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Article

Prevalence, Diversity of *Listeria* spp. Isolates from Food and Food Contact Surfaces and Occurrence of Virulence Genes

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Abstract: This study aimed to evaluate the hazards posed by foodborne bacteria of the *Listeria* genus by analyzing prevalence, diversity and virulence of *Listeria* spp. in food and food manufacturing plants.
Seventy five isolates obtained from the routine analysis of 653 samples by three diagnostic laboratories in Northern Italy were genotipically differentiated by Repetitive Extragenic Palindrome (rep) PCR with the GTG5 primer, identified by sequencing the 16S rRNA gene and examined by specific PCR tests for the presence of *L. monocytogenes* virulence determinants occasionally found to occur in other species of the genus. The identity of the amplification products was confirmed by sequencing.
Fifty seven isolates were identified as *L. innocua*, 12 as *L. monocytogenes*, 5 as *L. welshimeri* and one as *L. seeligeri*.
All *L. monocytogenes* isolates belonged to the serotype 1/2a and were predicted to be virulent for the presence of the *inl*J internalin gene.
Potentially virulent strains of *L. innocua*, *L. seeligeri* and *L. welshimeri*, carrying the *L. monocytogenes inl*A gene and/or *hly* gene, were identified, and most isolates were found to possess the toxin-antitoxin system *maz*EF for efficient adaptation to heat shock.
Results indicated the need to reinforce food contamination prevention measures against all *Listeria* species by efficiently defining their environmental distribution.

Keywords: *Listeria* spp.; Food; Food contact surfaces; Genotyping; Virulence genes; toxin-antitoxin system *maz*EF

1. Introduction

Bacteria belonging to the low G+C facultatively anaerobic, Gram-positive *Listeria* genus are ubiquitous and can contaminate food products of both animal and plant origin [1].

The human pathogen *Listeria monocytogenes* can cause severe foodborne infections with fatality rates of 20–30%, including septicemia, meningitis and endocarditis, in the elderly, immunocompromised persons and pregnant women, with resulting abortion or neonatal infections [2]. It grows at refrigeration temperatures, under vacuum or modified atmosphere, tolerates low pHs and forms sanitization resistant biofilms in food processing plants. These characteristics make contamination prevention the main defense against this pathogen [3]. Ready-to-eat foods are those with higher risk to transmit the bacterium. In these products *L. monocytogenes* must not reach levels higher than 100

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CFU/g during the shelf life and it must be absent in foods intended for infants and special medical purposes [4].

Beyond *L. monocytogenes*, *L. ivanovii* is pathogenic in animals and humans [5]. In addition, illnesses caused by virulent strains of *L. innocua* and *L. seeligeri* [6-8], have been reported. Meningitis was caused by a *L. innocua* strain possessing *L. monocytogenes* internalin genes *inl*A and *inl*B and genes *prf*A, *hly* and *plc*A of the pathogenicity island 1 (LIPI1) [8], considered relics left after divergence from *L. monocytogenes* [9]. Atypical *L. innocua* strains possess different combinations of *L. monocytogenes* LIPI-1, *inl*A and *inl*B genes [10]. Strains harboring only LIPI-1 are hemolytic but not virulent, while those expressing InlA enter non phagocytic epithelial cells expressing InlA receptor E-cadherin (Ecad) as efficiently as *L. monocytogenes*. However, *L. innocua* expressing *inl*A, but not LIPI-1 genes, was not able to escape from the vacuole and polymerize actin in the cytosol and was, therefore, less virulent than the counterpart expressing LIPI-1 genes in a mouse model [10]. Other hemolytic *L. innocua* strains possess the *L. monocytogenes* pathogenicity island LIPI-3, comprising the hemolysin S *lls*A gene, frequently found in lineage I of *L. monocytogenes* [11].

Not much is known about the occurrence of virulent non-*monocytogenes Listeria* strains in food so that this study was carried out to analyze the prevalence, diversity and virulence traits of *Listeria* spp. isolated during routine analysis of food products and swabs from production plant surfaces in contact with food.

Molecular typing by rep-PCR with the GTG5 primer, allowing rapid *L. monocytogenes* strain differentiation [12], was applied to select representative isolates to be identified by 16S rRNA gene sequencing.

The presence of the *L. monocytogenes* genes *inl*A and of *hly* and *lss*A, indicators of *L. monocytogenes* LIPI-1 and LIPI-3 presence, respectively, was analyzed in non-*monocytogenes Listeria* isolates to identify potentially virulent strains.

