disease may often be favored by a host's immunosuppressive state. In contrast, some *Lm* strains isolated from food belonged to hypervirulent CCs and presented good adhesive and invasive properties, highlighting the significant health risk for the consumer.

The combined approach of WGS and phenotypic assays can provide new insights establishing connections with variation in genetic information and phenotypes that influence *Lm* virulence.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ongoing studies

In vitro assessment of disinfectants' effectiveness on *Lm* strains

In order to assess *in vitro* the sensitivity of *Lm* strains to commercial sanitizers used in food industry, a part of the research activity that is still ongoing involved the development of an *in vitro* microplate assay evaluating the bactericidal effectiveness of different concentrations of sanitizers and its application on strains previously identified as persistent and/or carrying disinfectants resistance genes. All this was in order to verify if among the mechanisms responsible for persistence there was a lower sensitivity to sanitizers and to demonstrate the phenotypical expression of the carried tolerance genes. The method was drawn up basing on what reported by Cruz and Fletcher (2012) [88] with minor modifications as reported below.

Materials and Methods

Disinfectant dilutions

A commercial food-industry sanitizer based on BC (20%; corresponding to 200.000 μg/ml) and supplied by a local company, was tested in this study. The use concentrations recommended by the supplier were 200-600 ppm. Immediately before testing, the sanitizer was diluted in sterile water with 200 ppm added hardness (200 ppm hard water) containing $MgCl_2$, $CaCl_2$ and $NaHCO_3$ according to the AOAC (Association of Official Agricultural Chemists) Official Method 960.09: Germicidal and Detergent Sanitizing Action of Disinfectants [136]. The concentrated sanitizer was firstly diluted to 2000 μg/ml and to 1600 μg/ml. Starting from this last concentration, two-fold dilutions were prepared in order to obtain a total range of eight concentrations that included the maximum concentration recommended by the manufacturer (Table 1). Diluted sanitizer was used within 15 min of preparation.

	Sanitizer dilution dispensed in the well (50 µl on a total volume of 100 µl)	Final concentration of sanitizer in the well (experimental condition-EC)
1	2000 µg/ml	1000 µg/ml
2	1600 µg/ml	800 µg/ml
3	800 µg/ml	400 µg/ml
4	400 µg/ml	200 µg/ml
5	200 µg/ml	100 µg/ml
6	100 µg/ml	50 µg/ml
7	50 µg/ml	25 µg/ml
8	25 µg/ml	12,5 µg/ml
9	50 µl of hard water	0

Table 1. Sanitizer dilutions used and final concentration of sanitizer in each well (Experimental condition-EC).

Bacterial suspension

Frozen (-20 °C) stock cultures were resuscitated in Agar Listeria acc. to Ottaviani & Agosti (ALOA) plating a cryovials bead and incubating plates at 37°C for 24h. *Lm* strains were then grown overnight at 37°C in Tryptone Soya Yeast Extract Broth (TSYEB) to achieve stationary phase. After the incubation, bacterial suspensions were centrifuged at 3200xg for 5 minutes and then cells were washed in sterile 200 ppm hard water, collected by centrifugation and re-suspended in a final volume of 10 ml of sterile 200 ppm hard water. The concentration of the obtained bacterial suspensions was adjusted to an OD_{600} of ~ 0.125±0.05 corresponding to 10^8 CFU/ml and then diluted 1:5 to obtain a final concentration of $2x10^7$ CFU/ml. To verify the number of viable cells in the inoculum, it was enumerated by 10-fold serially diluting in 0.85% saline solution and using the drop plate counting method (4 x 25 µl) on TSYEA agar plates which were incubated at 37°C for 24h.

Determination of the minimal effective concentration (MEC) of sanitizer on *Lm* suspension

An aliquot (50 ml/well) of each sanitizer dilution was added to the wells of a microplate, followed by 50 ml/well of the final planktonic cell suspension which was dispensed using a multichannel pipette in order to guarantee the same contact time for each condition. After mixing gently, the mixed suspension was left for 5 min (contact time) at 25 °C. In Table 1 all

the experimental conditions (EC) tested are shown; EC 9 represented the negative control wells containing only 200 ppm hard water and the bacterial suspension.

