RESEARCH ARTICLE

Genomic and phenotypic characterisation of *Listeria monocytogenes* strains isolated from pig feces on farm and from pork meat at retail in France

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Abstract

Listeria monocytogenes is an ubiquitous foodborne pathogen transmissible to humans through the consumption of contaminated products, including pork. In this study, we characterised the *Listeria monocytogenes* population circulating from the primary reservoir, pigs, to consumers in France. In 2008 and 2010, 147 strains from on-farm pig feces and 154 strains from retail pork meat were respectively isolated. Serogroups and clonal complexes (CC) were determined (n = 301), and 123 representative strains were further assessed for virulence, biofilm-forming ability, and resistance to Benzalkonium chloride (BC). Serogroup IIb, and CCs such as CC11, CC21, CC20, CC26, CC31, CC59, CC37, CC1 and CC77 strains predominated on farms, whereas serogroup IIc and IIa (respectively CC9 and CC121) were mainly recovered from retail meat. All 123 strains harbored the virulence-associated genes *inlA*, *inlC, inlJ, plcA*, *prfA*, *actA*, *hlyA* and *iap.* Overall virulence did not differ significantly between farm- and retail-derived strains; however, serogroup IVb strains were significantly more virulent, while serogroup IIc strains exhibited lower virulence. In our study, CC1 (mainly present in feces) and CC121 (present only on meat) showed the highest virulence level. Biofilm-forming ability was significantly higher in strains isolated from farm feces than in those from retail meat. Most meat strains, including CC121, CC9, CC14 and CC7, were more resistant to benzalkonium chloride than fecal strains. Our findings highlight the genetic and phenotypic evolution of the *Listeria monocytogenes* population along the pig-to-pork continuum. They suggest that strains persisting to the retail stage are more strongly associated with resistance to biocides than with robust biofilm formation in the food-processing environment.

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Contrary to expectations, our CC121 strains exhibited the highest level of virulence, even though this CC is reported hypovirulent.

Introduction

Listeria monocytogenes is an important foodborne pathogen responsible for listeriosis, a severe disease associated with high mortality rates, particularly among immunocompromised individuals, pregnant women, newborns, and the elderly [1]. All *L. monocytogenes* isolates have a core set of virulence determinants responsible for the key stages of the *Listeria* intracellular infection cycle: (i) host cell invasion, (ii) escape from the phagocytic vacuole, (iii) rapid intracellular proliferation, and (iv) actin-based motility and cell-to-cell spread [2; 3].

Pigs and pork products are recognized reservoirs for *L. monocytogenes* [4]. The presence of the pathogen in these systems represents a dual concern, as it acts both as a vector for human listeriosis and as potential threat to animal health [5]. Several outbreaks of *L. monocytogenes* linked to ready-to-eat pork meat, jellied pork, pork pies, and pork pâté have been reported highlighting the public health relevance of this contamination route [4].

A major factor contributing to the persistence of *L. monocytogenes* in food production environments is its remarkable ability to adhere to surfaces and form biofilms. These biofilms, which frequently develop on equipment and surfaces within processing plants, enhance the bacterium's resistance to environmental stressors and facilitate food contamination [6,7] and thereby increasing public health risks [8]. In addition to biofilm formation, the bacterium's persistence is further reinforced by its capacity to develop resistance to biocides—chemical disinfectants widely used in the food industry [9,10].

Among the diverse genetic lineages of *Listeria monocytogenes*, specific clonal complexes (CCs) have been identified as particularly virulent [11] and/or strongly associated with biofilm formation [4]. These clonal complexes exhibit distinct ecological and host-associated adaptations that influence their distribution and prevalence throughout swine production systems. Several *in vitro* and *in vivo* model are available to assess the level of virulence of the *Listeria monocytogenes* strains. Among them, the *Galleria mellonella* insect larvae model has proven to be a reliable and efficient system for evaluating pathogenicity of *Listeria monocytogenes* [3].

Resistance to biocide in *Listeria monocytogenes* can emerge from prolonged exposure to sublethal concentrations of biocides [12], particularly quaternary ammonium compounds (QACs). Such exposure may induce adaptive responses, including efflux pump activation and genetic mutations [13]. These mechanisms not only enhance bacterial survival but also reduce the efficacy of disinfection protocols, especially in the presence of mature biofilms. Within these biofilms, bacteria may display increased tolerance to commonly usedbiocides like benzalkonium chloride and peracetic acid [14].

The aims of this study were:

- to identify *L. monocytogenes* clonal complexes circulating from pig feces on farms to pork meat at retail,

- and, to characterize their ability to pass through the food chain and infect human.

We obtained data on their biofilm-forming capacity, their biocide resistance and their virulence level. All these data on these strains are essential for mitigating contamination risks and enhancing food safety.