The *L. monocytogenes* isolates were identified at the serotype level by the multiplex PCR test described by Chen and Knabel, 2007 [13] and their virulence was assessed based on the presence of the putative internalin gene *inl*J, corresponding to locus *lmo2821* in the complete genome of *Listeria monocytogenes* EGD-e (acc.no. AL591824), invariably associated with virulence in mice [14].

An analysis of the distribution of the *maz*F gene, a component of the type II toxin-antitoxin system *maz*EF, conferring increased tolerance to heat shock [15], was carried out to obtain indications on the tolerance of the isolates to a key stress factor applied in food production.

2. Materials and Methods

2.1. *Bacterial strains and culture conditions*

Reference strains *L. monocytogenes* ATCC 7644 and *L. innocua* ATCC 33090 were used for genotypic profile comparison. One hundred and two presumptive *Listeria* spp. isolates, obtained by three diagnostic laboratories (Lab 1, 2 and 3) that in years 2016 – 2017 analyzed 653 samples of food and food plant surfaces in contact with food in Northern Italy, were characterized in this study. All bacterial strains were sub-cultured in Tryptic Soy (TS) broth (Biolife Italiana, Milan, Italy), incubated in aerobiosis at 37°C for 18 h. For long term storage, the cultures were kept at -20°C in the same medium added of 20% glycerol.

Isolates identified genotypically as *Listeria* spp. were tested for hemolytic activity on blood agar medium (Liofilchem, Roseto degli Abruzzi, TE, Italy).

2.2. *DNA extraction*

DNA was extracted from 2 ml of fresh culture using the Genomic DNA Extraction kit RBC Bioscience (Diatech Labline, Jesi, AN, Italy) according to the instructions. The quantity and integrity of the extracted DNA were checked by electrophoresis on 1.5% w/v agarose gel in 1× TAE buffer (80 mM Tris-acetate, 2 mM EDTA, pH 8.0) at 120 V stained with 1× GelRed (Biotium, Società Italiana Chimici, Rome Italy) in the recommended quantity.

2.3. *PCR tests*

PCR primers used in this study, respective targets and references are reported in Table 1.

Table 1. Oligonucleotides used in this study, respective targets, amplicon size and references.

Primers	Sequence (5′→3′)	Usage	Amplicon size (bp)	Reference
7f 1492r	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	16S rRNA gene amplification	1494	[16]
GTG_5	GTGGTGGTGGTGGTG	Rep-PCR genotyping	n.a.	[12]
4bF 4bR	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	identification of the *L. monocytogenes* serotype 4b	597	[13]
12aF 12aR	GAGTAATTATGGCGCAACATC CCAATCGCGTGAATATCGG	identification of the *L. monocytogenes* serotype 1/2a	724	[13]
ECIF ECIR	AATAGAAATAAGCGGAAGTGT TTATTTCCTGTCGGCTTAG	identification of the *L. monocytogenes* epidemic clone ECI	303	[13]

ECIIF ECIIR	ATTATGCCAAGTGGTTACGGA ATCTGTTTGCGAGACCGTGTC	identification of the *L. monocytogenes* epidemic clone ECII	889	[13]
ECIIIF ECIIIR	TTGCTAATTCTGATGCGTTGG GCGCTAGGGAATAGTAAAGG	identification of the *L. monocytogenes* epidemic clone ECIII	497	[13]
HlyF HlyR	CATTAGTGGAAAGATGGAATG GTATCCTCCAGAGTGATCGA	detection of the *L. monocytogenes hly* gene	730	[17]
inlAF1 inlAR1	TAACATCAGTCCCCTAGCAGGT TAGCCAACCTGTCACTATTGGA	detection of the *L. monocytogenes inl*A gene	516	[9]
lmo2821F lmo2821R	TGTAACCCCGCTTACACAGTT TTACGGCTGGATTGTCTGTG	detection of the *L. monocytogenes inl*J gene	611	[14]
llsAFor llsARev	CGATTTCACAATGTGATAGGATG GCACATGCACCTCATAAC	detection of *L. monocytogenes lls*A gene	280	[11]

mazF_qPCR_Fw	ACGGCCTGTTCTCATCATTC	detection of the *Listeria* spp. *maz*F gene	103	[15]
mazF_qPCR_Rv	CGTTGGCAATTTTGCTTTTT			

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Primers mazF_qPCR_Fw and mazF_qPCR_rv were evaluated by BLASTn (https://blast.ncbi.nlm.nih.gov) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) for their ability to target *maz*F homologs in *Listeria* species other than *L. monocytogenes*.

