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A secretome-based methodology may provide a better characterization of the virulence of *Listeria monocytogenes*: Preliminary results

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ABSTRACT

Four strains of *Listeria monocytogenes* with different levels of virulence were studied. Two strains were consistently evaluated as virulent (strain 3077) and of low virulence (strain 3993), whereas the other two strains (3006 and 3049) originated conflicting results in what the evaluation tests were concerned: both were shown to exhibit low virulence when evaluated by *in vitro* assays, but virulent when the analyses were performed under *in vivo* conditions.

To clarify the virulence potential of the selected strains, a proteomic approach was used after incubating *L. monocytogenes* cultures under conditions favoring the expression of virulence factors (minimal medium, at 37 °C). Bacterial proteins present in the liquid culture media were precipitated from late exponential phase cultures, fractionated by SDS-PAGE and identified by MALDI-TOF-MS.

Three virulence factors differentially expressed were detected: protein p60, listeriolysin O (LLO) and internalin C (InlC). Clustering analysis of the four *L. monocytogenes* strains based on their secretome profiles allowed their categorization in two groups: the virulent group, composed by strains 3077 and 3049, and the low virulence group, containing strains 3993 and 3006. The results presented in this work suggest that the virulent potential of a particular *L. monocytogenes* strain may be predicted from the levels of both listeriolysin O (LLO) and internalin C (InlC) present in its secretome when the bacterium is grown under conditions favoring the expression of virulence factors. Following validation of this proposal through the analysis of a large array of strains, this methodology exhibits a great potential to be developed into an accurate and rapid method to characterize *L. monocytogenes* strain virulence.

1. Introduction

Listeria monocytogenes is a foodborne pathogenic bacterium for humans and animals, causing a serious infection named listeriosis. The disease affects mainly the group of the so-called YOPI (young, old, pregnant and immunocompromised) persons. The manifestations of listeriosis include septicemia, central nervous system infections, and maternofetal infections leading to stillbirths and abortions [1]. Although epidemiological investigations show that listeriosis is a rare disease, it remains a serious public health concern because of its high case-fatality (20–30%). In several member states of the EU, reported listeriosis cases have increased consecutively over the past five years [2].

To infect target cells, *L. monocytogenes* has evolved strategies to efficiently avoid host defence mechanisms, liberating extracellular proteins that play an important role in pathogenicity [1,3]. The secretome, an important subgroup of the total bacterial proteome, is characterized by its dynamic nature, undergoing variations and adjustments to the prevailing environmental conditions [4–6]. Some authors reported that several secreted virulence factors are preferentially synthesized under nutrient stress conditions [3,7,8]. Excluding the host risk factors, there is well documented evidence for the heterogeneity in virulence among *L. monocytogenes* strains [9–11]. Results from different subtyping techniques suggest three genetic divisions or lineages correlated with serovars in *L. monocytogenes* [12–14]. Lineage I consists of strains (flagellar antigen types b and d) that are more likely to cause human disease than isolates classified in lineages II (antigen type a or c) and III (rarely detected serovars 4a and 4c, more often associated with animal cases of listeriosis), both exhibiting low virulence for

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humans [13]. Nevertheless, none of these subtyping methodologies is able to predict accurately the ability of a strain to cause disease.

Commonly used methods for the evaluation of *L. monocytogenes* virulence include both *in vitro* and *in vivo* tests. The *in vitro* tests are based on the use of specific human cultured cell lines. Thus, when compared to *in vivo* animal models, these tests allow a greater control of environmental factors and the analysis of a larger number of isolates per test. However, *in vitro* tests cannot evaluate the effect of all virulence genes on different types of cells. On the other hand, *in vivo* tests use laboratory animals. Virulence tests carried out on people, even in a voluntarily way, are not performed because they would raise ethical questions [15]. The agreement between an *in vitro* test of virulence based on a plaque forming assay (PFA) with HT-29 human epithelial cells and the infection of immunocompetent mice was previously showed [16] and confirmed [10]. Nevertheless, discrepancies between the two models are sometimes encountered for specific isolates [10].

The availability of rapid, sensitive and precise tests for differentiation of virulent from low virulence strains will not only eliminate unnecessary food product recalls and reduce economic losses, but also provide an assurance to consumers on the safety of food products being marketed [17].