Materials and methods

Strains

The 301 strains of *Listeria monocytogenes* analysed in this study were previously isolated during two one-year surveys conducted in mainland France. The first survey, carried out in 2008 at the pig farm level [15] involved pooled fecal samples (sows and fattening pigs) collected from 73 pig farms. From the 34 positive farms, a total of 147 *L. monocytogenes* strains were recovered. The second survey conducted in 2010 at the retail level [16] involved 320 raw pork meats from supermarkets, yielding 154 strains from the 41 positive meat samples. All fecal and meat samples were analysed for *Listeria monocytogenes* in our laboratory using the same methodology based on a modified ISO 11290−1 protocol published in 2005 [15]. Characteristic colonies were streaked on Tryptone Soy Agar Yeast Extract (TSAYe) plates (Oxoïd, England) and stored at −80°C in glycerol broth after 24 h at 37°C.

Listeria monocytogenes strains were serogrouped by a multiplex PCR assay following the protocol previously described [17,18]. This multiplex PCR amplifies the genes, lmo0737, lmo1118, ORF2819, ORD2110, and prs (gene for *Listeria monocytogenes*). The combination of certain amplicons with the prs amplicon allows strains to be classified into serogroup IIa (including serotypes 1/2a, 3a), serogroup IIb (including serotypes 1/2b, 3b, 7), IIc (including serotypes 1/2c, 3c) and serogroup IVb (including serotypes 4b, 4d, 4e). DNA was extracted from fresh bacterial cultures grown on TSAYE plates using the InstaGene® Matrix (BioRad Laboratories, Marnes-la-Coquette France) according to the manufacturer's instructions.

Pulsed-field gel electrophoresis and clonal complex mapping (n=301 strains)

The 301 strains of *Listeria monocytogenes* were typed by pulsed-field gel electrophoresis (PFGE). DNA plugs were prepared from fresh bacterial cultures grown on TSAYe plates, and PFGE was performed in accordance with the CDC PulseNet standardized protocol for *Listeria monocytogenes* [19]. Genomic DNA was digested with two macrorestriction enzymes, *Apa*I and *Asc*I (Biolabs, Beverly, MA). The *Apa*I digestion was carried out at 30°C for 6 h, and the *Asc*I digestion at 37°C for 3 h.

Restriction fragments were separated on a 1% SeaKem Gold agarose gel (Cambrex Bio Science, Verviers, Belgium) using the CHEF method in a CHEF-DR III system (Bio-Rad SA). Electrophoresis was performed with a linear ramping factor, applying pulse times from 4.0 to 40.0 seconds at 14°C for 21 h, at a voltage of 6 V/cm. For normalization and reference, *Salmonella enterica* serotype Branderup H9812 DNA digested with *Xba*I (New England Biolabs, 6 h at 37°C) was included on every gel [20]. Electrophoretic patterns were analyzed with BioNumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium), and clonal complexes (CCs) were assigned using a mapping protocol [21,22].

Selection of the strains for virulence genes and phenotypic tests

To investigate the biofilm-forming ability, biocide resistance and virulence (both genes carriage and *in vivo* assay), we selected a representative panel of 123 *Listeria monocytogenes* strains based on their CC distribution across farm and retail stages. If two or more strains from the same farm or the same meat sample had the same CC, only one strain was retained. If several CCs were in a farm or on a meat, we retained one strain per CC. In addition, two strains with the same CC were retained, only if one was isolated from feces and the other one from meat. The panel comprised: 22 strains belonging to CCs found exclusively on farms, 10 strains belonging to CCs found exclusively at retail, and 91 strains from CCs present in both compartments (64 from farm and 27 from retail). In total, 86 strains originated from on-farm pig feces and 37 from retail pork meat. Two reference strains from the Pasteur Institute collection, ScottA (CIP 103575) and EGDe (CIP 107776), were included in all assays as positive controls.

Detection of virulence genes (n=123 strains)

The presence of internalin genes (*inlA*, *inlC*, and *inlJ*) and virulence-associated genes (*plcA*, *prfA*, *actA*, *hlyA,* and *iap*) was assessed individually by Real-Time PCR developed for this study, using primers previously cited [23] and published by various authors (Table 1). Some primers were slightly modified (one or two bases added or removed) to harmonize melting temperature (indicated by * in the Table 1). Additional primers were designed by our laboratory to generate amplicons ≤ to 800 bp for optimal amplification efficiency (indicated by [b] in the Table 1).

DNA was extracted from fresh bacterial cultures on TSAYE plates using InstaGene® Matrix (BioRad Laboratories, Marnes-la-Coquette France) according to the manufacturer's instructions. Each PCR reaction was performed using 2.5µl DNA extract (adjusted to 10ng/µl) in a total reaction volume of 25µl containing SYBR® Green JumpStart™ Taq ReadyMix™ from Sigma-Aldrich, and 1µl of each primer (10µM). Amplifications of these eight genes were carried out under identical cycling PCR conditions; an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 2 min, 56°C for 1 minute and 72°C for 2 min. The PCRs ended by a step from 50°C to 95°C with an increment of 0.5°C every 5 seconds, to obtain the fusion curve.

