ANDERSON CARLOS CAMARGO

UNCOVERING THE *Listeria monocytogenes* VIRULENCE TRAITS

Thesis presented to Universidade Federal de Viçosa as part of the requirements of the Graduate Program in Veterinary Medicine to obtain the title of *Doctor Scientiae*.

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PRESENTED IN 2017 AUGUST 25

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ABSTRACT

CAMARGO, Anderson Carlos, D.Sc., Universidade Federal de Viçosa, August, 2017. **Uncovering the *Listeria monocytogenes* virulence traits.** Adviser: Luís Augusto Nero.

Listeria monocytogenes is a Gram-positive bacterium commonly isolated from food processing environment, raw and processed foods. It is recognized as the causative agent of listeriosis, a serious disease that affects mainly individuals from risk groups. The present study aimed to characterize *L. monocytogenes* isolates recovered in Brazil from clinical sources, meat processing environmental and beef. Antibiotic resistance profiles, virulence genes, serotype diversity, ability to invade, replicate and spread in host cells, as well as cell death and IFN production induced by *L. monocytogenes* isolates were characterized. All isolates presented sensitivity to antimicrobials used for listeriosis treatment while most of them presented resistance or intermediate resistance to clindamycin and oxacillin. Molecular serogrouping produced contradictory results for seven isolated from serotype 1/2a, which were positive for the gene *lmo1118* and exhibited the profile IIc (serotypes 1/2c or 3c). In addition, fifteen isolates from serotype 4b amplified the gene *lmo0737*, being classified as atypical IVb-v1. The potential to invade Caco-2 cells was significantly affected by the presence of premature stop codons (PMSCs) in *inlA* gene of isolates from serotype 1/2c ($p < 0.005$). The isolates varied widely in their intracellular doubling times, and there was no clear relationship between serotype or source. There were significant differences between serotypes for cell-to-cell spread in Caco-2 cells, isolates from serotype 1/2a were generally impaired in their spreading ability while most os isolates from serotype 1/2b exhibited increased spreading ability ($p < 0.0001$). In addition, we identified three isolates from serotype 4b that spread nearly twice as much as the reference strain 10403S. No trends were observed regarding intracellular doubling times in iBMDM by comparing sources and serotypes; based on these results, intracellular growth seems to be determined by genetic characteristics of each strain. The cell death induced by some isolates after infection of iBMDM was Caspase 1 dependent, indicating that they induce pyroptosis. Some of these isolates also induced high INF production by iBMDM. Using primary macrophages, it was revealed that INF production was always STING dependent, and in some cases it is fully cGAS independent, partially cGAS dependent, or even mostly cGAS dependent. We have characterized *L. monocytogenes* isolates that could provide novel insight into infectivity of this pathogen that may not be revealed by studying common laboratory strains,

demanding further analysis of their genomes and other experimental approaches to reveal the mechanisms associated with the most extreme phenotypes exhibited.

RESUMO

CAMARGO, Anderson Carlos, D.Sc., Universidade Federal de Viçosa, agosto de 2017. **Revelando as características de virulência de *Listeria monocytogenes*.** Orientador: Luís Augusto Nero.

Listeria monocytogenes é uma bactéria Gram-positiva comumente encontrada no ambiente de processamento de alimentos, produtos *in natura* e processados. Esse patógeno é reconhecido como o agente causador da listeriose, uma doença grave que afeta principalmente indivíduos de grupos de risco. O presente estudo teve como objetivo caracterizar isolados de *L. monocytogenes* recuperados no Brasil a partir de fontes clínicas, ambiente de processamento de produtos cárneos, e de carne bovina. Os perfis de susceptibilidade aos antibióticos, genes de virulência, diversidade de sorotipos, capacidade de invadir, replicar e se disseminar em células hospedeiras, bem como a morte celular e a produção de IFN induzidas por isolados de *L. monocytogenes* foram caracterizados. Todos os isolados apresentaram sensibilidade aos antimicrobianos utilizados para o tratamento da listeriose, no entanto a maioria apresentou resistência ou resistência intermediária à clindamicina e à oxacilina. A sorotipagem molecular produziu resultados contraditórios para sete isolados do sorotipo 1/2a, que foram positivos para o gene *lmo1118* e exibiram o perfil IIc (sorotipos 1/2c ou 3c). Além disso, quinze isolados do sorotipo 4b amplificaram o gene *lmo0737*, e foram classificados como atipico IVb-v1. O potencial de invadir células Caco-2 foi significativamente afetado pela presença de codons de parada prematuros (PMSCs) no gene *inlA* do sorotipo 1/2c ($p < 0,005$). As cepas variaram amplamente em seus tempos de duplicação intracelular em celulas Caco-2, e não houve relação clara entre sorotipo ou fonte. Foram observadas diferenças significativas entre os sorotipos com relação a disseminação em células Caco-2; isolados do sorotipo 1/2a apresentaram defeito na disseminação celular, enquanto isolados do sorotipo 1/2b apresentaram maior habilidade de disseminação celular ($p <0,0001$). Além disso, identificamos três isolados de sorotipo 4b que se disseminaram em uma área aproximadamente duas vezes maior que a cepa de referência 10403S. Não foram observadas tendências quanto aos tempos duplicação intracelular em iBMDM, comparando fontes de isolamento e sorotipos. Os resultados indicam que a replicação intracelular é determinada pelas características genotípicas de cada isolado. A morte celular induzida por alguns isolados após a infecção de iBMDM foi dependente da enzima Caspase 1, indicando que eles induzem morte celular por piroptose. Alguns isolados também induziram alta produção de INF pela linhagem celular iBMDM. Usando macrófagos primários, foi revelado que a produção de INF foi sempre STING

dependente, em alguns casos totalmente cGAS independente, parcialmente cGAS dependente, ou mesmo cGAS dependente. Este trabalho caracterizou o perfil patogênico de isolados de *L. monocytogenes*, permitindo uma melhor compreensão dos mecanismos de infectividade desse patógeno, e levando a uma análise mais profunda dos genomas e outras abordagens experimentais para revelar os mecanismos associados aos fenótipos de virulência mais extremos.

INTRODUCTION

Millions of people suffer from episodes of diarrhea and other debilitating consequences due to ingestion of foodborne pathogens worldwide every year. *Listeria monocytogenes* is a major foodborne pathogen often isolated from food processing environments. Its persistence in the industry is associated with several factors, such as biofilm formation, multiplication at low temperatures, and resistance to environmental stress. Control of *L. monocytogenes* is a challenge and its persistence in the food processing facilities is often linked to food contamination.

The incidence of *L. monocytogenes* in raw foods is high when compared with processed foods such as ready-to-eat (RTE), but these foods are the most common vehicles linked to listeriosis outbreaks. *L. monocytogenes* is able to multiply even in high salt content RTE or those that are stored at refrigerated temperatures, making some countries adopt a zero tolerance policy for *L. monocytogenes* in RTE while others adopted tolerance up to 100 CFU/g in foods that are not suitable for *Listeria* growth.

In the United States, where there is an efficient network for monitoring foodborne diseases, the Center for Disease Control and Prevention (CDC) estimated an annual occurrence of 1,660 to 1,700 cases of invasive listeriosis, resulting in more than 1,500 hospitalizations and 16% of mortality rate. There are not many registers of *L. monocytogenes* isolation from clinical cases in Brazil, and listeriosis is not a disease under compulsory notification, making outbreaks investigation very often compromised. Listeriosis has a variable incubation period and can lead to clinical manifestations such as gastroenteritis, septicemia, encephalitis, meningitis and abortion in individuals from group risk, such as pregnant woman, newborn, elderly, and immunocompromised adults. The emergence of listeriosis occurred in the 1980s with the occurrence of several outbreaks and sporadic cases of the disease. Since then, *L. monocytogenes* has been the focus of much research conducted by surveillance systems of government agencies and food industries and also has been broadly used as model organism for host-pathogen interactions studies due its ability to enter and multiply in a variety of host cells.

Although all *L. monocytogenes* strains are considered virulent by regulatory bodies, genetic diversity in genes needed for infectivity presumably contributes to differential pathogenicity. The isolation and characterization by phenotypical and molecular techniques are crucial steps to assess the virulence potential of each strain. In the last decades, molecular methodologies have been applied in addition to phenotypical methods for a precise characterization, facilitating the understanding of the genetic diversity

between pathogenic and nonpathogenic isolates. *L. monocytogenes* is divided into 13 different serotypes that are grouped into four phylogenetic lineages (I, II, III, and IV). Serotypes 1/2a (lineage II), 1/2b and 4b (lineage I) are responsible for more than 95% of confirmed cases of listeriosis. Serotype 4b is prevalent worldwide, while serotype 1/2a has become more common in Northern Europe.

Here we characterized the serotypes distribution, antibiotic susceptibility, virulence genes profile, ability to invade, replicate and spread in Caco-2 cells, as well as induction of cell death and IFN production after infection of macrophages by *L. monocytogenes* isolates recovered in Brazil (from clinical sources, meat processing environment, and beef) in the last four decades. The present work revealed the virulence characteristics of *L. monocytogenes* recovered in Brazil and here we described isolates that can provide novel insight into infectivity that may not be revealed by studying common laboratory strains.

OBJECTIVES

The aim of this study was to reveal the virulence traits of *L. monocytogenes* strains obtained in different Brazilian regions from clinical sources, meat processing environment, and beef by phenotypic and molecular typing methods. Considering the main goal, specific objectives were:

- ✓ Review of literature about *Listeria monocytogenes*;
- ✓ Evaluate the antimicrobial susceptibility profiles of *L. monocytogenes* isolates;
- ✓ Evaluate the performance of different protocols for serotyping *L. monocytogenes*;
- ✓ Evaluate the virulence potential of selected strains;

CHAPTER 1 - The continuous challenge of characterizing the foodborne pathogen *Listeria monocytogenes*

Anderson Carlos Camargo, Joshua John Woodward, Luís Augusto Nero

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Title page

The continuous challenge of characterizing the foodborne pathogen *Listeria monocytogenes*

Running title: Characterizing *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is an important foodborne pathogen commonly isolated from food processing environments and food products. This organism can multiply at refrigerator temperatures, form biofilms on different materials and under various conditions, resist a range of environmental stresses, and contaminate food products by cross-contamination. *L. monocytogenes* is recognized as the causative agent of listeriosis, a serious disease that affects mainly individuals from high risk groups such as pregnant women, newborns, the elderly, and immuno-compromised individuals. Listeriosis can be considered a disease that has emerged along with changing eating habits and large-scale industrial food processing. This disease causes losses of billions of dollars every year with recalls of contaminated foods and patient medical treatment expenses. In addition to the immune status of the host and the infecting dose, the virulence potential of each strain is crucial for the development of disease symptoms. While many isolates are naturally virulent, other isolates are avirulent and unable to cause disease; this may vary according to the presence of molecular determinants associated with virulence. In the last decade, the characterization of genetic profiles through the use of molecular methods has helped to track and demonstrate the genetic diversity among *L. monocytogenes* isolates obtained from various sources. The purpose of this review is to summarize the main methods used for isolation, identification, and typing of *L. monocytogenes*, and also describe its most relevant virulence characteristics.

Keywords: *Listeria monocytogenes*, food, listeriosis, virulence potential, typing methods

Introduction

Listeria monocytogenes is the causative agent of listeriosis, a serious zoonotic disease resulting from the ingestion of food containing this microorganism. The disease can lead to clinical manifestations such as gastroenteritis, sepsis, encephalitis, meningitis and abortion (McLauchlin and others 2004). In the United States of America (USA), where there is an efficient network for monitoring diseases associated with food consumption, the Centers for Disease Control and Prevention (CDC) estimates an annual occurrence of 1,660 to 1,700 cases of invasive listeriosis, resulting in more than 1,500 hospitalizations and 16% of mortality rate (Scallan and others 2011). Some groups have a higher risk to develop the disease, including newborns, pregnant women, the elderly, transplant patients, those with immunodeficiency, including patients with Human Immunodeficiency Virus (HIV), and those diagnosed with cancer. Although listeriosis is relatively rare, it is considered a serious disease. *L. monocytogenes* is responsible for 19% of total deaths associated with the consumption of contaminated food in the USA (Scallan and others 2011).

Many factors make this foodborne pathogen a public health concern. These include changes in industrial processes highlighted as relevant for *L. monocytogenes*, such as use of refrigeration systems, large-scale industrial food processing, and changes in eating habits of the population towards consumption of ready-to-eat (RTE) products (Jay and others 2005; Swaminathan and Gerner-Smidt 2007). Several studies have been published, mainly in industrialized countries, to understand the mechanisms of *L. monocytogenes* persistence in the food processing environment, its contamination routes, and its potential to cause disease in the host.

In the last few decades, phenotypic and genotypic studies have led to new insights into the genetic evolution and virulence potential of *Listeria* species. This characterization has been possible due to the development of accurate techniques for typing of this pathogen. Here, we present an updated description of the main methods described for isolation and identification of *Listeria monocytogenes*, the use of typing methods for surveillance and outbreaks tracking, and also present the most pathogenic features and its importance for each step of the virulence cycle.

Isolation methods and species identification

To date, 17 species and 6 subspecies have been described for genus *Listeria* (*http://www.bacterio.net/listeria.html*), and many of them are widely distributed in the food processing environment. Only two were described as pathogenic: *L. ivanovii*, which has epidemiological importance restricted to ruminants, and *L. monocytogenes*, which can infect a variety of animal species including humans (Ryser and Marth 2007; Swaminathan and Gerner-Smidt 2007).

Conventional microbiological methods are indispensable for the isolation and identification of *Listeria* spp. from environmental sources, as well as from food products and clinical samples. Standard methods for isolation from different sources have been described by the International Organization for Standardization (ISO), the US Food and Drug Administration (FDA), International Dairy Federation (IDF), United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS), and Health Protection Branch (HPB) (Ryser and Marth 2007).

For all methods, samples are pre-incubated in basal medium for the recovery of injured cells, followed by enrichment in selective/differential supplemented broths, such as Buffered Listeria Enrichment Broth (BLEB), Half-Fraser Broth (HFB), and Fraser Broth (FB). After the primary selective enrichment, the obtained cultures are streaked on selective agars; the most common culture media used in this step are the Palcam Agar Base (*Listeria* spp. develop small brown/black colonies, surrounded by black halos due to aesculin hydrolysis), Listeria Selective Agar (OXFORD, with the same characteristic colonies observed as in Palcam), and the selective Chromogenic Listeria Agar (ALOA, in which *Listeria* spp. develop blue/green colonies, and *L. monocytogenes* presents an additional feature: an opaque white halo due to its lecithinase activity).

Alternative enrichment broths and culture media can also be considered, and indications may vary in accordance with the adopted protocol (Table 1) (AOAC 2000.; Farber and others 1994; Hitchins and Jinneman 2011; Scotter and others 2001a; Scotter and others 2001b; USDA 2013). After isolation, when incubated in Trypticase Soya Agar (TSA), pure cultures appear as non pigmented, translucent, and present bluish color when viewed under oblique lighting (Jay and others 2005).

Table 1. Standard protocols for isolating *Listeria monocytogenes* from different sources[1].

Protocol	Source	Pre-enrichment/conditions	Enrichment/conditions	Selective/differential agar/conditions	Reference
AOAC/IDF	ES and food	BLEB, Acriflavin and naladixic acid (48h at 30°C)		OXA (up to 48 h at 35°C)	AOAC 1996; AOAC 2000
ISO 11290 Part 1	Cheese, minced beef, dried egg powder	HFB (24 h at 30°C)	FB (48 h at 37°C)	OXA, PALCAM (up to 48 h at 30°C or 37°C, respectively)	Scotter and others 2001a
ISO 11290 Part 2	Cheese, meat, dried egg powder	BLEB or HFB (24 h at 35°C)	FB or ONE (24 h at 35°C)	OXA, PALCAM /ALOA (up to 48 h at 35°C)	Scotter and others 2001b
FDA/BAM	Food	BLEB (24 h at 30°C)	BLEB (48 h at 30°C)	OXA, MOX, PALCAM, LPM / BCM, ALOA, CHROMagar *Listeria*, or Rapid' *L. mono* Medium (up to 48 h at 35°C)	Hitchins and Jinneman 2011
USDA-FSIS	ES, red meat, poultry, egg products	UVM (20 - 26 h at 30°C)	MOPS-BLEB, FB (18-24 h at 35°C)	MOX (up to 48 h at 35°C)	USDA 2013
HPB	ES and food	UVM (24 and 48 h 30°C)	FB (24 - 48 h at 35°C)	LPM or PAL (up to 48 h at 30°C)	Farber and others 1994

ALOA, Agar Listeria Ottaviani and Agosti Medium; AOAC, Official Methods of Analysis; BAM, Bacteriological Analytical Manual; BCM, Biosynth Chromogenic Medium; BLEB, Buffered Listeria Enrichment Broth; ES, environmental Samples; FB, Fraser Broth; FDA, Food and Drug Administration; HFB, Half-Fraser Broth; HPB, Health Protection Branch; IDF, International Dairy Federation; ISO, International Organization for Standardization; LiCl, lithium chloride; LPM, Lithium Chloride–Phenylethanol–Moxalactam Medium; mFB, modified Fraser Broth; MOPS-BLEB, Morpholine-Propanesulfonic Acid–Buffered Listeria Enrichment Broth; MOX, Modified Oxford Listeria Selective Agar; ONE, Oxoid Novel Enrichment Broth; OXA, Oxford Medium; PALCAM, PALCAM Listeria Selective Agar; USDA-FSIS, U.S. Department of Agriculture—Food Safety and Inspection Service; UVM, Modified University of Vermont Broth.