After the contact time, 150 ml of a neutralizer solution containing 5% egg yolk emulsion (Difco), 1% sodium thiosulphate (AnalaR, BDH Chemicals Ltd, Poole, England) and 0.5% Tween-80 (Spectrum, Gardena, CA) in TSYEB was applied to each well to neutralize the antimicrobial effect of the sanitizers and was mixed.

The number of viable cells in the suspension contained in each well was enumerated by 10-fold serially diluting in 0.85% saline solution and using the drop plate counting method (4 x 25 µl) on TSYEA agar plates. The plates were incubated at 37 °C overnight. The most diluted suspension of the tested sanitizer to show a viable bacterial reduction of 5-log10 CFU/ml (99.999% reduction), compared to the control, was considered as the minimal effective concentration (MEC) (UNI EN 1040:2006; UNI EN 1276:2019).

Preliminary Results

In this study six *Lm* strains were tested which belonged to the set of strains previously analysed in the Original Research Article 1 [55]. In particular, all the selected strains were isolated from the Meat_A plant: two of them belonged to the long-term persistent cluster A (*Lm*_1353; *Lm*_1791), two were from the persistent cluster B (*Lm*_2211; *Lm*_2266) and two from cluster C (*Lm*_2275; *Lm*_2268). Among these strains only those belonging to cluster C carried specific genetic determinants for resistance to BC and in particular the Tn6188_*qac*.

The obtained results expressed as $\log_{10}$ reduction are showed in Table 2. In all the strains the sanitizer gave a total reduction of the inoculated viable cells up to a concentration of 50µg/ml. The reduction in viable cell number resulted from the lower concentrations of 25µg/ml and 12.5µg/ml was similar between strains belonging to the same cluster. In particular, the reduction showed by the sanitizer at 25µg/ml was higher in strains belonging to cluster C, following by those of cluster B while in strains from cluster A this concentration produced the lowest reduction values. The concentration of 12.5µg/ml gave similar results in the strains belonging to clusters B and C and lower values in strains from cluster A.

ID	Cluster	log_{10} reduction CFU/ml			
		50µg/ml	40µg/ml	25µg/ml	12.5µg/ml
*Lm*_2275	C	Total reduction	4.0	3.1	0.7
*Lm*_2268	C	Total reduction	2.8	2.8	0.7
*Lm*_1353	A	Total reduction	2.5	1.3	0.2
*Lm*_1791	A	Total reduction	4.9	1.5	0.4
*Lm*_2211	B	Total reduction	4.8	2.1	0.8
*Lm*_2266	B	Total reduction	4.9	2.2	0.7

Table 2. Log_{10} reduction in viable cell number (CFU/ml) produced by different sanitizer dilutions. The concentrations higher than 50µg/ml are not shown.

In order to identify the exact MEC value, an additional intermediate dilution of 40µg/ml was tested (Table 2). For *Lm*_1791, *Lm*_2211 an *Lm*_2266 the additional dilution of the sanitizer showed a reduction of about 5-log_{10} CFU/ml and so represented the exact MEC. For the remaining strains this concentration proved to be insufficient to produce an effective reduction of viable cells with *Lm*_2275 showing a reduction value of 4-log_{10} CFU/ml and *Lm*_1353 and *Lm*_2268 of about 3-log_{10} CFU/ml (Table 2).

Discussion and future perspectives

The preliminary results indicated that all the recommended use concentrations of the commercial sanitizer tested were effective on *Lm* being significantly higher than the MEC. However, traces of highly diluted sanitizer can reach niches harbouring *Lm*, where isolates less susceptible might have a growth advantage [72]. Very interestingly, both the strains belonging to the long-term persistent cluster A were the least sensitive to the lowest sanitizer concentrations (12.5 and 25µg/ml) with the *Lm*_1353 also showing a smaller log-reduction after exposure to a BC concentration of 40µg/ml which was found to be the MEC, or close to it, for most of the other strains. These isolates did not carried specific genetic determinants for resistance to BC, they only presented the same determinants for different multidrug efflux-pumps (*sugE*, *mdrl*, *lde*, *norM* and *mepA*) as all other strains [55].