Virulence of the strains using *Galleria mellonella* model (n=123 strains)

We assessed the virulence of *Listeria monocytogenes* strains using the *Galleria mellonella* larvae model, by measuring larval survival after inoculation of each strain. Each *Listeria monocytogenes* strain was streaked on TSAYE plates, and incubated at 37°C for 24 h. A single colony was then inoculated into 20 mL of Brain Heart Infusion (BHI) broth (Biokar), and incubated at 37°C for 24 h. On the day of larvae infection, 5 ml of this culture was transferred into a 10 mL falcon tube and centrifuged at 4,000 rpm for 5 min. The bacteria pellet was washed with one ml of physiological water (distilled water with 0.9% sodium chloride) followed by centrifugation for one minute; this washing step was repeated twice.

The optical density (OD) of the 1 ml bacterial solution was measured at 600nm and adjusted to an OD of 0.2 corresponding to approximately 10^8 colony-forming unit (CFU)/mL. For each *Listeria monocytogenes* strain, 10 µL of the

Table 1. Primer sequences and size of the amplicons.

Target gene	Primer sequence (5'-3')	Forward/ Reverse	Amplicon size(bp)	References
inlA	ACG AGT AAC GGG ACA AAT GC	F	800	[24]
	CCC GAC AGT GGT GCT AGA TT	R		
inlC[a]	G CGG GAA TGC AAT TTT TCA CTA	F	518	[24]
	AAT TCC CAC AGG ACA CAA CC	R		
inlJ	*GT AAC CCC GCT TAC ACA GTT	F	237	[24]
	AGC GGC TTG GCA GTC TAA TA	R		
plcA	ATG TAG GGA TTT TAT TGC TCG T	F	800	designed in this study [25]
	*GGG TTT CAC TCT CCT TCT AC	R		
prfA[a]	AAC CAA TGG GAT CCA CAA GAA	F	733	designed in this study [25]
	*CTC TTC TTG GTG AAG CAA TCG	R		
actA	CTT AGA TTC TAG CAT GCA GTC	F	800	designed in this study [26]
	*G AAG GAA CCG GGC TGC TAG	R		
hlyA	*TT GCA AGC GCT TGG AGT GAA	F	448	[27]
	*CG TAT CCT CCA GAG TGA TCG	R		
iap	*CA AGC TGC ACC TGT TGC AG	F	130	[28]
	TGA CAG CGT GTG TAG TAG CA	R		

[a]: Forward and Reverse primers interchanged with respect to the paper of Soni *et al*. (2014); [b]: primers designed in this study; *: modified primers from published primers.

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bacterial suspension were injected into the hindmost left proleg of 10 larvae of *Galleria mellonella* (20 mm size) using a 1 mL syringe connected to a syringe pump inoculator (KD Scientific, USA). In each trial, 10 larvae received an injection of 10µl of physiological water in order to monitor any mortality in the absence of *Listeria*. Inoculated larvae were placed in plastic Petri dishes and placed at 37°C for 7 days. Larval survival was monitored daily, and at the end of the incubation period, the number of surviving larvae was recorded to calculate a survival percentage for each strain. Each assay was performed in duplicate for all strains. The higher the percentage of surviving larvae, the less virulent the *L. monocytogenes* strain was considered to be, and inversely.

Biofilm-forming ability of *Listeria monocytogenes* strains (n=123 strains)

We tested the ability of the strains to form biofilm on 96-well plastic microplates using a method previously described [29] with slight modifications. Each *Listeria monocytogenes* strain was streaked on TSAYe plates and incubated at 37°C for 24 h. A single colony was then transferred into 10 ml of Tryptone Soy Broth Yeast Extract (TSBYe) (AES, Combourg, France) and incubated at 37°C for 24 h. The bacterial suspension was adjusted in TSBYe to an optical density at 600nm of 0.07 corresponding to 10^8 UFC/ mL, and then diluted 20-fold prior to inoculation into the microplates.

For each strain, 100 µL of the 1:20 bacterial suspension were deposited in four wells of a flat bottom lidded 96-well microplate (Nunclon™ ΔSurface, Danemark). Four wells per microplate filled with 100 µL of TSBYe served as negative control (to account for assay background noise). The microplates were incubated at 37°C for 48h. Bacterial growth was assessed by measuring the optical density (OD) at 600nm. Suspended bacteria were removed by inverting the microplate, followed by three successive washes with 200 µl sterile water per well. The bacterial biofilm was fixed by placing the microplate in an oven at 55°C for 20 min. Next, 125 µL of 1% crystal violet (Sigma-Aldrich, USA) were added to each well and left in contact with the biofilm for 15 min at room temperature. Unbound crystal violet was removed by inverting the microplate, followed by three additional washes with 200 µL of sterile water per well. Microplates were then dried for 15 min at 55°C.

The stained biofilm was solubilized with 200 µL of 95% ethanol for 15 min at room temperature. Then, 125 µL from each well were transferred to another 96-well microplate with flat bottom (Costar 3590, USA). Optical densities (OD) were measured at 600 nm. The final OD values for each strain were determined by subtracting to OD value obtained from the negative controls (wells containing TSBYe without strain). The assay was repeated three times yielding a total of 12 OD values per strain.