All of the PCR tests were carried out with the EmeraldAmp GT PCR Master Mix Takara Clontech (Diatech, Jesi, Italy) under the conditions described in the respective literature (Table 1). The amplification products were separated on 1.5% (w/v) agarose gels.

2.4. *Numerical analysis of genotypic profiles*

Genotypic profiles obtained by rep-PCR were analyzed by the BioNumerics V5.10 software (Applied-Maths, Belgium), using the Dice coefficient for pairwise comparison and the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) clustering.

2.5. *Sequencing and sequence analyses*

Before sequencing PCR products were purified using the HiYield Gel/PCR Fragment Extraction Kit RBC Bioscience (Diatech) according to the instructions. Sequencing of both amplicon strands was carried out by Eurofins Genomics (Ebersberg, Germany), with the same primers used for amplification. Sequencing was carried out for species-level identification based on 16S rRNA gene identity and to confirm PCR product identity in *inl*A, *inl*J, *hly*, *lls*A and *maz*F genes targeted assays. Sequencing of the 16S rRNA gene was carried out for one isolate from each rep-PCR cluster separated at 85% similarity, a cut off value chosen on the basis of the lowest similarity exhibited by ten duplicate strains in preliminary experiments.

3. Results

3.1. *Prevalence of* Listeria *spp. in food and food plant surface samples*

The number of samples analyzed by each laboratory, none of which came from the same production facility, is reported in Table 2. The 102 presumptive *Listeria* spp. isolates initially examined were numbered from 1 to 30, from 31 to 69 and from 70 to 102 for Lab1, 2 and 3, respectively, but only those confirmed to belong to *Listeria* spp. by genetic tests are reported in Table 2.

Table 2. Number of samples analyzed by each laboratory, sample categories and respective *Listeria* spp. isolates as identified by molecular assays.

	Number of samples analyzed and respective *Listeria* spp. isolates*		
Sample category	**Lab1**	**Lab2**	**Lab3**
Bovine meat	102 samples	57 samples	98 samples
	L. innocua 8, 9, 11, 12, 13, 16, 18, 21, 23, 24, 26, 27; *L. monocytogenes* 10	*L. innocua* 42, 43, 46	*L. innocua* 79, 80, 82, 85, 89, 91, 92, 94, 95, 96, 98, 99; *L. monocytogenes* 87, 97, 102; *L. welshimeri* 81, 84, 86

Pork meat	31 samples *L. innocua* 4; *L. monocytogenes* 1, 2, 3, 7	9 samples	21 samples
Fresh fish	26 samples *L. innocua* 14, 15, 20	5 samples	14 samples
Pork raw cured products	16 samples *L. monocytogenes* 5, 6	13 samples *L. welshimeri* 44	14 samples *L. innocua* 73, 75
Frozen pizza	14 samples *L. innocua* 19, 25	13 samples	8 samples *L. innocua* 71, 72, 76, 77
Pizza dough	5 samples	2 samples *L. innocua* 32, 49	22 samples *L. innocua* 78
Frozen pasta	4 samples	11 samples *L. innocua* 40, 41	8 samples *L. innocua* 74
Roast peppers	3 samples	12 samples *L. innocua* 34, 35	0 samples
Swabs from cheese-making plants	53 samples *L. innocua* 28, 29, 30; *L. seeligeri* 22	19 samples *L. innocua* 31, 45, 47, 48	25 samples
Cheese aging boards	22 samples	12 samples *L. innocua* 54, 58, 69; *L. monocytogenes* 63, 67; *L. welshimeri* 61	14 samples

*Numbers missing in the series correspond to isolates for which identification as *Listeria* spp. was not confirmed by genotypic tests.

3.2. *Typing and identification of* Listeria *spp. isolates*

Numerical analysis of rep-PCR profiles allowed the distinction of three main clusters exhibiting comparable internal diversity, each comprising exclusively *L. innocua*, *L. monocytogenes* or *L. welshimeri* isolates, as ascertained by sequencing the 16S rRNA gene, with the exception of *L. welshimeri* 81 that fell outside the group formed by the other *L. welshimeri* isolates. One isolate not belonging to any of the three main clusters was identified as *L. seeligeri* (Figure 1). In all cases, identification at the species level was based on 99% sequence identity with entries in the public domain database.