The biochemical species identification is possible by the catalase reaction, carbohydrate fermentation profile, hemolytic activity, and other complementary tests such as Gram-staining and motility test at 25 ºC. Commercial kits, as the API-Listeria test kit (bioMerieux, Marcy-Etoile, France) have been designed for the genus *Listeria*, allowing easy biochemical differentiation in a microtube format, which targets presence or absence of arylamidase (DIM test), hydrolysis of aesculin, presence of α-mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-agatose (Allerberger 2003; Bille and others 1992; Jadhav and others 2012; Weller and others 2015).

To improve the assessment of hemolysis, and ensuring a more reliable differentiation between the hemolytic species *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, various authors recommend the use of the CAMP test (Christie, Atkins, Munch-Petersen) (Seeliger and Jones 1986). The CAMP test detects synergistic hemolysin activity of *Listeria* spp., with the beta toxin of *Staphylococcus aureus* and the exofactor of *Rhodococcus equi*, on a sheep blood agar plate. *L. monocytogenes* has a positive reaction with *S. aureus*, but a negative one with *R. equi*; and *L. ivanovii* displays the reverse results. Even more so, *L. seeligeri* can show a weak positive reaction with *S. aureus*. Therefore, skill is necessary to perform and interpret these results (Allerberger 2003).

L. monocytogenes can be identified by immuno-based techniques, such as Enzyme-Linked Immunosorbent Assays (ELISA) and Immunomagnetic Separation (IMS), but these methods haves a minimum detection limit of 10^5 cells/mL for bacterial detection from environmental samples by antigen-antibody reaction.

It is important to point out that in recent years several new species of *Listeria* has been described, and many of them have their own characteristics, distinct from other species. Considering this, molecular-based methods, using specific primers, and genetic sequencing are required to properly perform phylogenetic position analysis and species attribution (Liu 2006; Weller and others 2015). The polymerase chain reaction (PCR), have been widely used for *Listeria* identification. Many primers were designed for differentiation of *Listeria* species, and the most common targets to detect *L. monocytogenes* include the virulence-associated genes *hly, actA, plcA, plcB, inlA, inlB, inlC,* and *inlJ* (Liu 2006). The PCR procedure has proved to be a rapid and sensitive method for the routine analysis of different types of food (Aznar and Alarcón 2003).

According to Kaclıková and others (2003), *L. monocytogenes* can be identified by PCR equivalent to ISO 11290-1 or ISO 10560 in terms of detection limit (100 cfu per 25

or 10 g) and with 100% relative accuracy in cheese, smoked fish, and ready-to-eat meat products. Currently several multiplex PCR protocols are being used for the rapid detection of *Listeria* spp. and potentially pathogenic *L. monocytogenes* from different sources. Rawool and others (2007) developed a multiplex PCR to detect the 4 virulence-associated genes *plcA, hlyA, actA,* and *iap* in enrichment milk samples using previously described primers. Liu and others (2007) standardized a multiplex PCR to detect the *inlA, inlC,* and *inlJ* genes, that also provide identification regarding virulence potential.

Ryu and others (2013) developed a multiplex PCR for the rapid and simultaneous detection of 6 *Listeria* species (*L. grayi, L. innocua, L. ivanovii, L. monocytogenes, L. seeligeri,* and *L. welshimeri*) from meat processed foods. The authors suggested that this method can be useful for the detection of *Listeria* species in contaminated foods and clinical samples. Recently, Liu and others (2015) developed a new multiplex PCR method which is able to discriminate among *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii,* and *L. grayi* in deli meats. This method allowed accurate detection of *Listeria* spp., but pre-enrichment procedures are important to increase sensitivity.

Furthermore, real-time PCR-based methods have been developed for the rapid identification and quantification of *Listeria* species from a variety of sources (Barbau-Piednoir and others 2013; Gianfranceschi and others 2014; Le Monnier and others 2011; Quero and others 2014). Real-Time PCR was designed for the diagnosis of meningoencephalitis caused by *L. monocytogenes* using the *hly* gene target, and it provided reproducible results over a wide range of concentrations (Le Monnier and others 2011).

Recently in Europe, a study was conducted at 12 laboratories in 6 countries by using real-time PCR-based methods for *L. monocytogenes* detection in soft cheese, and the results were compared with those of the ISO 11290-1 standard method. The observed limit of detection after pre-enrichment in Half Fraser Broth by real-time PCR was down to 10 CFU per 25 g of sample. The excellent agreement observed among the laboratories suggests an option soon to be implemented by the authorities and the food industry (Gianfranceschi and others 2014). However, the equipment necessary for this analysis is still not available in many laboratories. Mass spectrometry based methods for identification and typing of *Listeria* spp. were already described and allows differentiation of pathogenic strains and even clonal lineages (Barbuddhe and others 2008).

In addition, sequencing of the 16S rRNA gene, 23S rRNA gene, or *iap* gene has been used by many authors for accurate phylogenetic analysis and species identification (Liu 2006). Genetic sequencing has more become popular in the last decade, and currently there are a several public and private companies providing these services at relatively low cost.

L. monocytogenes typing

Typing methods are useful for tracking the origins of the pathogen and to characterize its virulence potential. The typing can be performed by phenotypic and by molecular methods, and several methods have been described in last decades for epidemiological investigations and genetic characterization (Boerlin and Piffaretti 1991; Brosch and others 1994; Doumith and others 2004; Salcedo and others 2003; Schönberg and others 1996; Wernars and others 1996). In addition to the epidemiological data, the combination of different typing methods provides a proper characterization of isolates obtained from different sources.

L. monocytogenes strains are grouped into 4 phylogenetic lineages, which can be differentiated by conventional and molecular techniques. The serotypes 1/2b, 3b, 3c, and 4b constitute the lineage I; serotypes 1/2a, 1/2c, and 3a constitute the lineage II; and, rarely, serotypes 4a, 4c, and atypical 4b form the lineage III. Recently a new lineage was described (lineage IV), which is also represented by serotypes 4a, 4c, and atypical 4b, but is rarely found (Table 2) (Orsi and others 2011; Ward and others 2008).

One of the first phenotypic techniques used to type *L. monocytogenes* was serotyping (Seeliger and Höhne 1979). Based on the expression of surface proteins; namely somatic antigens (O factor) and flagellar antigens (H factor), 13 *L. monocytogenes* serotypes have been described (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (Allerberger 2003; Seeliger and Höhne 1979). Serotypes 1/2a, 1/2b, and 4b are involved in 95% of all listeriosis cases, with serotype 4b responsible for higher hospitalizations rates and deaths (Cartwright and others 2013; Swaminathan and Gerner-Smidt 2007).

The conventional agglutination method (Seeliger and Höhne 1979; Seeliger and Jones 1986) is the reference protocol for serotyping *L. monocytogenes* isolates obtained from clinical and food samples, however, it is time-consuming and expensive. Palumbo and others (2003) developed an ELISA-based assay for serotyping *L. monocytogenes*; however, it was also time-consuming and it was still dependent on producing high-quality antisera.

Table 2. Information regarding environmental distribution, genetic characteristics, and associated lineages of *L. monocytogenes* isolates.

Lineage	Initial identification	Serotypes	Genetic characteristics	Distribution
I	Piffaretti and others 1989	1/2b, 3b, 3c, 4b	Lowest diversity among the lineages; lowest levels of recombination among the lineages	Commonly isolated from various sources; overrepresented among human
II	Piffaretti and others 1989	1/2a, 1/2c, 3a	Most diverse, highest recombination levels	Overrepresented among food and food-related as well as natural environments; Serotype 1/2a is becoming common in human
III	Rasmussen and others 1995	4a, 4b atypical, 4c	Very diverse; recombination levels between those for lineage I and lineage II	Most isolates obtained from ruminants
IV	Described by Roberts and others 2006; first reported as lineage IV by Ward and others 2008	4a, 4b atypical, 4c	Few isolates analyzed to date.	Most isolates obtained from ruminants

Source: Orsi and others (2011), adapted from Piffaretti and others (1989), Rasmussen and others (1995), Roberts and others (2006), Ragon and others (2008), Ward and others 2008

Due to practical reasons, molecular methodologies have been widely used for the rapid screening of *L. monocytogenes* strains based on serogroup divisions. These methods provide rapid and low-cost results, but the exact identification of the serotype is not possible, since these protocols propose a serogroup categorization that includes different serotypes; usually the most prevalent serotype, and other non frequent serotypes (Borucki and Call 2003; Doumith and others 2004; Kérouanton and others 2010; Vitullo and others 2013; Zhang and Knabel 2005).

Serogrouping proposed by Doumith and others (2004) differentiates *L. monocytogenes* into 4 molecular serogroups: IIa corresponded to serotypes 1/2a and 3a; IIc to 1/2c and 3c; IIb to 1/2b, 3b, and 7; IVb to 4b, 4d and 4e (Doumith and others 2005). This is now the main protocol considered for serogrouping isolates obtained from food and clinical samples. However, various similar PCR-based methods have been described in the last decade (Chen and Knabel 2007; Kérouanton and others 2010; Vitullo and others 2013; Zhang and Knabel 2005), and all of them propose a similar serogroup categorization.

Recently, Nho and others (2015) developed a new multiplex PCR to differentiate serotypes 1/2a, 1/2c, 3a, and 3c, but few isolates have been tested. The great advantage of these methods is to obtain fast results at low cost; however, some isolates from serotypes 1/2a and 4b may present atypical profiles, as described before (den Bakker and others 2014; Kérouanton and others 2010; Leclercq and others 2011). This problem is related to the target genes in the protocol, which are not serotype-specific surface antigens (Camargo and others 2016; Huang and others 2011; Kérouanton and others 2010; Leclercq and others 2011; Lee and others 2012).

Phage typing is an alternative method for clustering *L. monocytogenes*, and it has also helped to link listeriosis as a foodborne disease (Fleming and others 1985). A number of phage sets have been developed, and typable strains vary from 52% to 78%, according to the number of phages used in these investigations and the susceptibility of each strain (Audurier and Martin 1989; Van Der Mee-Marquet and others 1997).

A significant number of *Listeria* strains are untypable by this technique, limiting its scope in *L. monocytogenes* typing. On the other hand, in recent years its application has been further explored, how being considered as a biocontrol tool with foods (Chibeu and others 2013; Oliveira and others 2014), diagnostics (Hagens and others 2011; Tolba and others 2012), immobilization and detection (Habann and others 2014), and as an antimicrobial (Schmelcher and others 2012). According to Hagens and Loessner (2014)

phages that are specific for *Listeria* provide numerous novel tools for various interesting approaches.

Although phenotypic methods have been useful to typing *L. monocytogenes*, currently molecular methods appear to be more reliable and sensitive, and these tools are widely used worldwide. Molecular typing methods can be based on use of restriction enzymes. Ribotyping method is based on the restriction fragment length polymorphisms (RFLPs) associated with the ribosomal operons, and it was used by Wiedmann and others (1997) for lineage-specific differentiation of *L. monocytogenes* isolates, and to also assess their pathogenic potential. Ribotyping has been successfully used to type *L. monocytogenes* from various sources, offering the best discriminatory power when associated with serotyping (De Cesare and others 2007; Kabuki and others 2004; Sant'Ana and others 2012).

According to Wiedmann (2002), the use of enzymes *Eco*RI and *Pvu*II by 2 distinct restriction reactions provides better strain discrimination. However, it cannot efficiently differentiate strains from serotypes 1/2b and 4b, thus limiting its use for epidemiologic investigations (Jadhav and others 2012). Its discriminatory power is similar to Multilocus Enzyme Electrophoresis (MLEE). MLEE divides the *L. monocytgenes* into 2 primary subgroups: one represented by serotypes 1/2b, 4a, and 4b, and another by serotypes 1/2a and 1/2c. The bacterial isolates are differentiated according to the electrophoretic mobility variation of a large number of metabolic enzymes. However, this method is labor-intensive, suffers from inter-laboratory non-reproducibility, and presents less discriminatory power when compared with others molecular methods (Boerlin and Piffaretti 1991; Ryser and Marth 2007).

Pulsed Field Gel Electrophoresis (PFGE) is widely used for genetic characterization of microorganisms, and the method standardized for *L. monocytogenes* uses a combination of 2 restriction enzymes: *Apa*I and *Asc*I (Graves and Swaminathan 2001). This method is based on RFLPs of bacterial DNA, resulting for *L. monocytogenes* fragments ranging from 30 to 600 kb, which appears in 6 to 25 bands in agarose gel electrophoresis (Graves and Swaminathan 2001; Jadhav and others 2012; Liu 2006). PFGE is widely used and is considered the gold standard to track *L. monocytogenes* from food processing facilities, foods, and clinical samples. The CDC and Association of Public Health Laboratories have created a network for tracking *L. monocyogenes* in epidemiological studies and foodborne outbreaks using PFGE as a standardized method for subtyping this pathogen.

The European Center for Disease Prevention and Control (ECDC) also uses the PFGE as a standard procedure for typing *L. monocytogenes* isolates. PFGE is more discriminatory than serotyping and other methods based on restriction enzymes, and it shows the possibility that cultures belonging to the same serotype have different genetic profiles (Fugett and others 2007; Galvão and others 2012; Graves and Swaminathan 2001; Gudmundsdóttir and others 2005; Hächler and others 2013). The analysis of genetic band profiles is done by computer software, which enables a quick and easy comparison of the data and may involve the profiles of isolates obtained from food, with profiles of strains involved in outbreaks. This method is useful for epidemiological studies, implementation of corrective actions, and for monitoring of critical control points. Major disadvantages of PFGE are the time required to complete the procedure (minimum 24 hours), high costs of restriction endonucleases, and specialized equipment for electrophoresis.

Several PCR-based methods also have been used as an alternative for typing *L. monocytogenes* strains, such as Molecular Serogrouping (previously described), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Repetitive Element PCR (REP-PCR), and Fluorescence Amplified Fragment Length Polymorphism (fAFLP), and Multilocus Variable Number of Tandem Repeat Analysis (MLVA). In addition, DNA sequencing-based subtyping techniques, as Multilocus Sequence Typing (MLST), Multi-Virulence locus Sequence Typing (MVLST), and Single Nucleotide Polymorphism (SNP) analysis have been demonstrated (Jadhav and others 2012; Liu 2006).

The RAPD method allows a single arbitrarily selected primer, generally 10 bp in length, to anneal with complementary sequences on the target DNA, creating a genetic profile (Lawrence and others 1993). This protocol has been used for fast typing of *L. monocytogenes* from foods and can provide valuable information about possible persistence of strains, also indicating potential sources of cross-contamination when used combined with serotyping (Aurora and others 2009; Vogel and others 2001). In addition, according to Hadjilouka and others (2014) the combination of REP-PCR with any primer used in the RAPD analysis can provide an optimum discrimination of strains, but alone, the technique does not have high discriminatory power.

In the AFLP method, the total DNA is subject to digestion by 2 restriction enzymes, fragments are amplified using PCR, and the products are subjected to gel electrophoresis. The described protocols were based on different restriction enzymes, being *Bam*HI and *Eco*RI for AFLP I, and *Hind*III and *Hha*I for AFLP II. According to Parisi and others

(2010), AFLP and MLST methods produced similar results in terms of discriminating power in *L. monocytogenes* from food and environment, generating 62 and 66 types from isolates used in the study, respectively. The authors suggest that these 2 methods can be associated in order to provide high-resolution results. Lomonaco and others (2011) compared AFLP and PFGE methods. Both protocols grouped *L. monocytogenes* strains into 2 main clusters, and the results showed a similar discriminatory power. According to the authors, AFLP can be successfully used to type *L. monocytogenes* strains obtained from foods and food processing facilities where PFGE is not available; however it is also expensive and time-consuming.

PCR-RFLP consists of PCR amplification of *L. monocytogenes* housekeeping or virulence genes, followed by digestion with selected restriction enzymes (*Hha*I, *Sac*I or *Hin*fI) and separation by gel electrophoresis. The distinct band patterns allow differentiation among *L. monocytogenes* subtypes (Wiedmann and others 1997). As advantages, this method requires only a small amount of starting DNA, and may be used in epidemiological investigations to track *L. monocytogenes* (Liu 2006). Strydom and others (2013) subjected *L. monocytogenes* isolates to PCR-RFLP and PFGE, and the results indicated that both molecular subtyping methods were sensitive and specific enough to assess the genetic diversity.

REP-PCR targets dispersed repetitive sequence elements, such as repetitive extragenic palindromes (REPs) of 35–40 bp, which are found in the extragenic regions of the genome in direct or reverse orientation. The REP sequences represent useful single primer set binding sites for PCR amplification, and different locations of these REP sequences in the genome produce variable fragment sizes. According to Harvey and others (2004), PFGE presented greater discriminatory power for typing *L. monocytogenes* isolates from foods than REP-PCR and MEE, although the 3 methods were able to differentiate closely related *L. monocytogenes* strains. Hadjilouka and others (2014) subjected 121 strains from food products to RAPD and REP-PCR, and the last method provided better differentiation among isolates. Apparently, REP-PCR possesses a discriminatory power similar to PFGE and ribotyping; as REP-PCR is faster and cheaper than these typing techniques, it can be considered as an important alternative for typing *L. monocytogenes*.

fAFLP is a variant of AFLP, done by using fluorescent PCR primers. fAFLP is done by a modified protocol previously described for typing *Campylobacter*. By this protocol, *Listeria* genomic DNA is digested by using the enzyme pair *Hind*III and *Hha*I. Roussel and others (2013) showed that 109 *L. monocytogenes* isolates from human clinical cases,

foods, food processing environments as well as animal cases, reference strains and isolates associated with outbreaks and sporadic cases were divided by fAFLP and PFGE into 3 clearly distinguishable lineages, and both methods showed equal discriminatory power. The authors suggested that fAFLP is a good alternative to PFGE for *L. monocytogenes* typing, and it can be used for investigations of listeriosis outbreaks and tracking contamination sources in food processing environments.