Previous comparative studies between *L. monocytogenes* genome and proteome with those of the closely related, but non-pathogenic species, *L. innocua*, suggested that the main differences between these species are related to the secretory and surface proteins [3,18].

In this study, four *L. monocytogenes* strains with different levels of virulence, previously, evaluated by *in vitro* and *in vivo* tests, were used. Two of these strains produced conflicting results in those tests. To assess the pathogenic potential of the four strains, a simplified methodology based on their secretome profiling, obtained under conditions favoring the expression of virulence factors, was utilized.

The methodology proposed in this work may be developed into an accurate method to the rapid identification of virulence protein markers in order to better characterize the pathogenic potential of *L. monocytogenes* strains.

2. Materials and methods

2.1. Strains used

The characterization of the four *L. monocytogenes* strains used in this study is presented in Table 1. The four strains were selected based on their genetic diversity and on their differential levels of virulence, previously evaluated using *in vivo* tests on mice and *in vitro* tests on human cultured cells (adenocarcinoma cell line HT-29). The three genetic lineages of this bacterial species (I, II and III) [13] are represented (Table 1). Two strains were consistently evaluated as virulent (strain 3077) [10] and of low virulence (strain 3993) [19], whereas the other two strains (3006 and 3049) originated conflicting results in what the evaluation tests were concerned: both were shown to exhibit low virulence when evaluated by *in vitro* assays, but virulent when the analyses were performed under *in vivo* conditions (Table 1) [9,10,20].

2.2. Bacterial cultures

Bacterial culture supernatants were obtained following the method described previously by Trost et al. [3] with modifications: strains were cultured overnight at 37 °C on Tryptone Soya Yeast Extract Agar (Biokar Diagnostic, Beauvais, França). Single isolated colonies were suspended in 25 mL of a minimal medium (Modified Welshimer Broth—MWB) [21] and incubated overnight at 37 °C with shaking. Overnight cultures were adjusted with fresh MWB to an $A_{600} = 0.05$ (final volume of 50 mL). Incubation proceeded for about 20 h at 37 °C with shaking. The cells were harvested, at late exponential phase, by centrifugation (3,000 × *g*, 10 min, 4 °C). After filtering the supernatant through Millex-GP filters, with 0.22 μm Millipore Express Membranes (Millipore, Carrigtwohill, Ireland), 0.2 mM phenylmethylsulphonyl fluoride (PMSF) (AppliChem, Darmstadt, Germany) was added.

2.3. Protein precipitation

The secreted proteins present on supernatants were precipitated by incubation with 0.2 mg/mL sodium deoxycholate (DOC) (Merck, Darmstadt, Germany) for 30 min at 4 °C, followed by addition of 6% (w/v; final) trichloroacetic acid (TCA) (Panreac, Barcelona, Spain) and overnight incubation, at 4 °C. After centrifugation (17,900 × *g*, 15 min, 4 °C), the precipitate was washed with ice-cold acetone (JT Baker, Deventer, The Netherlands). The protein concentrations were determined by a modification of the Lowry method [22]. After protein precipitation, the pellet was dissolved in 1 mL of a mixture of a solution A (2% (w/v) Na_2CO_3, 0.4% (w/v) NaOH, 0.16% (w/v) sodium tartrate, 1% (w/v) SDS) with a solution B (4% (w/v) $CuSO_4 \cdot 5H_2O$) in the proportion 1B:100A. The bovine serum albumin (BSA) (Sigma, MO, USA) was used as standard.

2.4. SDS-PAGE

Samples containing 50 μg total protein were dissolved in sample buffer: 80 mM Tris–HCl, pH 6.8, containing 2% (w/v) SDS (Merck, Darmstadt, Germany), 0.1 mM β-mercaptoethanol (Sigma, MO, USA), 15% (v/v) glycerol (Merck, Darmstadt, Germany) and 0.01% (w/v) *m*-cresol purple (Sigma, MO, USA). Samples were then heated at 100 °C for 4 min and submitted to SDS-PAGE in 15% (w/v) slab gels [23] with 8 cm × 7.3 cm × 0.75 mm, on a Mini-PROTEAN 3 Cell (BioRad, CA, USA).

Separated bands were visualized by colloidal Coomassie Brilliant Blue G-250 staining according to Neuhoff et al. [24]. Apparent masses were calculated based on the molecular masses of standards (14.2–66 kDa; Sigma, Saint Louis, MO, USA).