Susceptibility to Benzalkonium chloride (n=123 strains)

We tested the susceptibility of the strains to Benzalkonium chloride, as this quaternary ammonium compounds is among the most widely used disinfectants to control the growth and spread of *Listeria monocytogenes* in food processing facilities. The biocide susceptibility profiles of the 123 *Listeria monocytogenes* strains were determined by measuring the Minimal Inhibitory Concentrations (MIC) using a broth microdilution method adapted from NF EN 1040. *Listeria monocytogenes* ScottA (CIP 103575) was included as a reference in each experiments.

Benzalkonium chloride (BC 50%, Stepan Europe, N-CAS: 68391-01-5) was used in this study at final concentrations ranging from 1 to 6 µg/mL. All strains were streaked on TSAYe plates and incubated for 24 h at 37°C. Two to three colonies were inoculated into 4 ml of TSBYe and were incubated for another 24 h at 37°C. From these cultures, 300 µl of were transferred in 10 ml of TSBYe, and incubated at 37°C for only 3–4 h. The optical density (OD) of these bacterial cultures was measured at 600nm and adjusted with sterile tap water to an OD of 0.2 (+/- 0.02) corresponding to approximately 1.5 x 10^8 to 3 x 10^8 colony-forming unit (CFU)/mL. These resulting bacterial suspensions were adjusted with sterile tap water to an optical density (OD) of 0.2 (+/- 0.02) at 620 nm, corresponding to approximately 1.5 x 10^8 à 3 x 10^8 CFU/mL.

Each well of 96 well-microplates (Dutscher, PS, flat bottom, clear, Greiner bio-one) was filled in duplicates with 20 µL of each 10x concentrated biocide solution (10, 12.5, 15, 30, 40 and 60 µg/mL), and 180 µl of bacterial suspension at OD

0.2, diluted to 1:100, resulting in final concentration of 1.35 x 10^6 to 2.7 x 10^6 CFU/mL. Three wells containing only sterile tap water served as negative control, while wells containing bacteria cultures without biocide as positive controls. Minimal Inhibitory Concentrations (MICs) were determined after incubating the microplate for 24 h at 37°C. The experiment was performed three times on different days, and the median of the six MIC measurements was used for MIC distribution analysis.

Statistical analysis

All comparisons of means were performed in R software (version 3.2.4). Holm's correction was applied to compare the modalities two by two. The strains were subsequently clustered by Ascending Hierarchical Classification (AHC) using the "hclust ward D2" method in R resulting in three classes based of their ability to form biofilm (low L_BF, intermediate I_BF, and high H_BF), and in three classes based on their level of virulence (low L_V, intermediate I_V and high H_V). A Principal Component Analysis (PCA) was conducted in R on normalized data to explore the correlation among the three variables "ability to form biofilms (OD)", "biocide resistance" (MIC in µg/ml) and "virulence" (% of survival) and to visualize relationships in high-dimensional dataset. The data on the 123 strains are available in the supplementary S1 Table.

Results

Distribution of serogroups and clonal complexes in farm and retail (n=301 strains)

Listeria monocytogenes strain were distributed across four serogroups. The importance of these serogroups were significantly different between farm and retail (χ^2 test; p-value <to 0.05). Only 2% of the strains isolated from feces belonged to serogroup IIc, whereas this serogroup accounted for nearly half of the strains isolated from meat (48.7%) (Table 2). In contrast, 37.4% of the fecal strains were identified as serogroup IIb, while only 7.1% of meat strains belonged to this serogroup.

The strains were distributed across 20 clonal complexes; 18 identified at farm level and 14 at retail level. Of these, 12 CCs were identified at both stages (Table 3). Greater genetic diversity was observed among the fecal strain population (Simpson's index = 0.90 $_{IC95\%}$ [0.88–0.92]) compared to the meat strain population (Simpson's index = 0.73 $_{IC95\%}$ [0.66–0.80]).

We identified six clonal complexes (CC11, CC20, CC21, CC26, CC31 and CC59) that were exclusively isolated on farms, while CC121 (13.6% of the meat strains) was found only at retail. All strains belonging to serogroup IIc were classified as clonal complex CC9; which was also the most prevalent CC overall (n = 78) and was predominantly represented at retail (48.9% of the strains isolated at retail) (Table 3). CC37, from serogroup IIa, were mainly present in the feces (21.1% of the strains from pig farm), along with CC77 (10.9%) and CC1 (10.9%). The distribution of these three CCs between farm and retail differed significantly (p-value < 0.05).

Virulence of *Listeria monocytogenes* strains (n=123 strains)

PCR amplification of the internalin genes (*inlA*, *inlC* and *inlJ*) and virulence-associated genes (*plcA*, *prfA*, *actA*, *hlyA* and *iap*) were confirmed for all the 123 strains.

Table 2. Distribution of the 301 strains according their serogroup and origin (number and percentage).