MLVA focuses on the study of the variability of the number of tandem repeats (VNTRs) at specific loci of bacterial genomes. VNTRs are short segments of DNA that have variable copy numbers, and the difference in copy numbers at specific loci is used to measure relationships among strains. The primers are designed from the flanking regions of VNTRs. The PCR products may be separated on agarose gels (according to sizing of individual fragments the copy numbers are detected) and/or subjected DNA sequencing systems (Murphy and others 2007; Sperry and others 2008).

Chenal-Francisque and others (2013), evaluated 18 VNTRs loci and combined the 11 best ones into 2 multiplex PCR assays. The authors suggest that this protocol is useful for the characterization of *L. monocytogenes* strains, and that it represents an attractive first-line screening method to epidemiological investigations and listeriosis surveillance. Furthermore, it is of low cost, easy to perform, and provides rapid results (around 8 h), with portable (numerical) results. However, this method exhibits lower discriminatory power than PFGE based on *Apa*I and *Asc*I restriction enzymes, as observed by Sperry and others (2008).

According to Lindstedt and others (2008), MLVA was slightly more discriminatory than PFGE for isolates from Norway, consisting of 28 MLVA profiles and 24 PFGE profiles. The opposite was observed for isolates from Sweden, producing 42 MLVA profiles and 43 PFGE profiles; however, only *Asc*I enzyme was used for PFGE in this study. In addition, Chen and others (2011) reported the MLVA application to typing *L. monocytogenes* directly in food samples. This method successfully typed strains from cheese, roast beef, egg salad, and vegetable samples after a 48h enrichment, generating accurate, reproducible, and high-quality results from artificially contaminated food samples.

Many of the techniques described above have been widely used in phenotypic and genotypic studies and led to new insights into ecology, epidemiology, virulence potential, and genetic evolution. In the last decade, PCR-based sequencing methods have become available for many laboratories, providing several advantages, including electronic

portability of nucleotide sequences, which allows for quick comparisons between laboratories.

One of the first PCR-based sequencing techniques described for typing pathogenic microorganisms was MLST (Maiden and others 1998). By using this technique, it is possible to evaluate the DNA sequence variations of housekeeping genes, and compare the sequences with their original profiles. For each gene, different sequences found are assigned as alleles, and these provide a profile that defines unambiguously the type of sequence (ST) of each isolate. Changes in the nucleotide housekeeping genes occur relatively slowly, making this method suitable for epidemiological investigations (Cooper and Feil 2004; Enright and Spratt 1999).

L. monocytogenes typing by MLST was first demonstrated by Salcedo and others (2003). Nowadays, a protocol adapted by Ragon and others (2008) has become widely used to characterize *L. monocytogenes* isolates, targeting 7 housekeeping genes: *acb*Z (ABC transporter), *bgl*A (beta-glucosidase), *cat* (catalase), *dap*E (Succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (lactate deshydrogenase), and *lhk*A (histidine kinase).

In addition, Zhang and others (2004) developed a multi-virulence-locus sequence typing (MVLST) method that exhibits higher discriminatory power than ribotyping, PFGE and MLST. This method is based on the sequencing of 3 virulence genes (*prf*A, *inl*B, and *inl*C) and 3 virulence-associated genes (*dal*, *lis*R, and *clp*P), and it can improve the discriminatory power of MLST while also providing information about virulence potential of *L. monocytogenes* strains. In addition, the inclusion of the 2 virulence genes (*inl*A and *act*A) was proposed by Chen and others (2007) to complement the 6 previous genes used in the analysis. Recently, various studies have used MLST and MVLST combined and demonstrated success for epidemiological investigations, and also genetic/virulence evolution (Cantinelli and others 2013; Knabel and others 2012; Martín and others 2014; Yin and others 2015).

Another approach is to type *L. monocytogenes* by assessing SNPs at multiple locations on the genome. Unnerstad and others (2001) categorized 106 *L. monocytogenes* on the basis of SNPs in the *inl*B gene, and strains were clustered into 4 groups (serogroups 1/2a and 1/2c were clustered in one group, 1/2b and 3b in another group, and serotype 4b strains in 2 groups). Ducey and others (2007) designed Multilocus Genotyping (MLGT) by sequencing 23,251 bp of DNA from 22 genes distributed across 7 genomic regions in order to efficiently target SNP variation and type *L. monocytogenes* isolates from Lineage I. This protocol also provides information about a specific virulence-attenuated subtype

with a characteristic truncation mutation in *inl*A. Considering this, MLGT represents a significant new tool for pathogen surveillance, risk assessment, outbreak detection, and epidemiological investigations (Ducey and others 2007; Ward and others 2008).

Since the genomes of *L. monocytogenes* and *L. innocua* was published by Glaser and others (2001), various studies have been focused on comparative genomics to understand the genetic relationships among *L. monocytogenes* lineages and molecular mechanisms associated with virulence potential (Bécavin and others 2014; Gilmour and others 2010; Nelson and others 2004).

New technologies, as next-generation sequencing (post- Sanger sequencing technologies) by using Illumina MiSeq or NextSeq platforms have been described and successfully applied for extremely fast whole genome sequencing. To accurate data analysis, new bioinformatics devices are facilitating comparative genomics and data sharing among researchers, which improves the quality of epidemiological studies (Bergholz and others 2016; Stasiewicz and others 2015). These studies indicate that in a very near future, genome sequencing will replace many of the previously described methods due to its discriminatory power and portability of generated data (Kwong and others 2015).

Pathogenicity factors and virulence potential

L. monocytogenes has acquired a variety of virulence factors that allow it to successfully invade and survive within host cells. This bacterium has a characteristic way to colonize the human host, using phagocytic cells to be distributed in the body for cell-to-cell spread (Figure 1) (Chaturongakul and others 2008). After adhesion and invasion of the intestinal epithelium, *L. monocytogenes* spreads throughout the body, infecting macrophages, epithelial cells, endothelial cells, hepatocytes, fibroblasts, and cells of the nervous system (Figure 2). Translocation from the lumen into the intestinal epithelial cells is mediated by the surface-associated virulence protein internalin A (InlA) encoded by gene *inl*A; these proteins interact with specific receptors on host cells, leading to receptor-mediated internalization. InlA protein binds to glycoprotein E-cadherin present on the host cell, resulting in a rearrangement of the cell cytoskeleton and entry of the pathogen into the host cell (de las Heras and others 2011).

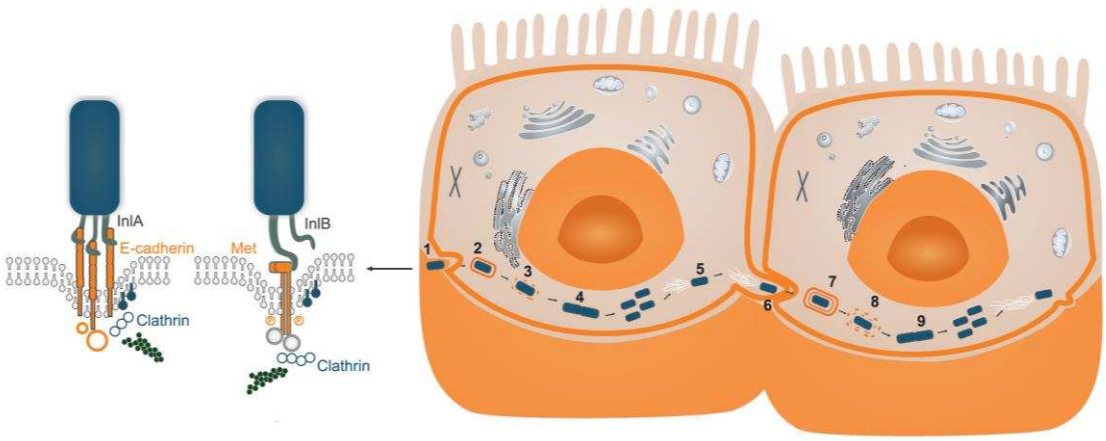

Figure 1. Steps of *Listeria monocytogenes* intracellular infection cycle**: (1)** attachment to the host cell surface and internalization via a "zipper mechanism" is promoted by two listerial surface proteins (InlA and InlB, encoded by *inlA* and *inlB* genes) with their respective epithelial cell surface receptors (E-cadherin and the receptor tyrosine kinase c-Met); **(2)** this results in engulfment of bacteria and endosome formation; **(3)** the scape from the vacuole is mediated by three membrane-damaging factors: a pore-forming toxin listeriolysin O (LLO), encoded by gene *hly*, and two phospholipases PlcA and PlcB, encoded by genes *plcA* and *plcB* (the latter need to be processed by the Mpl metalloprotease after secretion, which is encoded by *mpl* gene); **(4)** replication in the cell cytosol, by using cytosolic resources (Listeria adapt their metabolism and start the expression of several genes, such as the hexose transporter Hpt or the lipoate protein ligase LplA1, for multiplication; **(5)** cell-to-cell spread mechanism, which allows intracellular motility, is mediated by the surface protein ActA (encoded by the gene *actA*), which induces actin polymerization, providing *Listeria* motility through the cytoplasm; **(6)** at the end of this cycle, *Listeria* reach the plasma membrane and induce the formation of pseudopod-like structures (listeriopods), allowing the invasion of the neighbor cell: this activity is mediated by InlC protein (encoded by *inl*C gene); **(7)** phagocytosis and formation of a double-membrane vacuole; **(8)** rupture of the two membrane vacuole and *Listeria* escape, initiates a new round of proliferation, actin-based motility, and intercellular spread.

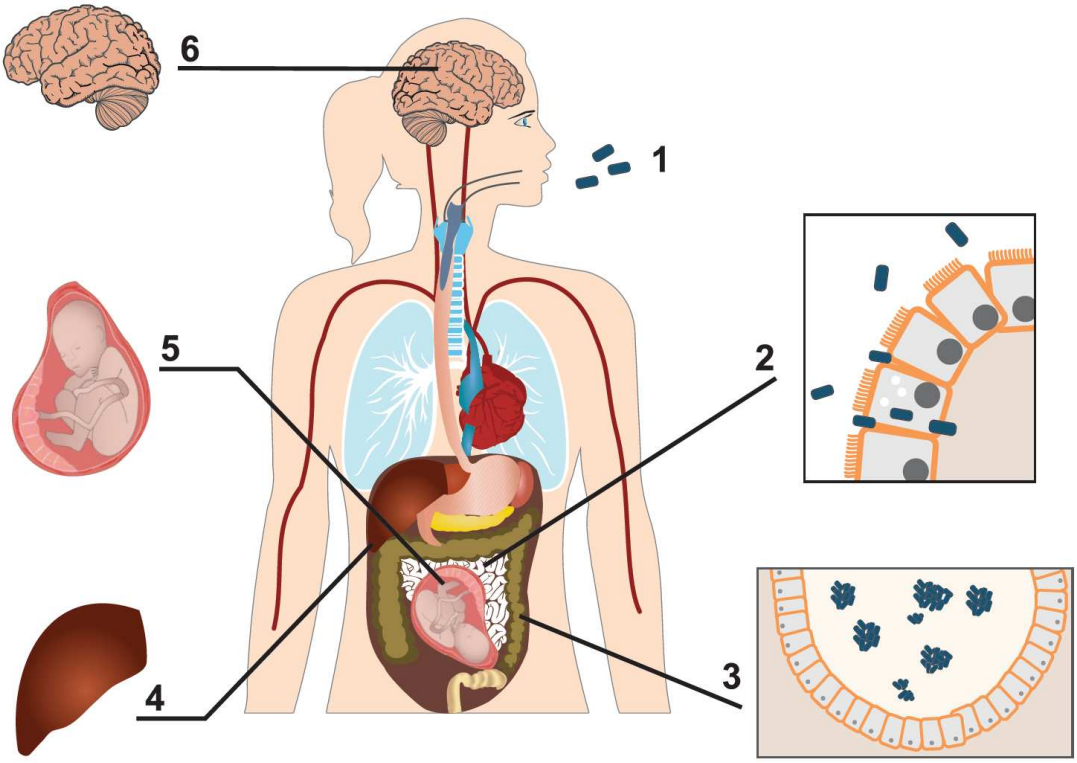

Figure 2. Human infection by *Listeria monocytogenes*: (1) ingestion of contaminated food; (2) *Listeria* colonization of the digestive tract can lead to symptoms including diarrhea and fever; the bacteria cross the intestinal barrier, due to the production of InlA, reaching the mesenteric lymph nodes and accessing the circulatory system; (3) *Listeria* can form aggregates, ActA-dependent, favoring the long-term persistence in the cecum lumen; (4) the first organs affected are the liver and spleen (which are reservoirs of bacterial persistence): these organs release this bacteria into the blood system, resulting in septicemia; (5) *L. monocytogenes* may cross the placental barrier leading to abortion, or generalized neonatal infection (causing pneumonia, sepsis, or meningitis); (6) in addition, *L. monocytogenes* can cross the blood–brain barrier and reach the brain, causing meningitis, meningoencephalitis, and rhombencephalitis.

After internalization, other virulence proteins such as pore-forming cytolysin listeriolysin O (LLO) encoded by gene *hly*, and 2 phospholipases C (PlcA and PlcB) encoded by genes *plc*A and *plc*B, help the bacterium to escape from phagosomes formed during the invasion process. The protein encoded by the *hly* gene causes pores in the membrane of the phagossome, resulting in lysis of the membrane and bacterial escape.

Furthermore, this protein is responsible for hemolytic activity in virulent isolates. The proteins encoded by the genes *plc*A and *plc*B operate in synergy within the vacuole, being activated by a metalloprotease (Mpl) which is encoded by *mpl* gene. For replication in the host cell cytoplasm, sugar phosphate permease HPT protein is required. Then, the spreading to neighboring cells is fully dependent on the actin polymerizing protein ActA, encoded by gene *act*A, that guarantees the movement through the cell by induction of

actin polymerization (Figure 1). In addition, the actA protein also participates in the maintenance of *Listeria* aggregates in the cecum lumen (de las Heras and others 2011; Vázquez-Boland and others 2001).

L. monocytogenes cells can spread by the bloodstream to mesenteric lymph nodes, liver, and spleen, where they multiply within macrophages and endothelial cells. Several genes encoding proteins related virulence, such as *act*A, *plc*A, *plc*B, *hly*A, *mpl*, and *prf*A, that are located in the "Listeria pathogenicity island" (LIPI-1) (Schmidt and Hensel 2004), which is regulated by the protein PrfA. In order to avoid unnecessary expression in the environment, the PrfA regulon is selectively activated during infection (de las Heras and others 2011).

Another important virulence-associated locus comprises the *inl*AB gene locus. The *L. monocytogenes* genome encodes 27 proteins now known as a family of internalins, however, InlA and InlB are the only internalins that have been directly implicated in host cell internalizations (Pizarro-Cerdá and others 2012). In addition, the Listeria genomic island (LGI1), initially identified by Gilmour and others (2010), consists of genes that encode putative translocation, resistance, and regulatory determinants. These characteristics suggest that this locus plays an important role in bacterial persistence and/or pathogenicity. The internalin protein encoded by the gene *inl*A is critical to the invasion of the bacteria into the intestinal barrier during the initial stage of infection (Bonazzi and Cossart 2006).

Several studies have shown that mutations in the *inl*A gene can lead to premature stop codons (PMSCs), resulting in the expression of a non functional truncated protein (Tamburro and others 2010). Although PMSCs are found in isolates from lineages I and II, they are most commonly observed in isolates from lineage II (serotypes 1/2a and 1/2c) that are frequently associated with environmental and food sources.

In a study conducted by Van Stelten and others (2010), it was confirmed that mutations in isolates from foods are more common than in isolates from clinical cases. Associated with this, strains with mutations had reduced invasion of Caco-2 cells, consistent with the critical role of internalins in the infection process. In this way, sequencing data of the *inl*A gene provide important information regarding virulence potential of *L. monocytogenes* strains obtained from the food production chain and clinical cases. In addition, sequencing *inl*A gene helps to estimate the risk of exposure to pathogenic strains through the consumption of food (Olier and others 2005; Rousseaux and others 2004; Van Stelten and others 2010).

Several factors may contribute to the development of listeriosis, such as the number of ingested bacterial cells, the host immunity, and the virulence potential of each strain. Despite being naturally virulent, some *L. monocytogenes* strains are avirulent and unable to cause disease. The evaluation of the virulence potential should be made by appropriate laboratory methods, such as sequencing of virulence genes and evaluation of the expression of virulence factors and their effects on host cell invasion through cell culture studies (Liu 2006). Such assessment may contribute to a better understanding of the virulence potential of strains isolated from the food production chain and clinical cases, and it provides input to implement programs to promote the prevention of listeriosis.