Table 1
Characterization of *L. monocytogenes* strains used in this study.

Isolate (reference CBISA)[a]	Serovar	Genetic lineage	Source[a]	Virulence potential	
				In vivo	*In vitro*
3006	4c	III	Animal strain CIP 78.39 (=ATCC 19116)	High virulent [20]	Low virulence (Gallo et al., unpublished results)
3049	1/2a	II	Milk	High virulent [9,10]	Low virulence *in vitro* [10]
3077	4b	I	Cheese	High virulent [10]	High virulent [10]
3993	4d/4e	I	Food product	Low virulence [19,10]	Low virulence [19,10]

[a] CIP—Collection de l'Institut Pasteur; ATCC—The American Type Culture Collection; CBISA—Colecção de Bactérias do Instituto Superior de Agronomia.

2.5. Image scanning and data analysis

To determine the experimental variation among assays, for each strain, three biological replicates were performed. From each biological assay, samples were subjected to three independent SDS-PAGE experiments. Electronic images of the SDS-PAGE gels were acquired with a calibrated densitometer ImageScanner (Amersham Biosciences, Buckinghamshire, UK) operating with the software LabScan (Amersham Biosciences, Buckinghamshire, UK). Secretome patterns and band volumes were analysed using program GelCompar II version 5.1 (Applied Maths, Kortrijk, Belgium). For cluster analysis of the patterns, the unweighted-pair group matching algorithm (UPGMA) was used with an optimization value of 0.0% determined by the program. The levels of similarity were based on the Pearson product–moment correlation coefficient (Pearson correlation).

In order to assess the repeatability of the assay, for each strain a clustering analysis of the replicas of the secretome profiles was performed. The minimum level of similarity among replicates was calculated as a cut-off value for identifying identical patterns.

The band volumes, i.e., digitized staining intensity of the bands, integrated over the corresponding areas, were analysed. For each band considered, the program automatically calculates a best-fitting Gaussian curve, which makes the quantification reliable. The volumes of the bands were expressed as absolute values (arbitrary units). The relative intensity for each band, i.e., the volume of the individual band divided by the total volume of all correspondent bands, was used for band semi-quantification. Quantification of data was performed by calculating means ± SD ($n = 3$). The homogeneity of variance was confirmed and the significant differences between the amounts of each selected protein secreted by the four strains was determined using one-way ANOVA with Tukey's HSD (honest significant difference) multiple comparison test ($\alpha = 0.05$) by running the program STATISTICA for Windows, version 6 (StatSoft, Inc., Tulsa, OK, USA).

2.6. MS protein digestion

A Simplicity 185 apparatus (Millipore, Milan, Italy) was used to obtain the Milli-Q water used throughout the experiments. After in-gel protein separation, gel bands were manually excised and treated using a slight modification of the method of Galesio et al. [25]. Only bands that were consistently present among replicates were analysed. Protein bands were washed, first with 25 mM NH_4HCO_3 (99.5%, Fluka, Steinheim, Germany) and then with 100% acetonitrile (ACN) (MALDI-MS grade, Sigma, Steinheim, Germany), in an ultrasonic bath model Transsonic TI-H-5 (Elma, Singen, Germany) operating at 35 kHz (60% amplitude) for 5 min for each step. The ultrasonic bath was used to speed up gel washing and the subsequent steps of protein reduction and protein alkylation. Protein reduction and alkylation steps were achieved with 10 mM dithiothreitol (DTT) (Sigma, Steinheim, Germany) and 100 mM iodoacetamide (Sigma, Steinheim, Germany), respectively, 5 min for each step. After reduction and alkylation steps, the gel was submitted to the washing procedure in the same way as described above, followed by a drying step of 30 min. A vacuum concentrator centrifuge model UNIVAPO 100H (UniEquip, Martinsried, Germany) with a refrigerated aspirator vacuum pump model Unijet II was used for sample drying and sample preconcentration. The dried protein bands were incubated with trypsin sequencing grade (Sigma, Steinheim, Germany) (375 ng in 25 μL 12.5 mM NH_4HCO_3/10% (v/v) ACN) at 37 °C, overnight. Trypsin activity was stopped by the addition of 20 μL of 5% (v/v) formic acid (Fluka, Steinheim, Germany). The supernatants were transferred into new tubes. Then, a solution with 50% (v/v) ACN/0.1% (w/v) trifluoroacetic acid (TFA) (Riedel-de-Haën, Seelze, Germany) was added to the gel bands to extract more peptides. The extraction step was performed in a sonoreactor bath model UTR200 (Dr. Hielsher, Teltow, Switzerland) at 50% amplitude, 2 min. The sonoreactor was used to accelerate the peptide extraction from gel pieces. After the extraction step, the extraction solution was added to the correspondent supernatant.