	IIa		IIb		IIc		IVb		
serogroup	**n°**	**%**	**n°**	**%**	**n°**	**%**	**n°**	**%**	**Total**
Farm (pig feces)	58	39.5	55	37.4	3	2.0	31	21.1	147
Retail (pork meat)	52	33.8	11	7.1	75	48.7	16	10.4	154
Total	**110**	**36.5**	**66**	**21.9**	**78**	**25.9**	**47**	**15.6**	**301**

N: number of strains.

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Table 3. Distribution in percentage of the 301 strains according their origin, serogroup and clonal complex.

serogroup	CC	Farm (pig feces)	Retail (pork meat)	% on Total
IIc	CC9	2.0	48.7	25.9
IIa	CC37	21.1	7.1	14.1
IIa	CC121		13.6	7.0
IVb	CC4*	6.8	6.5	6.6
IIb	CC59	12.9		6.3
IIa	CC8	6.1	5.8	6.0
IIb	CC77	10.9	1.3	6.0
IVb	CC1	10.9	0.6	5.6
IIb	CC224	6.8	1.3	4.0
IVb	CC6	3.4	3.2	3.3
IIb	CC5	4.1	1.9	3.0
IIb	ST191	2.7	2.6	2.7
IIa	CC14	1.4	3.2	2.3
IIa	CC20	3.4		1.7
IIa	CC7	0.7	2.6	1.7
IIa	CC26	2.7		1.3
IIa	CC11	2.0		1.0
IIa	CC21	1.4		0.7
IIa	CC91*		1.3	0.7
IIa	CC31	0.7		0.3
	Total of strains	**147**	**154**	**301**

Note: CC4* = CC4-CC217; CC91* = CC91-CC14-ST360.

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All the larvae with injected water (control) survived during the assays. Analysis of the percentage of survival showed not significant different between strains isolated from feces and those isolated from meat (χ^2 Kruskal-Wallis, p-value = 0.977). However, significant difference was observed between serogroups (χ^2 Kruskal-Wallis, p-value <to 0.05) with strains belonging to serogroup IVb exhibiting higher virulence (61% of the IVb strains) and strains from serogroup IIc showing lower virulence.

By hierarchical clustering, the 123 strains were distributed in three classes according their level of virulence, with 17, 59 and 47 strains in the low (L_V), intermediate (I_V) and high (H_V) virulence groups, respectively (Table 4). Fecal strains and meat strains were evenly distributed across these classes. Among the serogroup IVb strains, 60.8% were classified as highly H_V strains (Table 4), while 46.3% of the IIa strains and 54.3% of IIb strains were classified into the intermediate virulence category (I_V).

Regarding clonal complexes, CC121 (found only on meat) and CC1, CC4 and CC6 (from serogroup IVb and predominantly from feces) were mostly associated to highly virulent class (Table 4). Strains belonging to CC37 (n = 16), the most prevalent CC, mainly isolated from feces, were mostly identified as intermediate virulent (56.2%).

Ability of *Listeria monocytogenes* strains to form biofilm (n=123 strains)

Analysis the OD values revealed that the biofilm-forming capacity of *Listeria monocytogenes* strains isolated from feces was significantly higher than that of strains isolated from meat (χ^2 Kruskal-Wallis, p-value <to 0.05). In addition, strains belonging to serogroup IVb exhibited significantly lower biofilm formation compared to other serogroups (χ^2 Kruskal-Wallis, p-value <to 0.05).

Table 4. Distribution of the strains according their origin, serogroup, clonal complex, biofilm classification and virulence classification.

			Biofilm			Virulence		
		Total	H_BF	I_BF	L_BF	H_V	I_V	L_V
FECES	**CC**	**86**	**35**	**33**	**18**	**32**	**43**	**11**
IIa		**36**	**12**	**22**	**2**	**11**	**19**	**6**
	CC37	14	1	12	1	4	8	2
	CC8	9	3	5	1	3	4	2
	CC20	3	1	2			2	1
	CC26	3	3			2	1	
	CC11	2	1	1			1	1
	CC21	2	1	1		1	1	
	CC14	1		1		1		
	CC31	1	1				1	
	CC7	1	1				1	
IIb		**31**	**16**	**8**	**7**	**10**	**17**	**4**
	CC59	11	6	1	4	2	7	2
	CC77	8	6	1	1	4	4	
	CC224	6	1	5		3	3	
	CC5	3	1	1	1		2	1
	ST191	3	2		1	1	1	1
IIc		**2**	**2**				**1**	**1**
	CC9	2	2				1	1
Ivb		**17**	**5**	**3**	**9**	**11**	**6**	
	CC1	9	3		6	6	3	
	CC6	5	2	1	2	3	2	
	CC4*	3		2	1	2	1	
MEAT	**CC**	**37**	**6**	**13**	**18**	**15**	**16**	**6**
IIa		**18**	**5**	**5**	**8**	**10**	**6**	**2**
	CC121	9		3	6	7	1	1
	CC8	3	3			2	1	
	CC14	2	1		1	1	1	
	CC37	2	1		1		1	1
	CC7	1		1			1	
	CC91*	1		1			1	
IIb		**4**	**1**	**1**	**2**	**1**	**2**	**1**
	CC224	1	1				1	
	CC5	1			1			1
	CC77	1		1		1		
	ST191	1			1		1	
IIc		**9**		**7**	**2**	**1**	**6**	**2**
	CC9	9		7	2	1	6	2
IVb		**6**			**6**	**3**	**2**	**1**
	CC4*	3			3	2	1	
	CC6	2			2	1		1
	CC1	1			1		1	
Total		**123**	**41**	**46**	**36**	**47**	**59**	**17**

Note: CC4* = CC4-CC217; CC91* = CC91-CC14-ST360.