Concluding remarks

The intracellular foodborne pathogen *L. monocytogenes* is ubiquitous in nature. Genetic profiles obtained by molecular methods has helped to demonstrate the genetic diversity of this foodborne pathogen from various sites. The best methods are those that are specific, sensitive, fast, simple, reproducible, and low cost. Also, they must provide information about the pathogenic potential of strains. By applying these methods, it has been possible to detect various foods involved in outbreaks of listeriosis and allow proper control by food inspection agencies and the food industry. These methods can be useful for monitoring critical control points in the food-handling environment, in order to provide subsidies to apply corrective measures regarding cleaning and sanitization procedures to eliminate *L. monocytogenes* in the environment.

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CHAPTER 2 - *Listeria monocytogenes* in food processing facilities, food contamination and human listeriosis: the Brazilian scenario

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Title page

Listeria monocytogenes in food processing facilities, food contamination and human listeriosis: the Brazilian scenario

Running title: *Listeria monocytogenes* in Brazil

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Abstract

Listeria monocytogenes is a foodborne pathogen that contaminates food-processing environments and persists within biofilms on equipment, utensils, floors and drains, ultimately reaching final products by cross-contamination. This pathogen grows even under high salt conditions or refrigeration temperatures, remaining viable in various food products until the end of their shelf life. While the estimated incidence of listeriosis is lower than other enteric illnesses, infections caused by *L. monocytogenes* are more likely to lead to hospitalizations and fatalities. Despite the description of *L. monocytogenes* occurrence in Brazilian food processing facilities and foods, there is a lack of consistent data regarding listeriosis cases and outbreaks directly associated with food consumption. Listeriosis requires rapid treatment with antibiotics and most drugs suitable for Gram-positive bacteria are effective against *L. monocytogenes*. Only a minority of clinical antibiotic resistant *L. monocytogenes* strains have been described so far; whereas many strains recovered from food processing facilities and foods exhibited resistance to antimicrobials not suitable against listeriosis. *L. monocytogenes* control in food industries is a challenge, demanding proper cleaning and application of sanitization procedures to eliminate this foodborne pathogen from the food processing environment and ensure food safety. This review focuses on presenting the *L. monocytogenes* distribution in food processing environment, food contamination, and control in the food industry, as well as the consequences of listeriosis to human health, providing a comparison of the current Brazilian situation with the international scenario.

Key words: *Listeria monocytogenes*, cross-contamination, listeriosis, antibiotic resistant, food safety

Introduction

Consumption of food and water contaminated by microorganisms is associated with the morbidity and mortality of humans worldwide (WHO 2007). *Listeria monocytogenes* is a foodborne pathogen that is frequently isolated from a variety of food processing environments and food products. Its prevalence in food processing facilities is attributable to its resistance to environmental stress, ability to multiply at low temperatures, and ability to form biofilms on the surfaces of different sites in processing facilities (Carpentier and Cerf 2011; Ryser and Marth 2007; Swaminathan and Gerner-Smidt 2007).

Ingestion of foods contaminated by *L. monocytogenes* can lead to listeriosis in people and other mammals. Although this disease can occur in healthy individuals, the invasive form is more likely to affect neonates, pregnant women, elderly, and immuno-compromised individuals. The incidence of listeriosis is lower than other foodborne pathogens, however, the disease presents a high fatality rate, placing *L. monocytogenes* among the main foodborne pathogens that cause deaths in the USA (Scallan and others 2015; Scallan and others 2011). In Brazil, however, the scenario is different: many studies demonstrate the presence of *L. monocytogenes* in food processing environment and in food products, but little is known about the incidence of human listeriosis, most probably due to the absence of an efficient surveillance and notification system (Blum-Menezes and others 2013; Lemes-Marques and others 2007).

Control of *L. monocytogenes* in food processing facilities is a challenge, in part because this pathogen can persist for years in these facilities. Nevertheless, the adoption of proper sanitization practices is the best way to control the pathogen in food processing facilities and to avoid cross-contamination to end products. This review focuses on describing the distribution of *L. monocytogenes* in the food processing environment, food contamination, the consequences for human health due to ingestion of contaminated foods, and the control of *L. monocytogenes* by food industries, comparing data obtained in Brazil with the international scenario.

L. monocytogenes distribution and food contamination

L. monocytogenes is widely distributed in the natural environment and it can be isolated from soil, water, drainage systems, vegetation, animal feed, farm environments, food processing facilities, various foods, and feces from healthy animals, including

humans (Ryser and Marth 2007; Sauders and others 2012). Several studies have documented the occurrence of *L. monocytogenes* in food processing facilities worldwide (Table 1). Many of these studies highlight the role of the food processing environment as sources of *L. monocytogenes* contamination of finished products.

Occurrence of *L. monocytogenes* on bovine carcasses, beef processing environment, and final cuts have been documented the in different regions of Brazil. Barros and others (2007) evaluated the occurrence of *Listeria* spp. and *L. monocytogenes* in 11 meat processing establishments, demonstrating that the pathogenic serotypes 1/2a and 4b are distributed on equipment and facilities in beef processing plants and meat products in the South of Brazil. Camargo and others (2015b) studied the diversity of *L. monocytogenes* in a beef processing environment in the Southeastern region, demonstrating by pulsed field gel electrophoresis (PFGE) that isolates recovered from final products shared the same profiles of isolates obtained from chopping boards and hands of employees.

Guerini and others (2007) also detected *Listeria* spp. at four cull cow and bull processing plants in the USA. *L. monocytogenes* was detected on carcasses in the chill cooler, including the pathogenic serotypes 1/2a, 1/2c, and 4b. Serotype 1/2c isolates that were recovered in the summer of 2005 and spring of 2006 from plant "A" shared a high similarity by PFGE, suggesting they were persistent, similar as observed by Camargo and others (2015b) in Brazil. Serotype 4b isolates that were found on hides and carcasses in the chill cooler presented different PFGE profiles, indicating they came from a different sources.

Regarding the occurrence of *L. monocytogenes* in the pork chain, various studies demonstrated that *L. monocytogenes* is ubiquitous in the Brazilian processing industry (Miyasaki and others 2009; Rossi and others 2011; Silva and others 2004; von Laer and others 2009). Interestingly, the pork processing environment in Brazil seems to be a source of atypical *L. monocytogenes* serotypes (Miyasaki and others 2009), as well as atypical hemolytic *L. innocua* (Moreno and others 2012).

An 2 years long study was conducted by Larivière-Gauthier and others (2014) in a pork slaughter and cutting plant in the Province of Quebec, Canada, in order to assess the distribution of *L. monocytogenes*. They found a high diversity of strains by PFGE within the entry areas of the processing plant (such as lairage pen environment, slaughtering, and evisceration), but over representation of one PFGE type in the cutting room environment. A similar 3-year study (2008 to 2011) was conducted in five pork plants in Sardinia, Italy. Among 211 samples, *L. monocytogenes* was detected from swine carcasses (33%), cecal

Table 1. Isolation of *Listeria monocytogenes* from food processing facilities, food, and associated serotypes.

Processing plant	Reference	Methodology*	Country	Samples	n of isolates	Serotypes or serogroups**
Beef	Camargo et al., 2015	ISO 11290-1 and 11290-2	Brazil	Tables	7	1/2c [1]
				Hand of employees	20	1/2c, 4b [1]
				Products	58	1/2b, 1/2c, 4b [1]
	Barros et al., 2007	USDA	Brazil	Equipaments	7	1/2a, 4b [1]
				Installations	2	1/2a, 4b [1]
				Products	10	1/2a, 4b [1]
	Zhu et al., 2012	ISO 11290-1	China	Feces	6	IIc [2]
				Hides	16	IIc [2]
				Pre-evisceration carcasses	16	IIc [2]
				Post-evisceration carcasses	12	IIc [2]
				Post-washed carcasses	19	IIa, IIc [2]
				Chilled carcasses	21	IIa, IIc [2]
				Meat	20	IIa, IIc [2]
				Environmental	6	IIc [2]
	Guerini, et al., 2007	Guerini et al. (2007)	USA	Hides	432	IIa, IIb, IVb [2]
				Preevisceration carcasses	150	IIa, IIb, IIc, IVb [2]
				Postintervention carcasses	88	IIa, IIc, IVb [2]
	Wieczorek et al., 2012	ISO 11.290-1	USA	Hides and carcasses	54	IIa, IIb, IIc, IVb [2]
	Camargo et al., 2014	ISO 11290-1 and 11290-2	Brazil	Hides	1	IIc [2]
				Post-washed carcasses	4	IIc [2]
	Loiko et al., 2016	ISO 11290-1 and 11290-2	Brazil	Carcasses	7	IIa, IVb [2]
Pork	Miyasaki et al., 2009	ISO 11290-1 and 11290-2	Brazil	*Linguiça* (fresh pork sausage)	82	IIa, IIb, IVb, 4a or 4c[2]
	von Laer et al., 2009	HPB	Brazil	*Linguiça*,processing environment	83	1/2b, 1/2c, 4b [1]
	Larivie`re-Gauthier et al., 2014	HPB	Canada	Lairage pens	30	IIa, IIb, IVb [2]
				Precutting room	13	IIa, IIb, IIc, IVb [2]
				Cutting room	86	IIa, IIb, IIc, IVb [2]
	Meloni et al., 2013	ISO 11290-1 and 11290-2	Italy	Carcass	24	IIa, IIc [2]
				Cecal material	5	IIa, IIc [2]
				Contact surfaces	12	IIa, IIc [2]
				Noncontact surfaces	9	IIa, IIc [2]

Table 1.

Processing plant	Reference	Methodology*	Country	Samples	n of isolates	Serotypes or serogroups**
Pork	Prencipe et al., 2012	EC decision 471/2001	Italy	Feces	1	1/2b [1]
				Carcasses	23	1/2a, 1/2b, 1/2c, 3c, 4b [1]
				Fresh hams	94	1/2a, 1/2b, 1/2c, 4b, 4e, 4c [1]
				dry-cured ham	13	1/2a, 1/2b, 1/2c [1]
	Chasseignaux et al., 2001	VIDAS assay	France	Meat	30	_ [3]
				Products	10	_ [3]
				Shelf-life pork products	32	_ [3]
Chicken	Chiarini et al., 2009	VIP test for *Listeria*	Brazil	Processing environment, products	210	IIa, IIb, IIc, IVb [2]
	Barbalho et al., 2005	USDA-FSIS	Brazil	Carcass, hands and gloves	7	1/2b, 1/2c [1]
	Berrang et al., 2010	FSIS	USA	Flor drains	56	_ [3]
				Raw Products	16	_ [3]
	Chasseignaux et al., 2001	VIDAS assay	France	Meat	17	_ [3]
				Poultry products	4	_ [3]
Fish						
	Gudmundsdóttir et al., 2005	FSIS	Iceland	Raw material	27	1/2a, 1/2b [1]
				Equipment and transporters	32	1/2a, 1/2b [1]
				Floors and drains	58	1/2a, 4b [1]
				Intermediate and final products	70	1/2a [1]
	Vongkamjan et al., 2013	FDA	USA	Smoked fish facility	80	_
Dairy	Almeida et al., 2013	VIDAS	Portugal	Cheese	23	IIa, IIb, IVb [2]
				Milk	7	IIa, IIb, IVb v
				Whey	1	IVb [2]
				Contact surfaces	21	IIb, IVb [2]
				Non-contact surfaces	37	IIa, IIb, IVb [2]
	Parisi et al., 2013	ISO 11290-1	Italy	Products	6	1/2a, 1/2b [1]
				Contact surfaces	13	1/2a, 4b/4e [1]
				Non-contact surfaces	6	1/2a, 1/2b, 3b, 4b/4e [1]
	Barancelli et al., 2014	FDA	Brazil	Products	4	1/2c, 4b [1]
				Contact surfaces	3	4b [1]
				Non-contact surfaces	21	1/2b, 4b [1]

* Abbreviations: ISO: International Organization for Standardization; VIDAS: Automated Immunoassay System; USDA: United States Department of Agriculture; FDA: Food and Drug Administration; FSIS: Food Safety and Inspection Service; EC: European Commission; HPB: Health Protection Branch
** serotyping method: [1] conventional; [2] molecular; [3] not available

material (7%), dehairing equipment, knives, carcass splitter surfaces (23%), floor drains and walls in the dirty zone (25%); only serotypes 1/2c (78%) and 1/2a (22%) were detected (Meloni and others 2013). In another study from Italy, 23 of 774 carcasses (3%) and 94 of 752 fresh hams (12.5%) were positive for *L. monocytogenes*. Nevertheless, only 14 of 708 (2.0%) dry-cured hams presented positive results for the pathogen, consistent with less risk associated with dried products (Prencipe and others 2012).

Despite having good manufacturing practices, control of *L. monocytogenes* in Brazilian poultry processing facilities remains a challenge (Barbalho and others 2005; Chiarini and others 2009). This challenge is not a particular problem only in Brazil: Berrang and others (2010) studied a newly constructed chicken processing plant in the USA, and did not detect *L. monocytogenes* before the beginning of operations; however, within four months of regular processing, different *L. monocytogenes* strains (as defined by PFGE) were identified in floor drains, both before and after cleaning and sanitizing operations. In another study, Chasseignaux and others (2001) tracked *L. monocytogenes* isolates obtained from poultry and pork processing plants where they detected *L. monocytogenes* at multiple locations: PFGE assays indicated that some strains were recovered from the plants for several months while others were not recovered on subsequent visits during the period studied.

The fish processing environment is also a source of *L. monocytogenes*, and the consumption of contaminated smoked fish has been involved in listeriosis outbreaks. Gudmundsdóttir and others (2005) detected *L. monocytogenes* contamination in cold-smoked salmon and the associated processing environment in Iceland, and found an overall prevalence of 11.3%, with 4% of final product being positive. The same strains, as identified by identical PFGE profiles, were also recovered from raw material, floors, and drains. Vongkamjan and others (2013) identified persistent *L. monocytogenes* strains from a smoked fish processing facility in USA. In another study conducted in USA, the authors selected four smoked processing plants for sampling and three were positive for *L. monocytogenes* in raw fish and processing environments (Thimothe and others 2004). Studies in fresh and processed “surubin”, a typical Brazilian fish, demonstrated low frequencies of *L. monocytogenes* and suggested the low risk of listeriosis associated with the consumption of this fish type (Alves and others 2005; Souza and others 2008).

L. monocytogenes has been a problem in dairy facilities (Ryser and Marth 2007), with sequential detection between years in some cases (Almeida and others 2013). Parisi and others (2013) reported the occurrence of *L. monocytogenes* in seven (20.6%) of 34 dairy plants studied in Italy. Barancelli and others (2014) evaluated the occurrence *L.*

monocytogenes in three cheese manufacturing plants in Brazil, and serotypes 4b, 1/2b, and 1/2c were detected in two plants, with serotype 4b being the most frequently recovered serotype: PFGE analysis indicated the presence of multiple strains and thus contamination may have arisen from multiple sources.

Studies over the past decades indicate that *L. monocytogenes* can contaminate and multiply in a variety of foods, even those with high salt content or that are stored at refrigerated temperatures. Ready-to-eat foods (RTE) are common vehicles involved in listeriosis outbreaks, stimulating many countries to create specific legislation aiming to control *L. monocytogenes* in these foods after the 1990s. In the USA there is a zero tolerance policy for *L. monocytogenes* in RTE (Shank and others 1996). Similar, the Brazilian Ministry of Agriculture Livestock and Food Supply (MAPA) adopted the criteria of the absence of *L. monocytogenes* in RTE in Brazil (MAPA 2009). In Canada and in some European countries the norms vary among food products: less than 100 CFU/g is tolerated in foods that are not suitable for *Listeria* growth, and a zero tolerance standard was adopted for foods that support growth and that possess extended shelf life (Canada 2011; Commission 2005; 2007).

Among 778 packaged smoked fish produced by 50 different manufacturers located in 12 European Union countries, 157 samples (20.2%) were contaminated by *L. monocytogenes*, however, only 26 samples (3.3%) at levels higher than 100 CFU/g (Acciari and others 2017). An integrative survey carried out in Italy tested 2,696 RTE samples (884 from soft and semi-soft cheese and 1,802 from cooked meat products), and the contamination of meat products was 1.66% at the arrival at the laboratory and 1.92% at the end of shelf-life, while the prevalence in cheese was 2.13% at the arrival and 1.01% at the end of shelf-life (Iannetti and others 2016). The occurrence of *L. monocytogenes* was analyzed in 2,864 RTE samples obtained in the Eastern region of Spain, with 3.8% tested as positive, the pathogen was more common in smoked salmon and dried pork sausages samples (Doménech and others 2015). These studies indicate that non-compliance with EU official criteria (100 CFU/g) were obtained only from few samples, the hygiene management strategies applied by different manufacturers appears to result to different levels of contamination in the final products.

In Brazil, Sant'Ana and others (2012) detected 3.1% (16/512) RTE vegetables marketed in São Paulo positive for *L. monocytogenes*, and only five samples presented countable levels, with counts ranging from 1.0 x 10^1 to 2.6 x10^2 CFU/g. A similar prevalence was observed by Byrne and others (2016) in RTE vegetables commercialized in Salvador. In another study carried out in Brazil, Martins and Leal Germano (2011)

found 6.2% salami samples and 0.8% ham samples positive for *L. monocytogenes*. Counts in salami samples ranged from < 10 to 1,900 CFU/g, and the results suggest that consumption of salami can be associated with high risk of listeriosis for the consumers. The distribution of *Listeria* spp. and *L. monocytogenes* serotypes in food samples collected in Brazil between 1990 and 2012 was analyzed recently by Vallim and others (2015), and the pathogenic serotypes 1/2a, 1/2b, and 4b were the most common in non-processed meat; while serotypes 1/2a and 4b were the most commonly found in processed meat and RTE.