The peptide solutions were evaporated to dryness. The pellets were resuspended in the ultrasonic bath for 5 min with 5 μl of 0.3% (v/v) formic acid (Fluka, Steinheim, Germany).

2.7. MALDI-TOF-MS analysis

A matrix-assisted laser-desorption ionization–time of flight-mass spectrometry (MALDI-TOF-MS) system model Voyager DE-PRO Biospectrometry Workstation equipped with a nitrogen laser radiating at 337 nm (Applied Biosystems, Foster City, CA, USA) was used to acquire the peptide mass fingerprintings (PMF). ProteoMass Peptide MALDI-MS Calibration Kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS. Prior to MALDI-TOF-MS analysis, the sample was mixed with the matrix solution. α-Cyano-4-hydroxycinnamic acid (α-CHCA) matrix puriss for MALDI-MS (Fluka, Buchs, Switzerland) was used throughout this work and was prepared as follows: 10 mg of α-CHCA was dissolved in 1 mL of 50% (v/v) ACN/0.1% (w/v) TFA solution. The matrix solution was then mixed with the sample (1:1). One microliter of each sample was applied into a well of a MALDI-TOF-MS sample plate and allowed to dry.

Measurements were done in the reflector positive ion mode, with a 20 kV accelerating voltage, 75.1% grid voltage, 0.002% guide wire and a delay time of 100 ns. The close external calibrations were performed with the monoisotopic peaks of the bradykinin, angiotensin II, P14R, and ACTH peptide fragments (m/z: 757.3993, 1046.5424, 1533.8582, and 2465.1989, respectively) (ProteoMass Peptide MALDI-MS Calibration (MSCAL2), Sigma). Monoisotopic peaks were manually selected from each of the spectra obtained. Mass spectral analysis for each sample was based on the average of 600 laser shots, using Voyager software for data collection and analysis. All the mass spectra were processed using Data Explorer™ software, version 4 (Applied Biosystems, Foster City, CA, USA): (1) baseline correction, to correct a curved baseline and eliminate broad artifacts; (2) noise removal, to remove background noise from the mass spectra; (3) peak deisotope, to reduce the isotope clusters to monoisotope peaks; (4) peak detection, with an average base peak intensity of 1.6%, to select the most significant peaks of the spectrum. Mass peaks corresponding to trypsin autolysis products and keratins were also removed before PMF. Peptide mass fingerprints were searched with the MASCOT search engine [http://www.matrixscience.com/search_form_select.html] with the following parameters: (i) Swiss-Prot/MSDB/NCBInr 2009 databases; (ii) one missed cleavage; (iii) fixed modifications, carbamidomethylation (C); (iv) variable modifications, oxidation (M); (v) peptide tolerance up to 150 ppm; (vi) minimum number of matched peptides, 11. The scores were obtained with Mascot against Firmicutes database. A match was considered successful when the protein identification score was located out of the random region, and the analysed protein scored first. Protein identification was only considered when the results, obtained in the three independent biological assays, were equal and a significant match corresponded typically to a score equal or greater than 70 ($P < 0.05$) [26] in *L. monocytogenes* databases.

3. Results and discussion

3.1. SDS-PAGE analysis of secreted proteins

The environmental modulation of secretory protein abundance has been well demonstrated before [6]. Extracellular proteins