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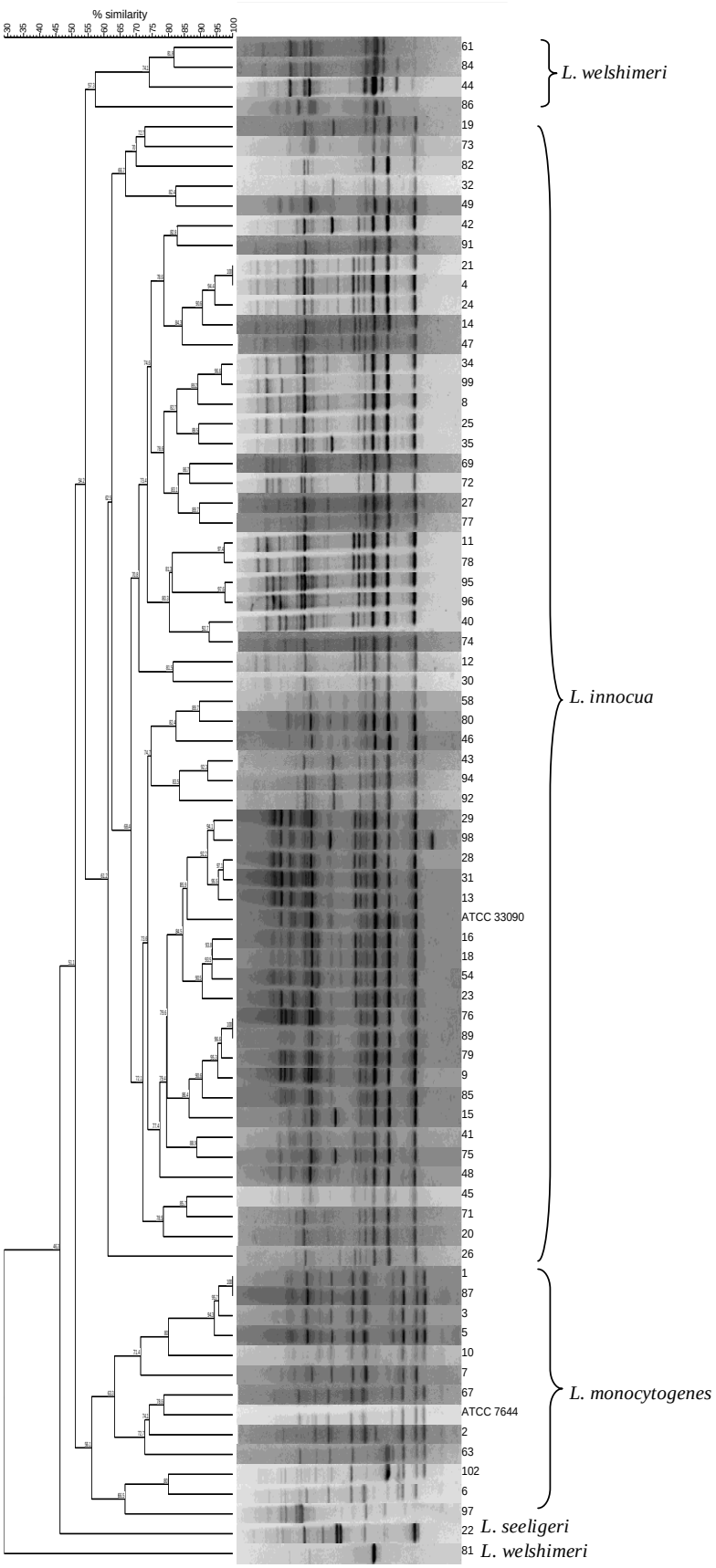

Figure 1. Clustering of *Listeria* spp. isolates from food and food production plants based on rep-PCR profiles.

From Figure 1 and Table 2, it can be observed that isolates deriving from the same sample type showed less than 85% profile similarity. Thus, different subtypes of *Listeria* spp. occurred in the same food category. Conversely, clusters composed of highly similar genotypes included isolates from different food categories, showing that some *Listeria* spp. subtypes are not associated with specific food products and are distributed across diverse environments.

It can be observed that isolate 5 from a sample of raw sausage appeared highly similar to isolates 1 and 3 from fresh pork (Figure 1, Table 2). These isolates might, therefore, represent an *L. monocytogenes* subtype able to survive in ready to eat cured pork products.

Based on genotypic identification, the prevalence of *Listeria* spp. was 13.6% for bovine meat, 8.1% for pork meat, 2.2% for fresh fish, 11.6% for raw cured pork products, 17.1% for frozen pizza, 10.3% for pizza dough, 13% for frozen pasta, 13.3% for roasted peppers, 8.2% for cheese-making plant swabs, and 27.2% for swabs from cheese-aging boards.