The Brazilian legislation allows reprocessing the RTE animal products positive for *L. monocytogenes*, since the procedure applied ensures the destruction of the pathogen. After the reprocessing, the establishments must carry out a microbiological analysis to ensure that *L. monocytogenes* is absent. In addition, after detection of *L. monocytogenes* in RTE foods, the supervised establishments shall review their self-control procedures (MAPA 2009).

Based on the presented information, different steps from the food processing environment can be considered as relevant sources of *L. monocytogenes* contamination in foods. Then, listeriosis development is clearly linked to consumption of contaminated foods, and also to particular characteristics of the consumer and virulence of *L. monocytogenes*.

Epidemiology of human listeriosis

L. monocytogenes incidence is much lower (0.2 per 100,000 people globally) compared to other enteric pathogens, such as *Salmonella enterica* (1,140 per 100,000), enterotoxigenic *E. coli* (1,257 per 100,000) and *Campylobacter* spp. (1,390 per 100,000) (Kirk and others 2015). The World Health Organization (WHO) evaluated the median rate of listeriosis in different regions in 2010 and estimated that the incidence of listeriosis is similar across different regions (was 0.1 per 100,000 people in Africa, Eastern Mediterranean, and South-East Asia; 0.2 per 100,000 people in Europe and the Western Pacific; 0.3 per 100,000 people in the Americas) (Kirk and others 2015). Interestingly, in some Northern European countries the incidence rate has been higher (0.6 to 1.6 cases per 100,000 people) than in other countries in recent years, maybe due to the increased consumption of RTE meat and fish products (EFSA 2012; Jensen and others 2010). Less is known about the outcome of listeriosis cases, although de Noordhout and others (2014) estimated that listeriosis resulted in 23,150 illnesses and 5,463 deaths globally in 2010.

Listeriosis cases and outbreaks have been described in multiple countries since 2000s (Table 2), with different food involved as vehicles, including cantaloupe, caramel apples, RTE salad, dairy products, diced celery, meat products, fish products, cooked ham, deli meat, among other foods. In 2013 alone the 27 member states of EU reported 1,763 confirmed human cases of listeriosis, resulting in 191 deaths (EFSA 2015). Listeriosis can manifest itself invasively or not, and it is responsible for a high hospitalization rate (95%). Mortality rates can range between 20 to 30%, and serotypes 1/2a, 1/2b, and 4b are involved in more than 95% of the cases and outbreaks. The invasive form demands rapid antibiotic treatment and is characterized by severe infection in persons belonging to risk groups (Swaminathan and Gerner-Smidt 2007). Unfortunately, hospitals themselves can be a significant source of listeriosis because of the combination of access to RTE foods and a higher probability of consumers who are immunosuppressed (Silk and others 2014).

According to Todd and Notermans (2011), products such as soft cheeses and deli meats were commonly involved in listeriosis outbreaks around the world. Similar transmission vehicles were observed by Cartwright and others (2013), where the authors reported changes in characteristics of outbreaks reported in the USA by CDC during 1998-2008. Earlier in the study period (1998 - 2003) the outbreaks were generally larger and longer in comparison with the late period (2005 - 2008). In the USA, declining cases may reflect the efforts of PulseNet to quickly identify and stop outbreaks. However, *Salmonella* and *L. monocytogenes* are still the major bacterial foodborne pathogens leading to death in USA (Scallan and others 2015; Scallan and others 2011).

The first documented North American outbreak of listeriosis (1981) linked to the consumption of contaminated coleslaw happened in Nova Scotia, Canada, with 41 cases and 18 deaths (Schlech and others 1983). In 1983 there was an outbreak in Massachusetts, USA, with 49 cases and 14 deaths, linked to pasteurized milk (Fleming and others 1985). A large listeriosis outbreak occurred in Southern California, USA, in 1985 with 142 cases and 48 deaths, caused by consumption of a soft cheese (Linnan and others 1988). Other relevant outbreaks happened before the 2000s, for example, between 1983 and 1987 in Switzerland (with 122 cases, 34 deaths), in United Kingdom (between 1987 and 1989, total of 366 cases), in France during 1992 (279 cases and 85 deaths), and in USA between 1998 and 1999, with 108 cases and 14 deaths (Swaminathan and Gerner-Smidt 2007).

Table 2. Report of the main listeriosis outbreaks and related food vehicles that occurred between 2007 and 2015 in different countries.

Food vehicle	Year	Cases	Deaths	Serotype	Country	Reference
Camembert cheese	2007	17	3	NR	Norway	Johnsen et al., 2010
Pasteurized milk	2007	5	3	4b	USA	CDC, 2008
Tuna salad	2008	5	3	1/2a	USA	Cokes et al., 2011
Jellied pork	2008	16		4b	Austria	Pichler et al., 2009
Deli meat	2008	57	24	NR	Canada	Currie et al., 2015
Mexican-style cheese	2008 - 2009	8		1/2a	USA	Jackson et al., 2011
Chicken wraps	2009	36	4	NR	Australia	Popovic et al., 2014
Contaminated beef	2009	8	2	NR	Denmark	Smith et al., 2011
Quargel	2009 - 2010	34	8	1/2a	Austria/Germany/Czech Republic	Fretz et al., 2010
Cheese	2009 - 2012	30	2[1]	4b	Portugal	Magalhães et al., 2014
Ricotta salata cheese	2012	20	4	NR	USA	CDC, 2012
Unrelated	2010	6	4	NR	Australia	Popovic et al., 2014
Rockmelons	2010	9	2	NR	Australia	Popovic et al., 2014
Diced celery	2010	10	5	NR	USA	Gaul et al., 2013
Cooked ham	2011	6		1/2a	Switzerland	Hächler et al., 2013
Cantaloupe	2011 - 2012	147	33	1/2a and 1/2b	USA	McCollum et al., 2013
ready-to-eat food	2013	3		1/2a	Scotland	Okpo et al., 2015
Truffles cheeses	2013	5	1	NR	USA	Choi et al., 2014
Unrelated	2013	5		1/2b	Spain	Pérez-Trallero et al., 2014
Ready-to-eat salad	2013 - 2014	32		4b	Switzerland	Stephan et al., 2015
Foie gras	2013 - 2014	10		1/2b	Spain	Pérez-Trallero et al., 2014
Meat products	2013 - 2014	41	17	NR	Denmark	Anonymous, 2015
Dairy products	2014	8	1	NR	USA	CDC, 2014
Caramel apples	2014 - 2015	35	7	4b	USA	Doyle, 2015
Ice cream	2014 - 2015	10	3	NR	USA	CDC, 2015

[1] fetal loss

Large outbreaks have become less frequent since 2000s, presumably due to implementation of *Listeria* control measures by the food industry and improved clinical diagnostics. Unlike the 1980s and 1990s, when *L monocytogenes* was commonly recovered from the central nervous system (Mylonakis and others 1998), currently the pathogen is most often detected in the blood, probably due to advances in medical diagnosis. The serotype 4b is the most often responsible for meningoencephalitis, and it has also becoming less prevalent (Swaminathan and Gerner-Smidt 2007), but according to Cartwright and others (2013), this serotype is still responsible for the largest number of outbreaks in the USA. However, in Canada and European countries the serotype 1/2a has becoming more common (Althaus and others 2014; Jensen and others 2016; Knabel and others 2012; Lomonaco and others 2015; Mammina and others 2013).

One factor that hampers investigations in many countries, including Brazil, is the absence of an effective surveillance system and a lack of reporting requirements. There are few registers of isolation *L. monocytogenes* from clinical cases in Brazil, and as listeriosis is not a disease under compulsory notification, the outbreak investigation is very often compromised. Reis and others (2011) reported that serotype 4b was the most frequently recovered serotype from clinical samples between 1969 to 2008 in Brazil, but serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 4a, and 4ab have all been found among clinical material of human origin in Brazil (Hofer and others 2006; Hofer and others 2000). A cluster of listeriosis in hospitalized patients (caused by serotypes 1/2b and 3b) was characterized for the first time in Brazil by Martins and others (2010), and the results indicate that the hospital kitchen was the possible source of contamination. To determine the true incidence of listeriosis in countries like Brazil, it is necessary the implementation of a laboratory surveillance system to track sporadic cases or outbreaks of human listeriosis and the sources of contamination (Blum-Menezes and others 2013; Lemes-Marques and others 2007).

Clinical manifestations, treatment and monitoring of resistance

The incubation period of *L. monocytogenes* ranges between 3 to 70 days according to observations by Linnan and others (1988) from a large 1988 listeriosis outbreak. Goulet and others (2013) recently demonstrated that incubation periods differed significantly by clinical form of the disease, being the longer incubation periods identified in pregnancy-associated cases (median 27.5 days), followed by infections in the central nervous system (median 9 days), sepsis (median 2 days), and febrile gastrointestinal disease (median 24

h). In pregnant women, infection is more frequent in the third trimester, generally asymptomatic or with mild symptoms. The consequences to the fetus or newborn are extremely serious, including stillbirth, premature birth, pneumonia, sepsis, and meningitis (Janakiraman 2008). Early-onset neonatal listeriosis occurs in infants infected *in utero.* The late-onset type may occur from one to several weeks after birth, as the result of transplacental transmission, cross-contamination, or ingestion of contaminated foods (Janakiraman 2008; Ryser and Marth 2007).

Among immunocompromised patients, such as the elderly, transplant patients, HIV carriers, and in people diagnosed with cancer, listeriosis can present as sepsis, meningitis, or meningoencephalitis. In immunocompetent individuals, non-invasive listeriosis is most common and manifests itself as febrile gastroenteritis, diarrhea, muscle pain, and headache (Cartwright and others 2013; Ryser and Marth 2007). Although *L. monocytogenes* is the only species considered pathogenic to humans, sporadic cases of gastroenteritis and bacteremia due to *L. ivanovii* (Guillet and others 2010), bacteremia due to *L. innocua* and *L. grayi* (Perrin and others 2003; Rapose and others 2008), and meningitis due to *L. innocua* (Favaro and others 2014) and *L. seeligeri* (Rocourt and others 1986) have been reported in the last few years.

Regarding the treatment with antibiotics, *L. monocytogenes* is sensitive to most drugs suitable for Gram-positive bacteria with the most common treatment involving β-lactam antibiotics (penicillin or ampicillin), potentially in conjunction with gentamicin. Vancomycin and trimethoprim/sulfamethoxazole are alternative drugs for patients allergic to penicillin. In addition, erythromycin, tetracycline, chloramphenicol, rifampicin, and linezolid can be used. The choice of antibiotic will vary according to the symptoms of infection and patient risk group (Janakiraman 2008; Salamano and others 2005; Swaminathan and Gerner-Smidt 2007).

In Brazil, Reis and others (2011) reported only 1 strain (1.5%) resistant to rifampin, and 2 (3%) were resistant to trimethoprim-sulfamethoxazole among 68 human strains isolated between 1970 and 2008, but increase in the minimum inhibitory concentration (MIC) values reinforce the need of continuing resistance surveillance. Lemes-Marques and others (2007) observed two clinical strains resistant to sulfamethoxazole. In addition, a large number of strains resistant to clindamycin and oxacillin were reported by Camargo and others (2015a) among isolates obtained from meat-processing environments, beef products, and clinical cases in Brazil.

L. monocytogenes recovered from food and food processing environment can be resistant to antimicrobial agents, including drugs used for listeriosis treatment (Table 3).

But similar as observed in Brazil, few strains obtained in European countries (from food, food processing environments and clinical) have demonstrated resistance to key antimicrobials (Conter and others 2009; Granier and others 2011; Khen and others 2015; Korsak and others 2012; Vitas and others 2007). However, description of resistance to antimicrobials that are not commonly used for listeriosis treatment appears to be increasing (Alonso-Hernando and others 2012; Khen and others 2015). In addition, multi-drug resistant strains have been isolated from RTE in different regions of the world, creating an concern about dissemination of these strains in the food processing chain (Chen and others 2014; Doménech and others 2015).

Table 3. Antibiotic resistant *Listeria monocytogenes* isolated from a variety of sources.

Antibiotic	**Source**	**Reference**
Ampicillin	Fish	Conter et al., 2009
	Beef chain	Khen et al., 2015
Chloramphenicol	Poultry meat	Miranda et al. 2008
	Fish	Obaidat et al., 2015
	Human	Morvan et al., 2010
	Salad	Jamali et al., 2014
Ciprofloxacin	Fish, fish env[b].	Conter et al., 2009
	Smoked salmon, smoked salmon fingers	Kovacevic et al, 2012
	Poultry meat (1993 and 2006)	Alonso-Hernando et al., 2012
	Human	Morvan et al., 2010
Clyndamicin	Fish, meat, meat env.	Conter et al., 2009
	Fish	Obaidat et al., 2015
	Beef chain	Khen et al., 2015
	Smoked salmon, smoked salmon fingers	Kovacevic et al, 2012
	Environment, foods, clinical	Camargo et al., 2015
	Seafood	Jamali et al., 2014
Doxycycline	Poultry meat	Miranda et al. 2008
	Food	Vitas et al., 2007
Erythromycin	Poultry meat	Miranda et al. 2008
	Fish	Obaidat et al., 2015
	Seafood, chicken	Jamali et al., 2014
	Human	Morvan et al., 2010
Gentamicin	Poultry meat (2006)	Alonso-Hernando et al., 2012
	Beef chain	Khen et al., 2015
	Fish	Obaidat et al., 2015
Linezolid	Fish, fish env., meat	Conter et al., 2009
Penicillin	Beef chain	Khen et al., 2015
	Fish	Obaidat et al., 2015
	Seafood, egg, chicken, salad	Jamali et al., 2014
Oxacillin	Fish, fish env., meat, meat env.	Conter et al., 2009
	Beef chain	Khen et al., 2015
	Envinronment, foods, clinical	Camargo et al., 2015
Sulfisoxazole	Poultry meat	Miranda et al. 2008
Trimethoprim/ sulfamethoxazole	Fish	Obaidat et al., 2015
Vancomycin	Fish	Conter et al., 2009
	Beef chain	Khen et al., 2015
	Fish	Obaidat et al., 2015
	Seafood, salad, egg,	Jamali et al., 2014

Table 3.

Antibiotic	**Source**	**Reference**
Streptomycin	Smoked salmon fingers	Kovacevic et al, 2012
	Fish	Obaidat et al., 2015
	Seafood	Jamali et al., 2014
	Human	Morvan et al., 2010
	Poultry meat (2006)	Alonso-Hernando et al., 2012
Tetracycline	Fish	Conter et al., 2009
	Food	Vitas et al., 2007
	Beef chain	Khen et al., 2015
	Fish	Obaidat et al., 2015
	Seafood, chicken, salad	Jamali et al., 2014
	Human	Morvan et al., 2010
Rifampicin	Meat, meat env.	Conter et al., 2009
	Poultry meat (2006)	Alonso-Hernando et al., 2012
	Beef chain	Khen et al., 2015
	Fish	Obaidat et al., 2015

[a] Environment

According to Magalhaes and others (2014b), who characterized *L. monocytogenes* isolates from human clinical cases that occurred in Portugal between 2008 and 2012, resistance to ciprofloxacin, rifampicin, nitrofurantoin, and to streptomycin was observed, with 29 isolates (14.3%) resistant to 2 or more antimicrobials of different classes. Even though the incidence of antibiotic-resistant isolates is relatively low, it was significantly higher than in previous years (2003–2007) (Barbosa and others 2013). Morvan and others (2010) evaluated 4,816 *L. monocytogenes* isolates obtained from clinical cases in France, and the prevalence of resistance was estimated at 1.27%, being the resistance to tetracyclines and fluoroquinolones more common, and also the presence of resistance related genes *dfrD, tet*M, *int*-Tn, *cat,* and *lde*. In the same study, the authors described the first clinical isolate exhibiting a high-level of resistance to trimethoprim, and an increase in penicillin MICs, reinforcing the need of a continuous surveillance.

L. monocytogenes efflux pumps encoded by *lde* are associated with fluoroquinolone resistance (Godreuil and others 2003), while resistance to macrolides, cefotaxime, heavy metals and ethidium bromide are encoded by *mdrL* (Mata and others 2000). Mobile plasmids (pIP501, pAMβ1, pRYC16, pDB1, pIP811, pIP823, pUBX1, and pWDB100) and transposons (Tn*1545*- Tn*916* family) from enterococci and streptococci may transfer antibiotic resistance genes to *Listeria* spp. Some of these mobile elements can be transferred among *Listeria* species once they are in the same environment (Bertsch and others 2013; Biavasco and others 1996; Charpentier and Courvalin 1999; Charpentier and others 1995; Roberts and others 1996).