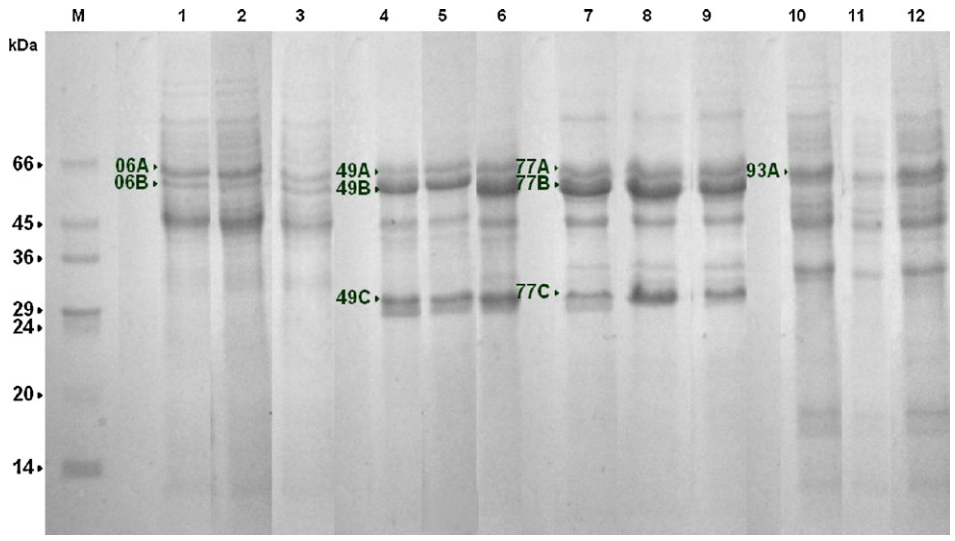

Fig. 1. SDS-PAGE profiles of proteins secreted by four *L. monocytogenes* strains, with three biological replicates for each strain. M: molecular marker; 1–3: strain 3006 (serovar 4a); 4–6: strain 3049 (serovar 1/2a); 7–9: strain 3077 (serovar 4b); 10–12: strain 3993 (serovar 4d/4e). Signed bands correspond to: protein p60 (06A, 49A, 77A and 93A); listeriolysin O (06B, 49B and 77B) and internalin C (49C and 77C).

of *L. monocytogenes* play an important role in its pathogenesis [1,3]. All *L. monocytogenes* strains are considered as potentially pathogenic. However, the virulence heterogeneity among isolates is well known. Some authors have found significant differences between secreted protein profiles of *L. monocytogenes* and those of the non-pathogenic species *L. innocua* [3]. Therefore, there seems to be no doubt that the secretome profile is species specific for *Listeria* species incubated under a given set of conditions. Furthermore, comparisons made among the secretome profiles of the more virulent *L. monocytogenes* serovars (1/2a, 1/2b and 4b) [27,28], or even among strains within serovar 4b [29], also showed differences. These observations raise a potentially important query: is the secretome related in some way with the virulence level of that particular species, serovar or strain? The absence of reliable methods to predict accurately the level of virulence exhibited by a particular *L. monocytogenes* strain prompted the identification of proteins secreted by *L. monocytogenes* strains from four serovars selected within the three genetic lineages of the species. Following growth in minimal medium at 37 °C until late exponential phase, the secreted proteins were precipitated from the culturing media for comparative SDS-PAGE protein profile analysis. The respective growth curves of these strains were compared and did not show any significant differences in what concerns growth rate, lag time or maximum absorbance (data not shown). Fig. 1 shows the electrophoretic profiles of the proteins secreted by the four strains. The observed secretome heterogeneity was particularly noticeable in the ranges of 29–36 kDa and 45–66 kDa, where absence or presence of particular bands was not consistent among isolates.

3.2. Clustering analysis based on secretome profiles

Fig. 2 shows the clustering analysis of *L. monocytogenes* based on the secreted protein patterns. Considering the established cut-off value of 0.93, the differentiation of the four strains was confirmed. At about 0.52 similarity, two clusters were found. Strain 3006 (serovar 4c) and the low virulence strain 3993 (serovar 4d/4e) belonged to one of these clusters with 0.71 similarity. The second cluster gathered the virulent strain 3077 (serovar 4b) and strain 3049 (serovar 1/2a) at 0.83 similarity. Dumas et al. [27], on a comparative analysis of the proteomes of *L. monocytogenes*, reported a correlation between proteome and serovar. However, in our study the secretome did not clustered the strains according to their serovars. In fact, the two strains representative of genetic lineage I (3077 and 3993) were grouped in different clusters with the other strains from lineage II (3049) and III (3006), respectively. Therefore, the results obtained thus far seem to indicate the absence of any relation between secretome and *L. monocytogenes* serovar.