Despite in most cases resistance to BC in *Lm* may be due to the acquisition of QAC-specific efflux pumps, several authors reported that multidrug efflux pumps were also involved in BC tolerance [137–139]. Previous studies observed an increased expression of *mdrl* in *Lm* strains

not carrying QAC-specific determinants when exposed to sublethal concentration of BC [137,138]. Moreover, Romanova et al., (2006) [137] observed that there were naturally resistant strains in which efflux pumps played no role in the innate resistance to BC. Furthermore, resistant strains that were negative for genetic determinants specific for tolerance to BC could presumably harbor novel resistance determinants [140].

Therefore, there may be more explanation for the less sensitivity of cluster A to low concentrations of BC. This may have contributed to the long lasting persistence of this cluster in the plant providing a survival and growth advantage in specific environmental niches.

The only two strains harbouring the Tn6188_*qac*, were completely killed by a BC concentration of 50 µg/ml with the lowest concentrations of 25µg/ml and 12.5µg/ml producing ~3-$\log_{10}$ and 1-$\log_{10}$ viable bacterial reduction respectively. For both these strains, the additional concentration of 40µg/ml was insufficient to reach an effective 5-log reduction of viable cells. If compared with recent studies, these results indicated a lower susceptibility towards BC. Indeed, Møretrø et al. (2016) [141] reported a reduction range of 0.6-2.2 $\log_{10}$ with 10µg/ml of BC and Andrade et al. (2020) [90] reported a 4-$\log_{10}$ reduction with BC concentrations from 12.5 to 20µg/ml.

One of the future perspectives of this study will be to extend the effectiveness assessment to other disinfectants, perhaps after asking the FBO for those specifically used in the plant where the strains were isolated. This would allow to support FBOs in contrasting *Lm* persistence in FPP, minimizing the risk of food contamination. Another important aspect, planned for the near future, is to evaluate the effectiveness of those sanitizers on *Lm* biofilm.

Moreover, it would also be very interesting to perform a genome-wide association study (GWAS) to identify genes associated with tolerance to sanitizers in *Lm*.

Finally, the next goal for the future is also to extend the study to other FPPs improving the monitoring activity of *Lm* in FPEs.

Conclusions

Many mechanisms may contribute to survival and persistence of hypo and hypervirulent clones of *Lm* in FPEs, with complex interactions of changing factors from case to case. A multidisciplinary approach based on both genomic and phenotypic analysis is required to better understand this phenomenon.

This study focused on evaluating the role of FPEs as reservoir of hypo- and hypervirulent clones of *Lm*, improving knowledge about persistence and virulence characteristics of *Lm* strains associated with small-scale food processing plants of Central Italy and identifying genetic biomarkers that can be used to predict their adaptation and long-term survival in food-processing facilities. A total of 205 *Lm* strains, isolated from foods and environmental surfaces in different FPEs of Central Italy, were studied combining genome analysis with different *in vitro* assays.

WGS was applied on all the strains since it provides the most comprehensive overview of the full bacterial genome with the highest possible microbial subtyping resolution compared to typing methods used in the past. Through the combination of different bioinformatics analysis, it was possible to identify *Lm* clones persisting over years in the same FPP as well as clones contaminating different FPP of Central Italy. In addition, the WGS approach provided insights into the dynamics of stress tolerance-related genetic markers promoting survival and persistence of *Lm* CCs in FPEs and gave information about their virulence potential.

On the other hand, despite their known limits, *in vitro* assays were applied on a selection of the *Lm* studied strains, adding important information about biofilm formation and sensitivity to BC as well as their adhesion and invasion abilities.

In particular, strains belonging to the same genetic cluster may exhibit a different biofilm-forming phenotype and the amount of produced biofilm did not seem to be decisive for long-term persistence in FPEs. Indeed, once a strain is able to produce biofilm, even just a thin layer of it, if formed in niches that are difficult to reach during sanitation procedures, represents a persistent source of contamination.