Tetracycline resistance from *Listeria* strains is most frequently associated with *tet*(M), which can be transferred to *Listeria* by mobile elements such as a family of

conjugative transposon Tn916. The *int*-Tn gene is a target to detect the transposons family Tn916-Tn1545 (Bertrand and others 2005; Bertsch and others 2014; Haubert and others 2016). In addition to the *tet*(M), others genes that mediate tetracycline resistance, such as *tet*(K), *tet*(L), and *tet*(S), can also be expected in *L. monocytogenes* (Facinelli and others 1993; Hadorn and others 1993; Poyart-Salmeron and others 1992; Yan and others 2010). The resistance genes *dfrD* (corresponding resistance to trimethoprim), *spc* (spectinomycin), and *erm*(A) (erythromycin or clindamycin) can be located on plasmids such as pDB2011 (Bertsch and others 2013). Similarly, chloramphenicol resistance can be encoded by the *cat* gene carried on plasmids. According to Poyart-Salmeron and others (1990), the genes carried in on plasmid pIP811 confer resistance to chloramphenicol, erythromycin, and streptomycin. Haubert and others (2016) recently identified *L. monocytogenes* isolates from food processing environment and food obtained in Brazil exhibiting multi-drug resistance phenotypes. Two multi-drug resistant strains harbored the *tetM* and *ermB* resistance genes, and the presence of resistance gene (*tetM*) in a plasmid indicates a potential risk of transferring multi-drug resistance to other microorganisms.

The differences in the frequency of antibiotic resistance of *L. monocytogenes* strains between studies could be in part explained by design of experiments, number of strains tested, and biological independence. It is important to keep in mind that in many studies that focus on resistance to antibiotics, the authors do not use techniques such as PFGE, capable of distinguishing the tested isolates (Camargo and others 2015a; Conter and others 2009; Loiko and others 2016; Miranda and others 2008; Obaidat and others 2015). Without attention to the possibility that investigators are sampling from clonal populations, estimates of the prevalence of antibiotic resistance over time (increasing or decreasing) may be biased by repeated characterization of the same strains. Sampling from dispersed premises and sources will likely limit this issue.

Control of *L. monocytogenes* in food processing facilities

Eliminating *L. monocytogenes* from food processing environmental is nearly impossible, however, the implementation of control procedures by Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) in 2009 was an important step to overcome the worse scenarios observed in the past (Catão and Ceballos 2001; Destro and others 1991; Padilha da Silva and others 2004; SILVA and others 1998). Despite the low prevalence of *L. monocytogenes* in RTE worldwide, samples with counts above the limit

allowed by the legislation can offer a high risk to consumers, and the risk tend to increases with the number of cells ingested.

The bacterial biofilms are important to the food industry because these structures provide an additional means to protect bacteria from sanitation efforts. The association between higher adherence and persistence of *L. monocytogenes* in industrial environments was identified by Norwood and Gilmour (1999). While some investigators have shown serotype-specific differences in biofilm formation, others have found no correlation, indicating that the ability to form biofilms varies by strain rather than by serotype (Borucki and others 2003; Di Bonaventura and others 2008; Djordjevic and others 2002; Lundén and others 2000; Nilsson and others 2011; Norwood and Gilmour 1999; Pan and others 2010). In addition, a multi-species biofilm can be more resistant than a biofilm composed only by *L. monocytogenes* (Møretrø and Langsrud 2004).

The best approach to avoid biofilm formation and food contamination is to adopt effective cleaning practices to remove organic matter that can be metabolized by the bacteria. Disinfection with quaternary ammonium compounds (QACs), sodium hypochloride, sodium dichloroisocyanurate, peracetic acid, and iodofors can be effective against *L. monocytogenes*. The processing environments must to be designed to accommodate complete removal of particulates during pre-wash, access of chemicals at proper concentrations, and the ability to completely rinse surfaces, such as interior of drains, floors, cut room, grinders, boxes, among others (Chaitiemwong and others 2014; Ryser and Marth 2007).

QACs are applied for sanitation of medical and food processing environments with benzal-konium chloride (BC) being a common formulation. The complete dissolution in water allows for the frequent use of chlorine as disinfectants in food processing facilities, these formulations are ease of use, low cost, and has high antimicrobial activity. However, it is very important to know their functions, effective concentrations, action mode, and the correct way to prepare them in order to optimize their application, avoid health risks and to obtain better effect with a minimal quantity. Silva and others (2016) studied the *Listeria* spp. contamination in a butcher shop located in Minas Gerais, Brazil. The authors evaluated the *L. monocytogenes* adhesion ability and sensitivity to food contact surface sanitizers (chlorine based and quaternary ammonium based compounds). After the adoption of both sanitizers in the cleaning routine of the butcher shop, *L. monocytogenes* was not detected, demonstrating *in situ* their effectiveness and the importance of the adoption of appropriate sanitation procedures for *L. monocytogenes* control.

While effective, To and others (2002) demonstrated that *L. monocytogenes* can survive sub-lethal concentrations of BC through the activity of efflux pumps; these mechanisms may contribute to higher tolerance of BC should advantageous mutations arise. QACs are not recommended for use on surfaces that come into direct contact with fermented food because small concentrations of residues can inactivate "starter" cultures. One alternative would be use peracetic acid, which does not have this shortcoming.

In addition to the use of chemicals, development of alternative strategies to eliminate *L. monocytogenes* has become an active field of research. There are studies that describe use of ultraviolet radiation, bacteriophages, lactic acid bacteria, purified bacteriocins produced by lactic acid bacteria, ultrasonic treatment, natural products (such oils), and surfactants to control *L. monocytogenes* in the food processing environment or directly in the food (Baños and others 2016; Camargo and others 2015c; Cotter and others 2013; Giaouris and others 2014; Møretrø and Langsrud 2004; Strydom and Witthuhn 2015).

Although many bacteriocins produced by lactic acid bacteria have been characterized, their application by food industries is still restricted and can vary by country (Cotter and others 2005; de Arauz and others 2009; Favaro and others 2015). In Brazil, studies have been describing bacteriocinogenic strains with antilisterial activity isolated from raw milk and cheeses (Cavicchioli and others 2017; Perin and others 2012), sausage (De Martinis and Franco 1998), salami (Barbosa and others 2014), charqui (Biscola and others 2013), pork products (de Carvalho and others 2010), rocket salad (Kruger and others 2013), and smoked fish (Alves and others 2005).

The restrictions for the use of these bacteriocins can be in part explained because some producer strains are often considered as non safe, or the data available about these bacteriocins is still insufficient to approve their use, since this characterization is expensive and time-consuming. Nisin was the first approved bacteriocin for control of *L. monocytogenes*. Currently, nisin (a lantibiotic, class I bacteriocin) and pediocin Pa1/AcH (clas IIa anti-Listerial bacteriocins) are approved and used to treat many types of food worldwide, while others bacteriocins produced by acid lactic bacteria offer promising perspectives to be approved for commercial use (Gálvez and others 2007; López Aguayo and others 2016).

Various phage preparations also have been successfully tested against *L. monocytogenes* in the last years. The ListShield™ was the first phage-based preparation approved by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). Since then, the product was approved by others countries to be used as

decontaminant of surfaces, equipment, and direct on food. In addition, the Listex™ P100 has been tested and approved in several countries (Carlton and others 2005; Food and Administration 2006; Gutiérrez and others 2017; Yang and others 2017). The efficiency of bacteriophage P100 (LISTEX™ P100) was evaluated by Rossi and others (2011), being demonstrated that this product was very effective for biocontrol of *L. monocytogenes* in Brazilian fresh sausage. Bacteriophages can be useful for *L. monocytogenes* biocontrol in food contact surfaces and in foods, however, dose, contact time, storage temperature, and pH potentially affect the effectiveness of the product (Oliveira and others 2014; Soni and others 2010).

Concluding remarks

L. monocytogenes is a microorganism that occurs ubiquitously in nature and can be constantly reintroduced in food processing facilities. Good sanitization procedures are crucial to eliminate *L. monocytogenes* from food processing environments and prevent bacterial adhesion and biofilm formation, which often result in cross contamination to food. Listeriosis was recognized as a serious disease in the last decades, probably as a function of advances in medical diagnosis, but also due to the changes in food processing (for example use of cold for food preservation) and eating habits (increasing consumption of RTE foods). Continuous surveillance is needed to understand *L. monocytogenes* contamination dynamics in Brazil, and development of an effective surveillance system and compulsory notification of listeriosis, to facilitate the outbreaks investigation in Brazil, then will be possible to design better strategies to control the pathogen in food processing environments and food, as well as monitoring the emergence of antibiotic resistance.

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CHAPTER 3 - Antibiotic resistance of *Listeria monocytogenes* isolated from meat processing environments, beef products, and clinical cases in Brazil

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Title Page

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Keywords: antibiotic resistance; Listeria monocytogenes; serogrouping; virulence markers

Running title: Antimicrobial resistance of *Listeria monocytogenes*

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Abstract

The present study aimed to assess the antimicrobial resistance and the presence of virulence markers in 137 *Listeria monocytogenes* isolates obtained from meat processing environments, beef products, and clinical cases. All isolates were subject to molecular serogouping, and their antibiotic resistance profiles were assessed against 12 antimicrobials. In addition, isolates were subjected to detection of virulence marker genes (*inlA, inlC, inlJ*). The isolates were classified into serogroups 4b, 4d, 4a, or 4c (46%), 1/2c or 3c (27%), 1/2a or 3a (13.9%), and 1/2b or 3b (13.1%). All tested isolates presented sensitivity to the majority of the tested antimicrobials, but most of them presented resistance or intermediate resistance to clindamycin (88.3%) and oxacillin (73.7%). Virulence markers were detected in all isolates, demanding further analysis to better characterize their pathogenic potential.

Introduction

Listeria monocytogenes is a Gram-positive intracellular foodborne pathogen that is widely distributed in the environment. This pathogen can survive on a diversity of surfaces, resulting in persistence in food processing environments and cross-contamination with end-products [15]. Listeriosis is the disease caused by *L. monocytogenes,* and it is considered an emergent illness since the 1980s, when a number of cases and outbreaks were reported and associated with the consumption of foods contaminated with the pathogen [8,20,23]. This disease is characterized by severe infections in high-risk groups requiring rapid treatment with antibiotics [31,32].

Serogrouping of *L. monocytogenes* isolates obtained from clinical and food samples is important to predict the possible risks to consumers. Strains from serotypes 1/2a, 1/2b, and 4b are considered the most virulent, and are frequently associated with human listeriosis cases and outbreaks [5,31].

A diversity of proteins are associated with the virulence activity of *L. monocytogenes,* such as internalins, listeriolysins, and phospholipases. [13]. The internalin-related genes (*inlA, inlC,* and *inlJ*) are involved with the passage through the intestinal barrier, cell adhesion and invasion, and their presence in *L. monocytogenes* isolates suggests a potential pathogenicity for consumers.

Most *L. monocytogenes* isolates are susceptible to the antibiotics that are usually employed to fight Gram-positive bacteria, and the first report of a multi-resistant strain was described in 1990, by Poyart-Salmeron, et al. [27]. Nowadays, several studies has shown resistance among isolates obtained from food processing environments and foods [17,18,21,25,34]. The emergence of antimicrobial resistance has serious consequences for public health, such as failures in the treatment of diseases and limitations in therapeutic choice, and may require the use of more modern drugs [2].

The present study aimed to assess the antimicrobial resistance and the presence of virulence markers among *L. monocytogenes* isolates obtained from different sources (environment, food, clinical cases) and different Brazilian regions.

Material and Methods

Microorganisms

A total of 137 *L. monocytogenes* isolates from 11 different States of Brazil were analyzed in this study. They were obtained from different food processing environments, foods,

and clinical cases, between the years 1978 and 2013. All isolates were identified by biochemical tests according Pagotto, et al. [26], and stored at -20°C in trypticase soya broth (TSB, Oxoid Ltd., Basingstoke, England) supplemented with glycerol at 20% (v/v). At the time of use, isolates were transferred to trypticase soya agar (TSA, Oxoid) and incubated at 35°C.

Molecular serogrouping

Isolated colonies from each isolate were transferred to TSB and incubated at 35°C for 24 h, and the obtained cultures were subjected to DNA extraction and purification using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). PCR serogrouping was conducted based on the assay described by Borucki and Call [5]. The amplification mix composed 12.5 µL of GoTaq Green Master Mix (Promega), 2 µL DNA, 40 µM of each of the primers D1 (F: CGATATTTTATCTACTTTGTCA; R: TTGCTCCAAAGCAGGGCAT) and D2 (F: GCGGAGAAAGCTATCGCA; R: TTGTTCAAACATAGGGCTA), and ultra-pure PCR water (Promega) to 25 µL. Two additional reactions were then conducted as described above, using the primers FlaA (F: TTACTAGATCAAACTGCTCC; R: AAGAAAAGCCCCTCGTCC) and GLT (F: AAAGTGAGTTCTTACGAGATTT; R: AATTAGGAAATCGACCTTCT). Amplification conditions were the same as that described by Borucki and Call [5]. Five µL aliquots of the PCR products were electrophoresed on 2.0% (w/v) agarose gels in 0.5× Tris/Borate/EDTA buffer (TBE), stained with GelRed (Biotium Inc., Hayward, CA, USA), and visualized in a transilluminator. For each target DNA region, the following PCR product sizes were observed: 214 bp for D1, 140 bp for D2, 538 bp for FlaA, and 483 bp for GLT. In all molecular assays, *L. monocytogenes* strains Scott A, ATCC 7644, and ATCC 15313 were tested in parallel as positive controls.

Detection of virulence markers

Multiplex PCR reactions were conducted in order to identify the presence of virulence markers genes involved in the processes of host-cell invasion and cell-to-cell spread: *inlA* (F: ACGAGTTAACGGGACAAATGC; R: CCCGACAGTGGTGCTAGATT), *inlC* (F: AATTCCCACAGGACACAACC; R: CGGGAATGCAATTTTTCACTA), and *inlJ* (F: TGTAACCCCGCTATCACAGTT; R: AGCGGCTTGGCAGTCTAATA) [19]. PCR reactions were composed of 12.5 µL GoTaq Green Master Mix, 2.0 µL DNA, 1.0 µM of each primer, and PCR ultra-pure water to a final volume of 25 µL. Amplification conditions were the same as described by Liu, et al. [19]. Five microliter aliquots of the PCR

products were electrophoresed on 1.5 % (w/v) agarose gels in 0.5× TBE, stained with GelRed, and visualized in a transilluminator. For each target DNA region, the following PCR product sizes were observed: 800 bp for *inlA*, 517 bp for *inlC*, and 238 bp for *inlJ*. *L. monocytogenes* ATCC 7644 was used as a positive control for the assessed genes.

Antimicrobial resistance

L. monocytogenes strains were subjected to phenotypical analysis to characterize their resistances against 12 antimicrobials (10 µg ampicillin, 10 units penicillin G, 1 µg oxacillin, 2 µg clindamycin, 15 µg erythromycin, 10 µg gentamicin, 10 µg imipenem, 5 µg rifampin, 30 µg chloramphenicol, 30 µg tetracycline, 25 µg trimethoprim/sulfamethoxazole, and 30 µg vancomycin) using the disk diffusion method (Oxoid). Cultures were transferred to brain and heart infusion (BHI, Oxoid), incubated at 35ºC overnight, and diluted in 0.85% NaCl (w/v) until turbidity similar to 0.5 MacFarland. Diluted cultures were swabbed onto the surface of Mueller-Hinton agar (Oxoid), and the antimicrobial disks were added (3 disks per plate). After incubation at 35ºC for 18 and 24 h, the results for each antimicrobial agent were recorded, and their resistance profiles were classified as sensitive, intermediate, and resistant, as described by Cockerill [10] to *Staphylococcus* spp. Reference strain *Staphylococcus aureus* ATCC 25923 was used as the control.

Results

Table 1 shows the different origins of *L. monocytogenes* isolates, and also the serogrouping results. Among 69 isolates from meat processing environments, a predominance of isolates from serogroups 4b, 4d, 4a, or 4c, and 1/2c or 3c, were observed, and among 43 isolates from beef products, most were identified as belonging to serogroups 1/2b or 3b, followed by 4b, 4d, 4a, or 4c, and 1/2c or 3c. Finally, among 25 clinical strains, the most common serogroups were 4b, 4d, 4a, or 4c.

Table 1. *Listeria monocytogenes* isolates obtained from meat processing environments, beef products, and clinical samples used in the study.

Source	Sample	n	Serogroup			
			1/2a or 3a	1/2b or 3b	1/2c or 3c	4b, 4d, 4a, or 4c
Processing environments	Bovine hides	9	3	1	2	3
	Bovine carcass	13	1	1	2	9
	Swine carcass	1	1	-	-	-
	Conductive mat chicken	9	-	-	-	9
	Plastic box	5	-	-	2	3
	Table	9	-	-	7	2
	Floor of refrigeration room	2	-	-	1	1
	Meat handlers	9	1	-	5	3
	Meat tenderizer	5	-	-	1	4
	Grinder	5	1	-	3	1
	Knife	2	-	-	1	1
Beef products	Refrigerated beef	25	2	6	11	6
	Frozen beef	13	4	5	2	2
	Minced beef	1	-	-	-	1
	Seasoned beef	2	-	2	-	-
	Cooked beef	1	-	-	-	1
	Frozen cooked beef	1	-	-	-	1
Clinical	Blood	7	1	1	-	5
	Cerebrospinal fluid	16	4	2	-	10
	Placenta	1	1	-	-	-
	Sheep brain	1	-	-	-	1
Total	-	137	19	18	37	63

All *L. monocytogenes* isolates presented positive PCR results for the internalin genes (*inlA, inlC,* and *inlJ*), and were susceptible to ampicillin (10 µg), penicillin G (10 units), erythromycin (15 µg), gentamicin (10 µg), imipenem (10 µg), rifampin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (25 µg), and vancomycin (30 µg). However, resistance to clindamycin (2 µg) and oxacillin (1 µg) was found in most of the *L. monocytogenes* isolates (Table 2). The detailed results for each isolated and related to each antibiotic are presented in the Supplementary table (supplementary results).