3.3. Invasion-associated protein p60

One virulence factor expressed by all strains was protein p60 (Fig. 1, Table 2 and Supplementary Table 2S), which showed no significant differences in band intensity among the four strains analysed (results not shown). The extracellular protein p60, which is encoded by the *iap* gene, increases binding of *L. monocytogenes* to and invasion of both 3T6 fibroblasts and intestinal epithelial human cells [30,31]. It also promotes cell division and actin tail formation, with subsequent influence on virulence expression [31]. The database search for band 06A (strain 3006, serovar 4c) identified this secreted protein as the virulence factor p60 (Table 2 Supplementary Table 2S) of another strain from serovar 4c [32]. The bands (49A, 77A and 93A) detected in each of the other three strains were identified as the protein p60 of a previously studied strain from serovar 1/2a [30,32,33]. The *iap* gene contains a specific central region characterized by an extended repeat segment encoding for threonine-asparagine (TN) repeat units [33]. Some authors showed that phylogenetic analysis of the nucleotidic sequences of the TN repeat region can cluster together strains from serovars 1/2a (lineage II) and 4b (lineage I) [34]. These results suggest that detection of this protein in *L. monocytogenes* secretome may neither be related to the serovar of each particular strain, nor explain its virulence level.

3.4. Listeriolysin O

Some of the most important virulence genes identified in *L. monocytogenes* are positively regulated by the transcriptional activator PrfA [35,36]. The thermoregulated PrfA acts synergistically on the expression of virulence genes under thermal conditions, such as those that exist in the eukaryotic host (37 °C) [37,38]. Moreover, under nutrient stress conditions, PrfA-dependent proteins (PdPs) are preferentially synthesized [8].

Listeriolysin O (LLO), a water soluble haemolysin, is a PdP [39], which in our study was consistently detected in the secretomes

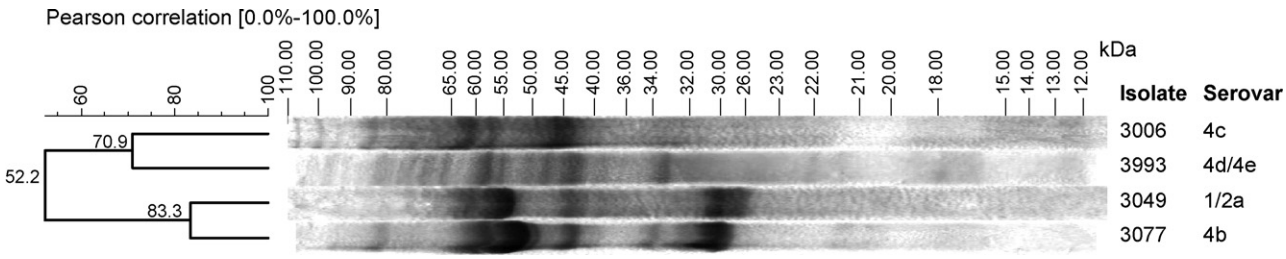

Fig. 2. Dendrogram (UPGMA clustering based on Pearson product-moment correlation) of SDS-PAGE profiles of proteins secreted by four *L. monocytogenes* isolates. GelCompar version 5.1 was used with optimization values of 0% for densitometric curves comparison. The reference of the isolates as well as their serovars are indicated.

Table 2
Proteins differentially expressed within the secretome of four *L. monocytogenes* strains.

Strain (serovar)	Band ID (Fig. 1)	Results						
		Protein description	Accession No.°	Mr (Da)	Score	Searched peptides	Matched peptides	Cov. (%)
3006 (4c)	06A	Protein p60	Q84DT7	47,381	113	131	21	58
	06B	Listeriolysin O	P13128	58,709	149	202	32	74
3049 (1/2a)	49A	Protein p60	P21171	50,613	97	191	24	46
	49B	Listeriolysin O	P13128	58,709	211	112	31	69
	49C	Internalin C	Q2PTV1	33,155	109	96	15	52
3077 (4b)	77A	Protein p60	P21171	50,613	113	73	17	43
	77B	Listeriolysin O	Q724L1	58,690	172	66	22	51
	77C	Internalin C	Q45NG6	17,735	111	59	11	72
3993 (4d/4e)	93A	Protein p60	P21171	50,613	93	198	18	38