The presence of non-*monocytogenes* listerial species in all sample types can indicate co-occurrence of *L. monocytogenes* [18]. The latter was detected in 1.5% fresh bovine meat samples, 6.5% fresh pork meat samples, 4.6% raw sausages, and 4.1% swabs from cheese-aging boards.

Prevalence variability among the three laboratories for different sample categories indicated an uneven distribution of *Listeria* spp. among sampling locations (Table 2).

3.3. Pathogenic potential of the Listeria *spp. isolates*

The multiplex PCR assay of Chen and Knabel 2007 [13] allowed to identify all of the *L. monocytogenes* isolates as serotype 1/2a, though none belonging to the major epidemic clone ECIII belonging to this serotype. *L. monocytogenes* isolates exhibited β-hemolysis and were all positive with the *inlJ* gene targeted PCR assay, being, therefore, classifiable as virulent according to Liu et al. 2003 [14].

Potentially virulent non-*monocytogenes Listeria* isolates represented the 0.93% of isolates. Indeed, an amplification product with 100% sequence identity with the *L. monocytogenes inl*A gene was obtained from *L. innocua* 74, *L. seeligeri* 22, and *L. welshimeri* 86. *L. innocua* 23, 45, and 95 and *L. welshimeri* 86 and 98 gave a *L. monocytogenes hly* gene specific amplification products confirmed by sequencing. Notably, *L. welshimeri* 86 endowed with both *L. monocytogenes inl*A and *hly* genes, could be highly virulent according to Moura et al. 2019 [10]. However, as all the other non-*monocytogenes* isolates, it did not exhibit hemolytic activity, indicating the inability to express *hly*. The *lls*A gene was not detected in any non-*monocytogenes* isolate.

The *maz*EF toxin-antitoxin system appeared to be absent in *L. innocua* isolates 31, 35 and 80 and to be most likely mutated at the primer annealing sites for *L. monocytogenes* isolates 6 and 10, for which amplification was inefficient with the *maz*F specific PCR test. However, most isolates were shown to possess this additional stress adaptation system.

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4. Discussion

This study presents the results of *Listeria* spp. prevalence based on the analysis of a rather high number of food products and food contact surfaces in manufacturing plants and, as such, provided a faithful representation of the diffusion of these bacteria and consumer exposure. Though with differences among the food categories, *Listeria* spp. appeared to be frequent in all food matrices and production plants, indicating that safety measures to prevent contamination must be improved since innocuous species are considered indicators of *L. monocytogenes* co-occurrence.

Rep-PCR with the GTG5 primer was chosen as genetic typing method for its rapidity, easiness and intra-species discriminating power. Its application to different species in this study highlighted the usefulness in differentiating isolates of *L. monocytogenes*, *L. innocua*, and *L. welshimeri*. The application of this technique in new studies regarding different *Listeria* species could lead to the definition of profile types to be used as references for a rapid preliminary identification of new isolates at the species level allowing, at the same time, intra-species clustering. The intra-species relationships defined by this genotyping method showed that some subtypes are not associated with specific food products and are distributed across diverse environments, purportedly for their better ability to persist in processing plants. These subtypes should be thoroughly characterized genotipically and physiologically and preferentially used to test the efficiency of sanitizing procedures.

A *L. monocytogenes* subtype, possibly derived from fresh meat, was identified in raw fermented sausages, a ready to eat food category. This highlighted that the risk of *L. monocytogenes* growth at unacceptable levels should be better assessed for this food category and that more efforts must be devoted to prevent the presence in pork meat and processing plants of *L. monocytogenes* by identifying specific contamination routes.

Isolation of *L. monocytogenes* serotype 1/2a in this study is in agreement with reports of its wide distribution in food and food-processing environments [12,19,20] and with the consequent numerous listeriosis outbreaks caused by this serotype until recently [21- 25].

In this investigation, the genetic analysis of virulence characters highlighted that food can be a source of potentially pathogenic strains of *Listeria* spp. belonging to species generally considered to be innocuous. It was found that the *L. monocytogenes inl*A and *hly* virulence determinants can be harboured not only, as previously reported, by atypical *L. innocua* strains but by *L. welshimeri* and *L. seeligeri* isolates as well. Therefore, species identification is not sufficient to estimate the risk associated with the presence of *Listeria* spp. in food and contamination prevention and identification of contamination sources should be extended to all *Listeria* species.

The presence in most isolates of the *maz*EF toxin-antitoxin system, indicating efficient heat shock adaptation capacity, underlines the need to reinforce contamination prevention to reduce the risk for consumers that these bacteria pose.

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