Table 2. Antimicrobial resistance profile of *Listeria monocytogenes* isolates tested in this study.

Antibiotic class	Antimicrobial	Number of isolates (%)		
		Resistant	Intermediate	Susceptible
Aminoglycoside	Gentamicin	-	-	137 (100.0)
Anfenicol	Chloramphenicol	-	-	137 (100.0)
Ansamycin	Rifampin	-	-	137 (100.0)
Carbapenem	Imipenem	-	-	137 (100.0)
Glycopeptide	Vancomycin	-	-	137 (100.0)
Lincosamide	Clindamycin	72 (52.5)	49 (35.8)	16 (11.7)
Macrolide	Erythromycin	-	-	137 (100.0)
Penicillin	Ampicillin	-	-	137 (100.0)
	Penicillin G	-	-	137 (100.0)
	Oxacillin	78 (56.9)	23 (16.8)	36 (26.3)
Potentiated Sulfonamide	Trimethoprim-sulfamethoxazole	-	-	137 (100.0)
Tetracycline	Tetracycline	-	-	137 (100.0)

Discussion

The conventional agglutination method is the reference protocol for serotyping *L. monocytogenes* isolates obtained from clinical and food samples. However, molecular methodologies have been proposed for identification of the main *L. monocytogenes* serogroups associated with listeriosis. These methods provide rapid and low-cost results, but the exact identification of the serotype is not possible, since these protocols propose a serogroup categorization that includes different serotypes, *i.e.*, usually the most prevalent serotype, and other non-frequent serotypes [5,14].

In this study, we showed that most isolates recovered from different Brazil States from food processing environments and foods belonged to serogroups 4b, 4d, 4a, or 4c (Table 1), which is in agreement with previous studies in Brazil [3,6,22]. Moreover, serotypes 1/2a or 3a, 1/2b or 3b, and 1/2c or 3c also have been identified in food processing environments and foods in different States, similar to previous studies [6,7,24]. All isolates from clinical cases belonged to serogroups 4b, 4d, 4a, or 4c, 1/2a or 3a, and 1/2b or 3b (Table 1), which are usually associated with the majority of outbreaks and sporadic cases of listeriosis [31].

The internalin-related genes play important roles in the virulence mechanisms of *L. monocytogenes* and their presence suggests potential pathogenicity. The *inlJ* gene is directly related to *L. monocytogenes* passage through the intestinal barrier; the *InlC* gene contributes to the post-intestinal steps of infection; and the *inlA* gene plays an important role to entry into host cells [19, 32]. In this study, all *L. monocytogenes* strains were positive for virulence markers *inlA, inlC,* and *inlJ,* as observed in a number of other studies with strains from serotypes 1/2a, 1/2c, 1/2b, and 4b [19,30,34]. However, additional characterization, such as the sequencing the entire *inlA* gene, is important to properly assess the virulence potential of *L. monocytogenes* strains, since some mutations are associated with the expression of truncated proteins, which can result in low virulence potential [28,30]. Treatment of listeriosis is done using β-lactam antibiotics (ampicillin or amoxicillin) in association, or not, with an aminoglycoside (gentamicin). However, other drugs can also be used, such as erythromycin, tetracycline, chloramphenicol, rifampicin, trimethoprim/sulfamethoxazole, and linezolid [31,33]. In our study, all isolates were susceptible to antibiotics used in listeriosis treatment, but a high level of resistance and intermediate resistance was observed against clindamycin and oxacillin. Resistance to lincosamides and penicillins has been described previously [12,16-18,29,34]. Antimicrobial resistance in microorganisms can occur due to endogenous and exogenous factors, and the environment plays an important role in allowing interactions with other bacteria and consequent gene or plasmid transfer [9,11,18,21]. Oxacillin and clindamycin resistance has been attributed to efflux-pumps or 23S ribosomal RNA modifications [1,18], as well as excessive use of both drugs in veterinary medicine [1]. However, Bertsch, et al. [4] suggested that *Listeria fleischmannii,* which is resistant to clindamycin, may possess a transferable transposon that remains to be identified. While these drugs are not used to treat listeriosis, the emergence of resistance demands attention due to the possibility of horizontal transfer to other bacteria [25].

This study showed the prevalence of pathogenic serogroups among isolates from food processing environments, foods, and clinical cases in Brazil. Despite having susceptibility to most of the antibiotics used to treat listeriosis, the presence of high antimicrobial resistance to oxacillin and clindamycin is a serious concern for public health, and more in depth research is needed to better understand the mechanisms of antimicrobial resistance. In addition, all isolates harbored virulence marker genes, demanding further analysis to proper characterize their pathogenic potential.

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Supplementary table. Additional information of 137 *Listeria monocytogenes* isolates included in the study of antimicrobial resistance. Presented information related to isolate code, sample source of isolate, Brazilian state in which the isolate was obtained, year of isolation, serogroup of isolate, and disk diffusion diameter for each isolate and tested antibiotic (in mm, and followed by indication of resistance, R, intermediary resistance, IR, or susceptibility, S).

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																	
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
1	refrigerated beef	MT	2009	1/2a or 3a	26	(S)	31	(S)	12	(IR)	29	(S)	14	(R)	31	(S)	30	(S)	35	(S)	35	(S)	38	(S)	25	(S)	40	(S)
2	frozen beef	SP	2006	1/2a or 3a	28	(S)	32	(S)	16	(S)	30	(S)	14	(R)	32	(S)	29	(S)	36	(S)	33	(S)	33	(S)	27	(S)	40	(S)
3	frozen beef	SP	2006	1/2a or 3a	29	(S)	35	(S)	13	(S)	33	(S)	13	(R)	35	(S)	30	(S)	36	(S)	33	(S)	36	(S)	27	(S)	39	(S)
4	frozen beef	SP	2006	1/2a or 3a	26	(S)	37	(S)	12	(IR)	34	(S)	13	(R)	34	(S)	29	(S)	38	(S)	31	(S)	32	(S)	25	(S)	36	(S)
5	frozen beef	SP	2003	1/2a or 3a	29	(S)	35	(S)	10	(R)	33	(S)	12	(R)	34	(S)	30	(S)	40	(S)	35	(S)	34	(S)	25	(S)	40	(S)
6	refrigerated beef	RS	2002	1/2a or 3a	27	(S)	29	(S)		(R)	34	(S)	17	(IR)	31	(S)	27	(S)	33	(S)	37	(S)	32	(S)	22	(S)	26	(S)
7	blood	SP	1986	1/2a or 3a	29	(S)	32	(S)		(R)	34	(S)	19	(IR)	35	(S)	31	(S)	39	(S)	36	(S)	34	(S)	24	(S)	42	(S)
8	cerebrospinal fluid	PR	1983	1/2a or 3a	22	(S)	40	(S)		(R)	31	(S)	12	(R)	30	(S)	25	(S)	38	(S)	32	(S)	31	(S)	26	(S)	41	(S)
9	placenta	PE	1978	1/2a or 3a	29	(S)	37	(S)	16	(S)	38	(S)	14	(R)	34	(S)	31	(S)	39	(S)	30	(S)	29	(S)	26	(S)	43	(S)
10	cerebrospinal fluid	SP	1978	1/2a or 3a	32	(S)	40	(S)	12	(IR)	37	(S)	21	(S)	39	(S)	32	(S)	44	(S)	37	(S)	36	(S)	33	(S)	48	(S)
11	cerebrospinal fluid	DF	1989	1/2a or 3a	25	(S)	37	(S)	11	(IR)	32	(S)	14	(R)	31	(S)	27	(S)	34	(S)	31	(S)	32	(S)	24	(S)	27	(S)
12	cerebrospinal fluid	PR	1983	1/2a or 3a	27	(S)	34	(S)	11	(IR)	30	(S)	17	(IR)	30	(S)	31	(S)	39	(S)	28	(S)	34	(S)	23	(S)	38	(S)
13	carcass of swine	SC	2004	1/2a or 3a	28	(S)	40	(S)	15	(S)	35	(S)	12	(R)	30	(S)	28	(S)	35	(S)	34	(S)	30	(S)	25	(S)	40	(S)
14	meat handlers	MG	2010	1/2a or 3a	23	(S)	31	(S)	12	(IR)	34	(S)	12	(R)	28	(S)	28	(S)	34	(S)	29	(S)	27	(S)	22	(S)	31	(S)
15	grinder	PR	2005	1/2a or 3a	24	(S)	35	(S)		(R)	29	(S)		(R)	28	(S)	25	(S)	35	(S)	30	(S)	32	(S)	23	(S)	35	(S)
16	bovine carcass	RS	2010	1/2a or 3a	24	(S)	32	(S)		(R)	33	(S)	13	(R)	32	(S)	30	(S)	34	(S)	30	(S)	32	(S)	23	(S)	42	(S)
17	bovine hide	RS	2010	1/2a or 3a	27	(S)	40	(S)	17	(S)	35	(S)	12	(R)	33	(S)	30	(S)	38	(S)	31	(S)	40	(S)	23	(S)	42	(S)
18	bovine hide	RS	2010	1/2a or 3a	26	(S)	31	(S)		(R)	30	(S)	11	(R)	29	(S)	25	(S)	36	(S)	24	(S)	30	(S)	24	(S)	26	(S)
19	bovine hide	RS	2010	1/2a or 3a	22	(S)	34	(S)		(R)	32	(S)	10	(R)	25	(S)	26	(S)	31	(S)	24	(S)	27	(S)	22	(S)	34	(S)
20	refrigerated beef	MT	2010	1/2b or 3b	25	(S)	32	(S)		(R)	29	(S)	22	(S)	31	(S)	21	(S)	31	(S)	25	(S)	31	(S)	25	(S)	37	(S)
21	refrigerated beef	MT	2007	1/2b or 3b	28	(S)	38	(S)		(R)	35	(S)	25	(S)	34	(S)	32	(S)	36	(S)	28	(S)	35	(S)	24	(S)	43	(S)

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																	
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
22	refrigerated beef	MT	2007	1/2b or 3b	24	(S)	33	(S)		(R)	29	(S)	19	(IR)	32	(S)	28	(S)	36	(S)	28	(S)	33	(S)	24	(S)	40	(S)
23	refrigerated beef	MT	2007	1/2b or 3b	25	(S)	30	(S)	20	(S)	36	(S)	19	(IR)	30	(S)	23	(S)	35	(S)	27	(S)	21	(S)	27	(S)	25	(S)
24	frozen beef	MT	2007	1/2b or 3b	29	(S)	35	(S)	15	(S)	34	(S)	25	(S)	32	(S)	28	(S)	36	(S)	25	(S)	35	(S)	24	(S)	40	(S)
25	frozen beef	MT	2007	1/2b or 3b	28	(S)	31	(S)	12	(IR)	30	(S)	20	(IR)	30	(S)	27	(S)	37	(S)	26	(S)	29	(S)	22	(S)	37	(S)
26	seasoned meat	SP	2002	1/2b or 3b	25	(S)	35	(S)		(R)	30	(S)	13	(R)	37	(S)	19	(S)	32	(S)	25	(S)	28	(S)	22	(S)	34	(S)
27	seasoned meat	SP	2002	1/2b or 3b	21	(S)	34	(S)	12	(IR)	33	(S)	19	(IR)	31	(S)	28	(S)	32	(S)	28	(S)	33	(S)	25	(S)	39	(S)
28	refrigerated beef	RJ	2004	1/2b or 3b	24	(S)	35	(S)	12	(IR)	35	(S)	20	(IR)	31	(S)	26	(S)	35	(S)	22	(S)	34	(S)	22	(S)	37	(S)
29	frozen beef	MT	2004	1/2b or 3b	27	(S)	34	(S)		(R)	29	(S)	16	(IR)	31	(S)	27	(S)	32	(S)	27	(S)	31	(S)	24	(S)	34	(S)
30	frozen beef	MT	2004	1/2b or 3b	27	(S)	34	(S)		(R)	33	(S)	16	(IR)	29	(S)	22	(S)	33	(S)	25	(S)	29	(S)	22	(S)	30	(S)
31	frozen beef	MT	2004	1/2b or 3b	23	(S)	33	(S)		(R)	32	(S)	12	(R)	25	(S)	31	(S)	31	(S)	28	(S)	28	(S)	21	(S)	37	(S)
32	refrigerated beef	MG	2009	1/2b or 3b	23	(S)	29	(S)		(R)	29	(S)	14	(R)	30	(S)	26	(S)	36	(S)	25	(S)	29	(S)	21	(S)	34	(S)
33	blood	RJ	2008	1/2b or 3b	25	(S)	34	(S)		(R)	31	(S)	17	(IR)	33	(S)	29	(S)	35	(S)	26	(S)	35	(S)	23	(S)	32	(S)
34	cerebrospinal fluid	SP	1982	1/2b or 3b	28	(S)	30	(S)		(R)	26	(S)	13	(R)	34	(S)	31	(S)	39	(S)	28	(S)	34	(S)	27	(S)	31	(S)
35	cerebrospinal fluid	PB	2001	1/2b or 3b	26	(S)	34	(S)	16	(S)	31	(S)	15	(IR)	32	(S)	27	(S)	34	(S)	31	(S)	31	(S)	22	(S)	40	(S)
36	bovine carcass	RS	2010	1/2b or 3b	26	(S)	31	(S)		(R)	32	(S)	10	(R)	32	(S)	30	(S)	37	(S)	32	(S)	35	(S)	22	(S)	37	(S)
37	bovine hide	RS	2011	1/2b or 3b	25	(S)	34	(S)	12	(IR)	32	(S)	25	(S)	31	(S)	33	(S)	33	(S)	32	(S)	33	(S)	23	(S)	40	(S)
38	frozen beef	SP	2003	1/2c or 3c	28	(S)	36	(S)	10	(R)	35	(S)	18	(IR)	37	(S)	32	(S)	41	(S)	35	(S)	37	(S)	24	(S)	45	(S)
39	frozen beef	SP	2003	1/2c or 3c	27	(S)	36	(S)	10	(R)	36	(S)	16	(IR)	34	(S)	29	(S)	35	(S)	33	(S)	33	(S)	23	(S)	42	(S)
40	refrigerated beef	MG	2009	1/2c or 3c	25	(S)	37	(S)	18	(S)	32	(S)	12	(R)	31	(S)	32	(S)	34	(S)	32	(S)	31	(S)	25	(S)	42	(S)
41	refrigerated beef	MG	2010	1/2c or 3c	26	(S)	35	(S)	14	(S)	36	(S)	14	(R)	31	(S)	33	(S)	34	(S)	31	(S)	30	(S)	24	(S)	42	(S)
42	refrigerated beef	MG	2010	1/2c or 3c	26	(S)	32	(S)	10	(R)	29	(S)	15	(IR)	31	(S)	27	(S)	35	(S)	28	(S)	31	(S)	22	(S)	37	(S)
43	refrigerated beef	MG	2011	1/2c or 3c	30	(S)	33	(S)	13	(S)	32	(S)	19	(IR)	31	(S)	29	(S)	34	(S)	30	(S)	33	(S)	22	(S)	40	(S)
44	refrigerated beef	MG	2012	1/2c or 3c	24	(S)	35	(S)	17	(S)	33	(S)	26	(S)	29	(S)	29	(S)	32	(S)	33	(S)	32	(S)	24	(S)	40	(S)
45	refrigerated beef	MG	2012	1/2c or 3c	24	(S)	32	(S)	12	(IR)	31	(S)	14	(R)	31	(S)	27	(S)	34	(S)	29	(S)	30	(S)	22	(S)	41	(S)