Data refer to parameters and results from database searches. "Accession no.°" refers to the UniProtKB accession number, except for 06A band (NCBInr accession no.°) and for 49C and 77C bands (MSDB accession no.°). The theoretical Mr is given. "Score" is reported as $-10\log_{10}(P)$, where P is the absolute probability that a match is random. The sequence coverage is expressed as a percentage of the complete sequence.

of strains from serovars 1/2a, 4b and 4c (Fig. 1 and Table 2 and Supplementary Table S2) [40,41]. However, the relative intensity of the LLO band (06B) from strain 3006 (serovar 4c) was significantly lower ($P < 0.05$) than the intensity of the corresponding band 49B from strain 3049 (serovar 1/2a) and of band 77B from strain 3077 (serovar 4b) (Fig. 3). The corresponding band from strain 3993 (serovar 4d/4e) did not show reproducibility among the biological replicates, and therefore was not analysed. This strain produced weak haemolysis [10], unlike the other three strains that exhibited strong haemolytic activity in horse blood-containing agar. Earlier studies, using a 1/2a strain, reported that some *prfA* mutations can reduce or abolish PrfA transcriptional activity [42], which may reduce the expression of virulence-associated proteins, such as LLO. Moreover, although mutations in *prfA* prevent the production of LLO, this virulence factor may continue to be expressed because its expression is also regulated by a PrfA-independent promoter responsible for small amounts of listeriolysin production [43]. This may be the case of strain 3993.

Once again, the results presented in this section do not support any correlation between genetic lineage and level of LLO in the secretome. However, considering LLO biological role as a virulent factor, we are tempted to speculate that strains 3049 and 3077 are virulent, whereas strains 3006 and 3993 exhibit low virulence.

3.5. Internalin C

The other PdP detected in virulent strain 3077 (serovar 4b) and in strain 3049 (serovar 1/2a), but absent in the low virulence strain 3993 (serovar 4d/4e) and in strain 3006 (serovar 4c) was internalin C (InlC) (Fig. 1 and Table 2 Supplementary Table 2S). The *inlC* gene, like most other known PrfA-regulated genes, is transcribed at a high rate at 37 °C [44] in minimal medium [45]. Several observations support the importance of InlC in host infection, including some structural features of the protein [46]. The *inlC* gene is present in pathogenic but not in non-pathogenic *Listeria* species [45,47]

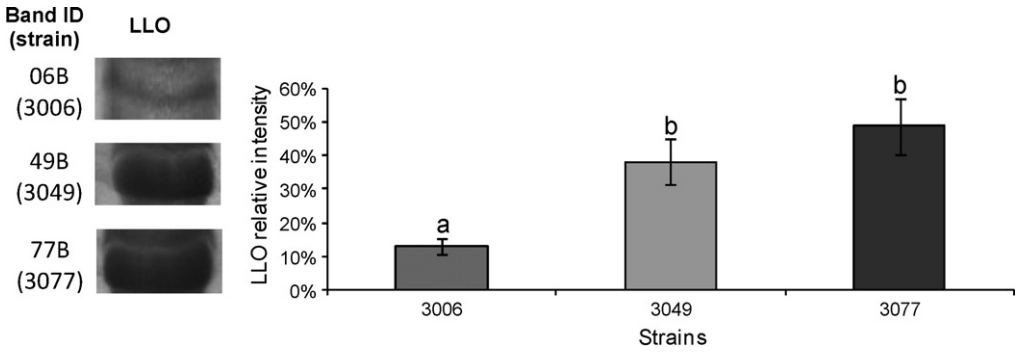

Fig. 3. Differences on listeriolysin O (LLO) production among three *L. monocytogenes* strains. The graph shows the mean relative intensity of the protein bands ± the standard deviation (error bars) ($n = 3$). Equal letters mean no significant differences between samples. Different letters mean that sample values are statistically different at $P < 0.05$. Representative subsections of SDS-PAGE images are shown.

and the corresponding deletion mutants have been reported as showing reduced virulence in a mouse model [45]. Previous studies have found *inlC* gene in lineage I and II isolates [47,48]. Although Doumith et al. [47] have detected the presence of the *inlC* gene in 4d and 4e strains (lineage I), in the present study the InlC protein was not detected in the supernatant of culture from serovar 4d/4e. Regarding lineage III strains, Tsai et al. [48] noticed that *inlC* gene was not amplified in a single isolate from serovar 4a. Some authors proposed the differentiation of strains 4a and 4c based on the lack of *inlC* gene in 4a strains [47]. Nevertheless, Liu et al. [49] analysed two 4c strains for the presence of *inlC* and reported that the gene was absent in strain ATCC 19116, which is equivalent to strain 3006 used in this work. Our results confirm the absence of InlC in this strain.