H_, I_and L_BF: high, intermediate, and low ability to form biofilm, respectively,

H_, I_and L_V: high, intermediate, and low virulence, respectively.

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After clustering by Ascending Hierarchical Classification (AHC), the 123 strains were distributed into three classes based on their biofilm-forming capacity: 36, 46, and 41 strains in the low (L_BF), intermediate (I_BF), and high (H_BF) biofilm-forming groups, respectively (Table 4). High biofilm-forming (H_BF) strains represented 40.7% of the strains isolated from feces and 16.2% of those isolated from meat. Conversely, low biofilm-forming (L_BF) strains accounted for 65.2% of the strains belonging to serogroup IVb (Table 4).

Among clonal complexes, CC8, CC59, and CC77 were predominantly high biofilm-formers. The most prevalent CC, CC37, mainly found in feces, was mostly classified as intermediate biofilm-forming (I_BF). CC1 and CC6 from serogroup IVb, which are involved in human infections in France, exhibited mainly low or intermediate biofilm-forming ability.

Susceptibility to Benzalkonium chloride

Most strains isolated from feces showed greater susceptibility to benzalkonium chloride (BC), with mean of MIC values equal to 1.4 µg/ml (Fig 1). Most strains isolated from feces showed greater susceptibility to benzalkonium chloride (BC), with mean of MIC values equal to 1.4 µg/ml ± 0.2 (χ^2 Kruskal-Wallis, p-value < 0.05). In contrast, strains isolated from retail meat exhibited higher MIC values, ranging from 1.5 to 4 µg/ml, with an mean of MIC values equal to 3.0 µg/ml ± 1.9. This indicates that meat-derived strains are generally more resistant to this biocide than those from fecal samples.

Strains belonging to serogroup IIc also demonstrated increased resistance to benzalkonium chloride compared to other serogroup (χ^2 Kruskal-Wallis, p-value < 0.05). Additionally, the 16 strains with MIC values ≥ to 3 µg/ml belonged to clonal complexes CC121 (n = 9), CC9 (n = 5), CC14 (n = 1) and CC7 (n = 1).

Interaction between phenotypic traits

A weak negative correlation (−0.238) was observed between the biofilm-forming (OD_Bf) and biocide resistance (p-value = 00081), while no correlation was found with virulence (% of survival). From the correlation circle (Fig 1A) and the map of individuals (Fig 1b) generated by the PCA (Table 5), we observed that strains isolated from feces (in red) would tend to exhibit a higher biofilm-forming capacity, whereas strains isolated from meat (in blue) would tend to exhibit an higher resistance to the biocide.

Discussion

Listeria monocytogenes is the causative agent of listeriosis, a zoonotic disease primarily transmitted to humans through the consumption of contaminated food [30]. Among the 13 known serotypes of *L. monocytogenes*, serotypes 1/2a, 1/2b, and 4b classified as serogroup IIa, IIb and IVb respectively, are most frequently associated with human listeriosis cases, with serotype 4b often linked to major outbreaks [31]. While serotype 4b strains are responsible for most listeriosis cases and outbreaks, the majority of strains recovered from food products and food-processing environments belong to serotype 1/2a [32].

In our study, serogroup IVb was equally present on farms and at retail, whereas serogroup IIb was more prevalent on farm and serogroup IIc predominated at retail. These results are consistent with previous observations showing that serogroup or serotype distribution varies along the pig and pork production chain [4].

All the 123 strains tested for virulence genes in our study carried internalin genes (*inlA*, *inlC* and *inlJ*) as well as virulence-associated genes (*plcA*, *prfA*, *actA*, *hlyA* and *iap*). These genes are commonly detected in *Listeria monocytogenes* strains [33–36]. They play essential roles in adhesion, cellular invasion, intracellular replication, and dissemination of the pathogen, thereby contributing to its pathogenicity in humans. However, the presence of all these genes for our strains does not provide information on their level of expression, which can vary between strains.