About *Lm* sensitivity to BC, despite the low number of tested strains, the microplate assay used in this study showed that strains belonging to a long term persistent cluster, despite not carrying specific genetic determinants for tolerance to QAC, were less sensitive to low sanitizer concentrations than the other strains. This suggested that there may be other mechanisms involved in tolerance to BC in these strains. On the other hand, if compared with what was reported in recent studies on *Lm*, our results indicated a lower susceptibility to BC for the CC121 strains harbouring the Tn6188_*qac*.

However, having been studied in a preliminary way and on a small number of strains, these aspects need further investigation.

For the *in vitro* assessment of *Lm* virulence three clinical *Lm* strains in addition to food isolates were also included. The obtained results showed that strains that were responsible for human listeriosis not necessarily exhibited a higher ability to invade Caco-2 cells when compared with food isolates, some of which in contrast, presented good adhesive and invasive abilities representing a relevant risk for the consumers' health. Similarly, strains presenting genetic features associated to increased virulence, not necessarily were characterized by high invasiveness if tested *in vitro* as well clones lacking specific virulence determinants could exhibit the ability to adhere and invade Caco-2 cells. These results could be explained considering that the presence of specific virulence-associated genes not necessarily indicates the expression of the relative virulence factors, but the detection of such determinants can be used to predict the virulence potential of *Lm*. Moreover, the small strain number and CC types tested in this study, hinder result generalization not allowing us to find significant correlation between CCs, virulence profiles and the ability to invade Caco-2 cells *in vitro*. On the other hand, once ingested, the success of a *Lm* strain in generating disease in the host derives from a fine balance between surviving in the gastrointestinal tract and successfully colonizing the host. Therefore, using a cellular model to assess *in vitro* the virulence of *Lm* strains presents limitations and does not allow having a complete picture with respect to an animal model. Moreover, evaluating *Lm* virulence *in vitro* on a large number of strains requires the use of cell culture models and is very laborious and expensive. In recent years, use of larvae of the greater wax moth *Galleria mellonella* has emerged as a promising model for the assessment of virulence of *Lm* as these larvae are cheap, do not require any specific caging, are easy to handle and their immune systems closely resemble that of mammals [62]. A future perspective could be to extend the study to a more representative group of strains using innovative biological models.

The spread, both at production and retail level, of hypovirulent CCs such as CC9 and CC121, more adapted to FPEs and able to persist after cleaning and sanitation represented a significant risk for food cross-contamination. On the other hand, in this study hypervirulent clones (CC1 and CC2) were also detected in FPEs and some of these strains warningly persisted for long time in the same plant. These findings demonstrated that persistence of *Lm* is not necessarily or exclusively the result of a contamination by strains having specific and unique genetic traits or phenotypic abilities. The fitness of a strain is relative to the environment with which it is interacting. Moreover, besides the specific characteristic of the

FPE (presence of ecological niches, non-compliant structures and equipment) and the survival abilities of the strains, other factors can influence *Lm* survival and persistence such as inappropriate processing, ineffective cleaning and sanitizing protocols and systematic reintroduction of contaminated raw materials. Identifying the main mechanisms promoting *Lm* survival and persistence in a specific FPP would allow providing FBOs with effective recommendations for remove or reduce resident *Lm*. Those corrective actions could include the use of different sanitizers in a rational combination or turning them, or increased attention in the sanitation of environmental niches and harbourage points (drains, doors, cleaning materials, sinks, porous or abraded surfaces ecc..).

Concluding, FPEs widely harbour hypo- and hypervirulent *Lm* representing potential sources of food contamination. Therefore, a systematic *Lm* monitoring of FPEs should be included in Italian food safety surveillance plans performed by the Competent Authorities, designing an effective, risk-based environmental monitoring program, and defining the guidelines for key design elements, such as the number, location, timing and frequency of sampling as well as standard criteria for classifying surfaces into specific categories. This will improve the management of the pathogen in the food industry minimizing risk of food contamination and recurrence of severe outbreak of listeriosis as that which occurred in Central Italy between 2015 and 2016.

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