The results described in this section fully support the working hypothesis formulated in the previous section: the levels of internalin C found in the secretome of *L. monocytogenes* strains strongly suggest a virulent nature for strains 3049 and 3077, but a nonvirulent character for strains 3006 and 3993.

4. Conclusions

L. monocytogenes is a foodborne pathogen capable of causing serious disease in humans and animals. In the United States and France, listeriosis is ranked among the most frequent causes of death due to foodborne illness, immediately after salmonellosis [50,51]. Nevertheless, despite numerous international efforts that have been undertaken to reduce the incidence of listeriosis, this ubiquitous bacterial pathogen continuous to endanger food safety. However, not all isolates are equally pathogenic and several isolates have been characterized as of low virulence. Extracellular proteins are well known to play a determinant role in host-pathogen interactions, and their detection should contribute to evaluate the effective risk posed by some *L. monocytogenes* strains.

In this study, virulence factor expression (minimal medium, at 37 °C) was optimized according to [3,7,8] and a simplified methodology was used, based on protein separation and identification, to analyse major differences in strain secretomes. The four strains used were previously evaluated, using *in vitro* and *in vivo* tests of virulence, as virulent (one strain), of low virulence (one strain) or originating inconclusive results (two strains).

Key secreted proteins (protein markers), whose abundance differed among strains, were identified and their role in relation to virulence was investigated and discussed. The strain from serovar 1/2a and the virulent strain from serovar 4b presented higher levels of internalin C and listeriolysin O. The previously evaluated low-virulence phenotype of the strain from serovar 4d/4e strain could be related with the absence of internalin C and with the low abundance of listeriolysin O, two important virulence factors in *L. monocytogenes*. Internalin C was also not detected in strain 3006 (serovar 4c).

In order to evaluate the virulence of *L. monocytogenes* strains, different methods have been used, including *in vitro* tests with human cells and *in vivo* tests with animals. In general, there is good agreement when the results produced by both types of tests are compared. However, disagreement often occurs with specific isolates, and it is not possible in such cases to accurately predict or evaluate the pathogenic potential of the strains under analysis.

In this study, the clustering analysis of the four *L. monocytogenes* strains based on their secretome profiles, enabled the identification of two groups of bacteria. One group included the virulent strain from serovar 4b and strain 3049 (serovar 1/2a) that had previously originated conflicting results regarding its virulence potential. The second group included the low virulence strain from serovar 4d/4e and strain 3006 (serovar 4c) that was shown as virulent in animal assays, but as of low virulence in *in vitro* assays. These results suggest that strain 3049 is potentially pathogenic for humans and that strain 3006 should be a low virulence strain for humans. To our knowledge, none of the listeriosis outbreaks occurred in Europe and America between 1979 and 2008 were due to strains from serovar 4c. In fact, all these outbreaks were related with the consumption of food contaminated with strains from serovars 1/2a, 1/2b and 4b, except in Finland (1999) where an outbreak was caused by butter contaminated with a strain from serovar 3a [52,53].

Proteomics is vital to characterize the behaviour of organisms since, unlike the genome (a static concept), the secretome (a dynamic concept) is highly dependent on environmental conditions. The study of *L. monocytogenes* secretome under conditions favoring the expression of virulence factors offers an additional tool for assessing *L. monocytogenes* pathogenic potential. Moreover, the results obtained in this study suggest that internalin C may act as a virulence protein marker in *L. monocytogenes*.

As a whole, the results presented in this work suggest that the virulent potential of a particular *L. monocytogenes* strain may be predicted from the levels of both listeriolysin O (LLO) and internalin C (InlC) present in its secretome when the bacterium is grown under conditions favoring the expression of virulence factors (minimal medium, at 37 °C). Validation of this proposal will certainly involve the analysis of a large array of *L. monocytogenes* strains. If the model proves correct, then it may be possible to develop a method, both of rapid and of simple application, capable of accurately predicting the ability of a strain to cause disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.09.039.

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