In addition, to further assess virulence, we used the *Galleria mellonella* insect larvae model- an established system for evaluating *Listeria monocytogenes* pathogenicity [37,38]. In our study, serogroup IV strains were the most virulent,

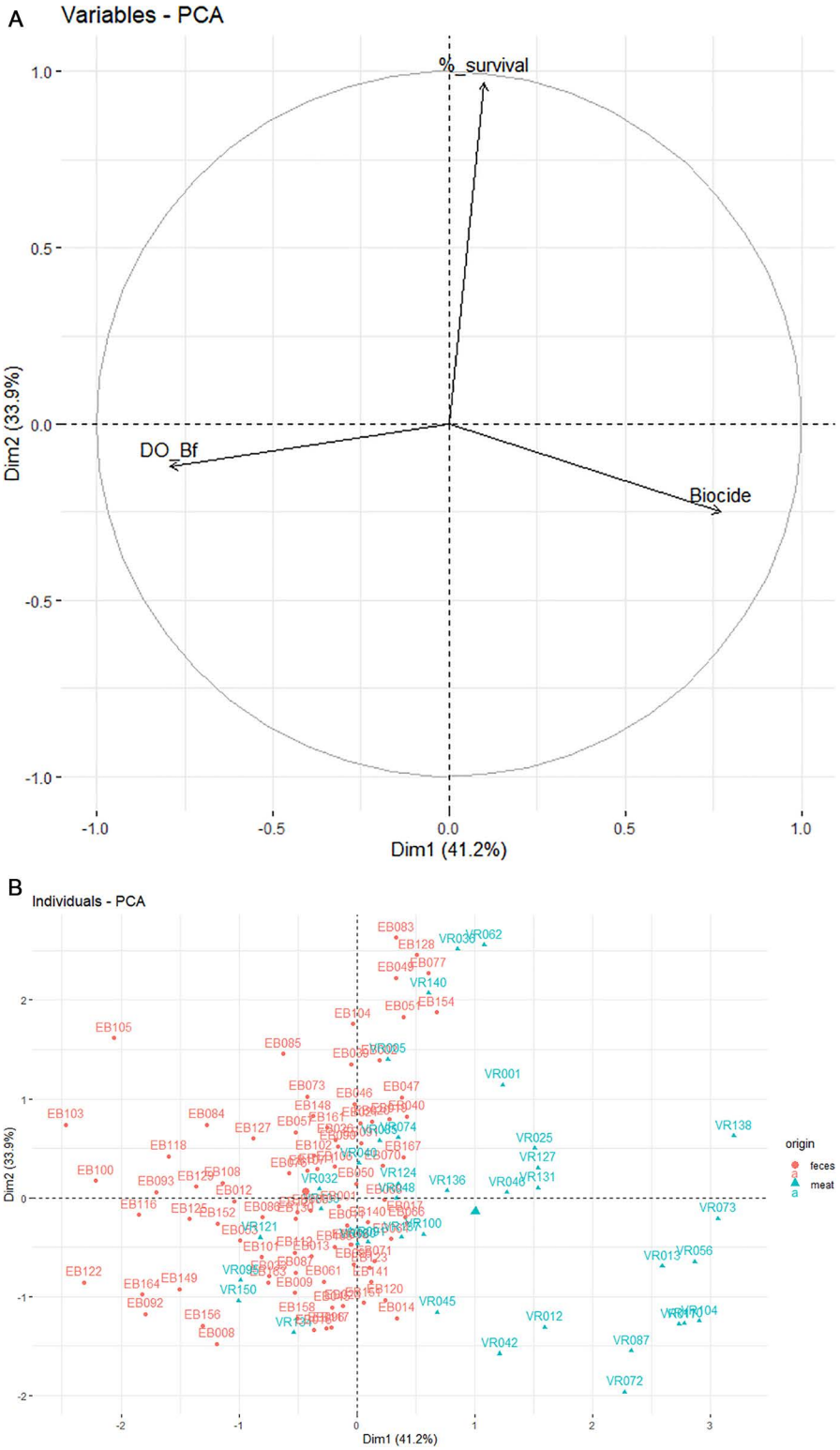

Fig 1. Principal component Analyses including the tree quantitative variables: Optical density of the biofilm, percentage of survival and Minimal Inhibitory Concentration against Benzalkonium chloride. a. Principal Component Analysis correlation circle with the three considered variables. **b.** Principal Component Analysis graph of individuals (strains).

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Table 5. Complementary data on ACP analysis.

variable coordinates	Dim.1	Dim.2	Dim.3	axis	eigenvalue percentage of variance	cumulative percentage of variance
DO_Bf (biofilm)	−0.79328988	−0.1220047	0.5964948	**axis 1**	41.22171	41.22171
% Survival	0.09936664	0.9691837	0.2254092	**axis 2**	33.88682	75.10854
Biocide	0.77296104	−0.2498051	0.5832055	**axis 3**	24.89146	100.00000

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whereas serogroups IIa and IIb displayed intermediate virulence. This observation aligns with previous findings reporting that serotype 4b strains cause higher *G. mellonella* lethality than other serotypes [39].

Consistently, CC1 and CC6 strains in our study were mainly classified as highly or intermediately virulent, corroborating earlier findings [11]. Indeed, hypervirulent CCs such as CC1, CC2, CC4 and CC6 are known to cause most severe listeriosis cases — particularly those affecting the central nervous system or maternal–neonatal infections —and are responsible for the majority of outbreaks and sporadic cases worldwide [11,40].

Interestingly, we also found that CC9 and CC121 strains — predominantly isolated from meat — were mostly classified as highly or intermediately virulent. This is unexpected, as these two CCs are generally regarded as hypovirulent, typically associated with infections in highly immunocompromised patients and showing limited virulence in humanized mouse models [11,41]. Previous studies [42,43] highlighted that CC2 strains carry a full-length *inlA* gene, whereas CC9 and CC121 strains often present a premature stop codon mutation (PMSC) in *inlA* correlated with reduced virulence. Liu et al., [44], reported that 99.2% of CC9 and 65% of CC121 strains exhibited the highest proportions of *inlA* with PMSC, though a minority retained the full-length *inlA* gene which may explain our observations. Alternatively, environmental or selective pressures within the pig and pork production chain could have favoured CC9 and CC121 strains with higher virulence than generally reported. Another possibility is that the *Galleria mellonella* insect model may not fully capture virulence variability among all *L. monocytogenes* CCs. However, no study to our knowledge has yet established a direct link between virulence in this model and strain CC.

First, in the initial population (n = 301 strains), we observed greater genetic diversity of *Listeria monocytogenes* on farms than at retail, and that six clonal complexes identified on farms were not detected in meat. Moreover, four other CCs differed significantly in distribution between farm and retail. These findings are consistent with Lagarde's review [4]. Such pattern may reflect the inability or ability of certain CCs to persist through the food chain and reach meat products. Their low prevalence on farms can lead to their gradual disappearance along the production chain, or their better adaptation to stressful conditions encountered in food processing environments and during cold storage can lead to their survival [45].

Indeed, *Listeria monocytogenes* strains circulating from pigs, the primary reservoir, to consumers can survive and multiply under conditions that are stressful for many other bacteria. In particular, their ability to adhere to surfaces and form biofilms allows long-term persistance for years on equipment and in food-processing facilities, as well as resistance to biocides commonly used in slaughterhouses and cutting plants [1,6,7,9,10]. Consequently, contamination of meat products by *Listeria monocytogenes* frequently occurs during food processing, mainly through cross-contamination from contaminated contact surfaces.

In our study, we observed that strains belonging to serogroup IVb exhibited significantly lower biofilm formation than other serogroups. This finding is consistent with a previous study using similar microplate-based biofilm quantification method [46] which showed that serogroup IVb strains produced significantly less biofilm than serogroup IIa and/or IIb strains.

We also observed that strains isolated from farms had a significantly greater ability to form biofilms than those isolated from retail. Our findings align with several studies reporting that *Listeria monocytogenes* strains recovered from slaughterhouse environments — such as conveyor belts [34] and the surfaces of equipment and utensils [47,48] — often show poor adhesion to surfaces and limited biofilm formation.

Among our strains, CC121, which was primarily isolated from meat, exhibited low biofilm-forming capacity. This contrasts with other studies reporting that CC121 strains are strong biofilm formers with increased tolerance to quaternary ammonium compounds [6]. Such intra-CC121 variability suggests that biofilm formation is strain-specific rather than strictly CC-dependent. Environmental and selective pressures along the pig-to-meat continuum — such as cleaning procedures, biocide exposure, and temperature conditions — may also modulate the expression of biofilm-associated genes, leading to phenotypic differences observed in our study compared to other [6].

Finally, we observed that strains isolated from meat were significantly more resistant to Benzalkonium chloride than those from feces, with four CCs (16 strains), including CC121, exhibiting high MIC values (≥ 3 µg/ml). Benzalkonium chloride is one of the most widely used disinfectants in the food industry. Similar findings, showing higher MIC values food-derived strains compared to animal isolates, have been reported previously [49]. This increased resistance may result from repeated exposure to sublethal concentrations of biocides, particularly in mature biofilms [12]. Prolonged contact with such concentrations can gradually increased tolerance to cleaning and disinfection over time when present in single-species biofilms, for both peracetic acid and quaternary ammonium disinfectants [50]. Moreover, persistence of *Listeria monocytogenes* in a pig slaughterhouse has been linked to the presence of Benzalkonium chloride resistance genes [10].

Conclusion

This study provide a genetic and phenotypic characterisation of the *Listeria mocytogenes* population along the pig-to-retail meat continuum in France. Conducted on the basis of two surveys carried out two years apart (2008 and 2010), the results should be interpreted with caution. Our findings demonstrate that the *Listeria monocytogenes* population would evolve between the reservoir (pigs) and consumer stage, suggesting that some strains may either be able—or unable—to persist throughout the food chain. This study is original in that it reports highly virulent CC121 strains, a feature rarely described in the literature. Furthermore, our results indicate that strains isolated from retail meat would be more strongly associated with resistance to biocides than with biofilm-forming ability within the food-processing environment. These observations highlight the importance of alternating the types of biocides used in the food industry to limit the emergence and spread of resistant *L. monocytogenes* strains.

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