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																	
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
46	refrigerated beef	MG	2012	1/2c or 3c	25	(S)	37	(S)	14	(S)	32	(S)	16	(IR)	31	(S)	30	(S)	32	(S)	30	(S)	34	(S)	23	(S)	43	(S)
47	refrigerated beef	MG	2012	1/2c or 3c	25	(S)	33	(S)	11	(IR)	30	(S)	10	(R)	31	(S)	29	(S)	34	(S)	29	(S)	31	(S)	22	(S)	40	(S)
48	refrigerated beef	MG	2012	1/2c or 3c	24	(S)	30	(S)		(R)	31	(S)	14	(R)	30	(S)	28	(S)	33	(S)	28	(S)	30	(S)	22	(S)	39	(S)
49	refrigerated beef	MG	2012	1/2c or 3c	23	(S)	29	(S)	10	(R)	30	(S)	12	(R)	31	(S)	26	(S)	34	(S)	29	(S)	30	(S)	23	(S)	37	(S)
50	refrigerated beef	MG	2012	1/2c or 3c	23	(S)	36	(S)	16	(S)	34	(S)	12	(R)	31	(S)	27	(S)	32	(S)	28	(S)	30	(S)	22	(S)	48	(S)
51	grinder	MG	2012	1/2c or 3c	32	(S)	42	(S)	15	(S)	44	(S)	15	(IR)	34	(S)	32	(S)	38	(S)	31	(S)	43	(S)	31	(S)	47	(S)
52	grinder	MG	2012	1/2c or 3c	22	(S)	38	(S)	11	(IR)	35	(S)	14	(R)	30	(S)	25	(S)	32	(S)	27	(S)	31	(S)	25	(S)	38	(S)
53	grinder	MG	2012	1/2c or 3c	25	(S)	30	(S)	11	(IR)	35	(S)	15	(IR)	31	(S)	26	(S)	32	(S)	28	(S)	32	(S)	22	(S)	37	(S)
54	meat tenderizer	MG	2012	1/2c or 3c	27	(S)	35	(S)	10	(R)	30	(S)	15	(IR)	31	(S)	33	(S)	35	(S)	32	(S)	34	(S)	22	(S)	40	(S)
55	plastic box	MG	2012	1/2c or 3c	24	(S)	32	(S)	10	(R)	29	(S)	13	(R)	30	(S)	27	(S)	31	(S)	28	(S)	29	(S)	23	(S)	40	(S)
56	plastic box	MG	2012	1/2c or 3c	24	(S)	36	(S)	10	(R)	29	(S)		(R)	29	(S)	25	(S)	33	(S)	25	(S)	28	(S)	22	(S)	35	(S)
57	table	MG	2012	1/2c or 3c	26	(S)	30	(S)		(R)	30	(S)	13	(R)	31	(S)	27	(S)	34	(S)	27	(S)	31	(S)	22	(S)	37	(S)
58	table	MG	2012	1/2c or 3c	25	(S)	34	(S)	14	(S)	31	(S)	21	(S)	29	(S)	32	(S)	31	(S)	28	(S)	32	(S)	23	(S)	39	(S)
59	bovine hide	MG	2010	1/2c or 3c	26	(S)	38	(S)	12	(IR)	36	(S)	13	(R)	31	(S)	33	(S)	34	(S)	32	(S)	32	(S)	24	(S)	45	(S)
60	meat handlers	MG	2009	1/2c or 3c	25	(S)	33	(S)	11	(IR)	30	(S)	12	(R)	28	(S)	30	(S)	33	(S)	29	(S)	29	(S)	23	(S)	41	(S)
61	meat handlers	MG	2010	1/2c or 3c	23	(S)	33	(S)	16	(S)	32	(S)	22	(S)	30	(S)	26	(S)	33	(S)	28	(S)	30	(S)	21	(S)	38	(S)
62	meat handlers	MG	2010	1/2c or 3c	25	(S)	36	(S)	19	(S)	34	(S)	16	(IR)	31	(S)	31	(S)	32	(S)	31	(S)	32	(S)	23	(S)	41	(S)
63	meat handlers	MG	2012	1/2c or 3c	29	(S)	31	(S)		(R)	29	(S)	14	(R)	32	(S)	28	(S)	33	(S)	28	(S)	31	(S)	23	(S)	40	(S)
64	meat handlers	MG	2012	1/2c or 3c	16	(S)	37	(S)	10	(R)	36	(S)	12	(R)	29	(S)	27	(S)	33	(S)	29	(S)	31	(S)	21	(S)	38	(S)
65	table	MG	2010	1/2c or 3c	23	(S)	32	(S)	12	(IR)	31	(S)	13	(R)	32	(S)	30	(S)	34	(S)	29	(S)	32	(S)	22	(S)	40	(S)
66	table	MG	2010	1/2c or 3c	25	(S)	35	(S)	17	(S)	35	(S)	14	(R)	32	(S)	30	(S)	33	(S)	29	(S)	30	(S)	22	(S)	40	(S)
67	table	MG	2010	1/2c or 3c	27	(S)	32	(S)	10	(R)	33	(S)	12	(R)	31	(S)	27	(S)	37	(S)	30	(S)	30	(S)	24	(S)	39	(S)
68	floor of refrigeration room	PR	2005	1/2c or 3c	28	(S)	37	(S)	14	(S)	32	(S)	14	(R)	32	(S)	31	(S)	41	(S)	30	(S)	31	(S)	24	(S)	38	(S)
69	bovine carcass	RS	2011	1/2c or 3c	26	(S)	35	(S)	14	(S)	33	(S)	14	(R)	32	(S)	29	(S)	34	(S)	29	(S)	31	(S)	23	(S)	40	(S)

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																					
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
70	bovine carcass	RS	2012	1/2c or 3c	25	(S)	34	(S)	10	(R)	30	(S)	15	(IR)	28	(S)	26	(S)	32	(S)	28	(S)	33	(S)	28	(S)	37	(S)
71	bovine hide	RS	2011	1/2c or 3c	26	(S)	33	(S)	13	(S)	30	(S)	12	(R)	31	(S)	29	(S)	35	(S)	31	(S)	30	(S)	25	(S)	35	(S)
72	knife	RS	2010	1/2c or 3c	25	(S)	38	(S)	16	(S)	33	(S)	26	(S)	31	(S)	26	(S)	35	(S)	30	(S)	30	(S)	28	(S)	38	(S)
73	table	RS	2010	1/2c or 3c	23	(S)	32	(S)	14	(S)	30	(S)	12	(R)	26	(S)	29	(S)	33	(S)	26	(S)	27	(S)	22	(S)	35	(S)
74	table	RS	2010	1/2c or 3c	26	(S)	34	(S)	14	(S)	33	(S)	15	(IR)	32	(S)	28	(S)	33	(S)	21	(S)	30	(S)	20	(S)	38	(S)
75	refrigerated beef	SP	2006	4b, 4d, 4a or 4c	30	(S)	37	(S)	10	(R)	33	(S)	14	(R)	34	(S)	29	(S)	40	(S)	29	(S)	35	(S)	26	(S)	40	(S)
76	cooked beef	SP	2004	4b, 4d, 4a or 4c	29	(S)	36	(S)		(R)	34	(S)		(R)	33	(S)	29	(S)	39	(S)	32	(S)	34	(S)	23	(S)	40	(S)
77	minced beef	RJ	2003	4b, 4d, 4a or 4c	28	(S)	30	(S)	10	(R)	32	(S)	12	(R)	34	(S)	27	(S)	38	(S)	29	(S)	33	(S)	24	(S)	39	(S)
78	refrigerated beef	RJ	2003	4b, 4d, 4a or 4c	27	(S)	31	(S)		(R)	33	(S)	14	(R)	34	(S)	30	(S)	36	(S)	30	(S)	33	(S)	29	(S)	40	(S)
79	frozen cooked meat	MT	2001	4b, 4d, 4a or 4c	28	(S)	38	(S)		(R)	35	(S)	17	(IR)	34	(S)	29	(S)	41	(S)	31	(S)	35	(S)	24	(S)	40	(S)
80	refrigerated beef	RJ	2000	4b, 4d, 4a or 4c	32	(S)	36	(S)	14	(S)	37	(S)	22	(S)	36	(S)	32	(S)	40	(S)	30	(S)	37	(S)	27	(S)	47	(S)
81	frozen beef	MT	2003	4b, 4d, 4a or 4c	26	(S)	32	(S)	14	(S)	32	(S)	15	(IR)	39	(S)	25	(S)	36	(S)	28	(S)	33	(S)	23	(S)	30	(S)
82	frozen beef	MT	2003	4b, 4d, 4a or 4c	25	(S)	38	(S)	10	(R)	32	(S)	13	(R)	32	(S)	21	(S)	35	(S)	27	(S)	30	(S)	21	(S)	38	(S)
83	refrigerated beef	MG	2012	4b, 4d, 4a or 4c	23	(S)	30	(S)		(R)	31	(S)	16	(IR)	31	(S)	29	(S)	32	(S)	29	(S)	32	(S)	23	(S)	41	(S)
84	refrigerated beef	MG	2012	4b, 4d, 4a or 4c	26	(S)	32	(S)		(R)	31	(S)	19	(IR)	32	(S)	29	(S)	35	(S)	27	(S)	33	(S)	24	(S)	39	(S)
85	refrigerated beef	MG	2012	4b, 4d, 4a or 4c	27	(S)	30	(S)	10	(R)	32	(S)	15	(IR)	32	(S)	27	(S)	35	(S)	29	(S)	30	(S)	24	(S)	39	(S)
86	cerebrospinal fluid	ES	2010	4b, 4d, 4a or 4c	29	(S)	43	(S)		(R)	30	(S)	13	(R)	25	(S)	31	(S)	40	(S)	23	(S)	35	(S)	25	(S)	38	(S)
87	blood	SP	1985	4b, 4d, 4a or 4c	27	(S)	33	(S)		(R)	33	(S)	20	(IR)	36	(S)	27	(S)	38	(S)	32	(S)	34	(S)	17	(S)	22	(S)
88	blood	SP	1985	4b, 4d, 4a or 4c	24	(S)	32	(S)	20	(S)	33	(S)	16	(IR)	27	(S)	22	(S)	32	(S)	24	(S)	27	(S)	23	(S)	21	(S)
89	cerebrospinal fluid	SP	1997	4b, 4d, 4a or 4c	29	(S)	29	(S)	19	(S)	29	(S)	17	(IR)	32	(S)	31	(S)	36	(S)	27	(S)	31	(S)	28	(S)	42	(S)
90	cerebrospinal fluid	RJ	2007	4b, 4d, 4a or 4c	28	(S)	36	(S)	12	(IR)	33	(S)	15	(IR)	33	(S)	29	(S)	39	(S)	31	(S)	32	(S)	23	(S)	41	(S)
91	blood	RJ	2004	4b, 4d, 4a or 4c	26	(S)	39	(S)	19	(S)	40	(S)	18	(IR)	26	(S)	20	(S)	33	(S)	21	(S)	27	(S)	27	(S)	23	(S)
92	cerebrospinal fluid	SP	1997	4b, 4d, 4a or 4c	24	(S)	34	(S)	18	(S)	32	(S)	10	(R)	29	(S)	24	(S)	27	(S)	20	(S)	24	(S)	20	(S)	23	(S)
93	cerebrospinal fluid	RJ	1990	4b, 4d, 4a or 4c	29	(S)	29	(S)	18	(S)	30	(S)	22	(S)	33	(S)	20	(S)	41	(S)	28	(S)	28	(S)	25	(S)	39	(S)

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																	
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
94	cerebrospinal fluid	DF	1989	4b, 4d, 4a or 4c	28	(S)	35	(S)		(R)	31	(S)	14	(R)	36	(S)	34	(S)	40	(S)	32	(S)	36	(S)	28	(S)	41	(S)
95	cerebrospinal fluid	PE	1989	4b, 4d, 4a or 4c	29	(S)	38	(S)	14	(S)	36	(S)	18	(IR)	37	(S)	30	(S)	42	(S)	32	(S)	36	(S)	26	(S)	42	(S)
96	blood	SP	1985	4b, 4d, 4a or 4c	27	(S)	30	(S)	14	(S)	32	(S)	22	(S)	30	(S)	26	(S)	34	(S)	29	(S)	25	(S)	25	(S)	30	(S)
97	cerebrospinal fluid	SP	1978	4b, 4d, 4a or 4c	27	(S)	32	(S)		(R)	30	(S)	19	(IR)	35	(S)	29	(S)	32	(S)	28	(S)	32	(S)	27	(S)	37	(S)
98	cerebrospinal fluid	SP	1980	4b, 4d, 4a or 4c	27	(S)	37	(S)		(R)	29	(S)	16	(IR)	36	(S)	33	(S)	40	(S)	31	(S)	36	(S)	24	(S)	39	(S)
99	sheep brain	RJ	1987	4b, 4d, 4a or 4c	28	(S)	37	(S)	12	(IR)	31	(S)	20	(IR)	27	(S)	38	(S)	40	(S)	32	(S)	25	(S)	26	(S)	43	(S)
100	cerebrospinal fluid	PR	2000	4b, 4d, 4a or 4c	27	(S)	30	(S)	10	(R)	32	(S)	13	(R)	36	(S)	31	(S)	35	(S)	31	(S)	38	(S)	25	(S)	40	(S)
101	blood	RJ	2007	4b, 4d, 4a or 4c	25	(S)	34	(S)	13	(S)	31	(S)	16	(IR)	32	(S)	27	(S)	32	(S)	28	(S)	30	(S)	22	(S)	41	(S)
102	meat handlers	MG	2012	4b, 4d, 4a or 4c	25	(S)	34	(S)	10	(R)	31	(S)	17	(IR)	32	(S)	27	(S)	34	(S)	28	(S)	30	(S)	23	(S)	39	(S)
103	bovine carcass	RS	1991	4b, 4d, 4a or 4c	30	(S)	37	(S)		(R)	33	(S)	16	(IR)	35	(S)	31	(S)	40	(S)	33	(S)	33	(S)	26	(S)	45	(S)
104	grinder	MG	2012	4b, 4d, 4a or 4c	25	(S)	29	(S)		(R)	29	(S)	12	(R)	32	(S)	25	(S)	37	(S)	26	(S)	30	(S)	24	(S)	37	(S)
105	knife	MG	2012	4b, 4d, 4a or 4c	26	(S)	30	(S)		(R)	29	(S)	14	(R)	30	(S)	25	(S)	33	(S)	25	(S)	30	(S)	22	(S)	37	(S)
106	meat handlers	MG	2012	4b, 4d, 4a or 4c	26	(S)	32	(S)		(R)	30	(S)	15	(IR)	32	(S)	30	(S)	35	(S)	28	(S)	35	(S)	25	(S)	38	(S)
107	meat tenderizer	MG	2012	4b, 4d, 4a or 4c	24	(S)	30	(S)		(R)	29	(S)	17	(IR)	30	(S)	26	(S)	34	(S)	27	(S)	32	(S)	24	(S)	37	(S)
108	meat tenderizer	MG	2012	4b, 4d, 4a or 4c	26	(S)	30	(S)		(R)	29	(S)	12	(R)	32	(S)	27	(S)	36	(S)	26	(S)	30	(S)	21	(S)	31	(S)
109	meat tenderizer	MG	2012	4b, 4d, 4a or 4c	25	(S)	32	(S)		(R)	30	(S)	16	(IR)	32	(S)	25	(S)	36	(S)	27	(S)	33	(S)	23	(S)	38	(S)
110	plastic box	MG	2012	4b, 4d, 4a or 4c	26	(S)	31	(S)		(R)	29	(S)	24	(S)	31	(S)	26	(S)	31	(S)	26	(S)	29	(S)	23	(S)	34	(S)
111	table	MG	2012	4b, 4d, 4a or 4c	24	(S)	31	(S)		(R)	29	(S)	15	(IR)	32	(S)	27	(S)	36	(S)	27	(S)	34	(S)	24	(S)	38	(S)
112	table	MG	2012	4b, 4d, 4a or 4c	24	(S)	29	(S)		(R)	30	(S)	12	(R)	31	(S)	27	(S)	36	(S)	23	(S)	30	(S)	24	(S)	37	(S)
113	meat handlers	MG	2012	4b, 4d, 4a or 4c	26	(S)	35	(S)	11	(IR)	31	(S)	21	(S)	32	(S)	26	(S)	31	(S)	27	(S)	33	(S)	22	(S)	38	(S)
114	bovine carcass	PR	2005	4b, 4d, 4a or 4c	21	(S)	31	(S)		(R)	31	(S)	11	(R)	31	(S)	27	(S)	35	(S)	31	(S)	31	(S)	25	(S)	39	(S)
115	bovine carcass	PR	2005	4b, 4d, 4a or 4c	24	(S)	37	(S)		(R)	30	(S)	24	(S)	39	(S)	26	(S)	36	(S)	28	(S)	30	(S)	23	(S)	35	(S)
116	bovine carcass	PR	2005	4b, 4d, 4a or 4c	23	(S)	37	(S)		(R)	29	(S)	15	(IR)	28	(S)	25	(S)	31	(S)	27	(S)	27	(S)	21	(S)	35	(S)
117	bovine carcass	PR	2005	4b, 4d, 4a or 4c	25	(S)	30	(S)		(R)	32	(S)	13	(R)	31	(S)	26	(S)	33	(S)	24	(S)	22	(S)	25	(S)	39	(S)

Isolate	Sample	Brazilian state	Year	Serogroup	Antibiotics																					
					CN		AMP		OX		P		DA		E		C		SXT		RD		TE		VA		IPM	
118	bovine carcass	PR	2005	4b, 4d, 4a or 4c	30	(S)	34	(S)		(R)	34	(S)	15	(IR)	35	(S)	35	(S)	39	(S)	29	(S)	33	(S)	28	(S)	40	(S)
119	floor of refrigeration room	PR	2005	4b, 4d, 4a or 4c	28	(S)	31	(S)		(R)	30	(S)	22	(S)	30	(S)	27	(S)	34	(S)	27	(S)	31	(S)	24	(S)	38	(S)
120	meat tenderizer	PR	2005	4b, 4d, 4a or 4c	28	(S)	32	(S)	11	(IR)	33	(S)	22	(S)	39	(S)	28	(S)	37	(S)	30	(S)	32	(S)	31	(S)	41	(S)
121	plastic box	PR	2005	4b, 4d, 4a or 4c	27	(S)	33	(S)	10	(R)	29	(S)	11	(R)	31	(S)	27	(S)	36	(S)	26	(S)	31	(S)	23	(S)	35	(S)
122	plastic box	PR	2005	4b, 4d, 4a or 4c	32	(S)	33	(S)	11	(IR)	35	(S)	14	(R)	38	(S)	32	(S)	36	(S)	32	(S)	35	(S)	27	(S)	40	(S)
123	bovine hide	RS	2011	4b, 4d, 4a or 4c	22	(S)	29	(S)		(R)	28	(S)	12	(R)	29	(S)	24	(S)	33	(S)	26	(S)	30	(S)	23	(S)	31	(S)
124	bovine hide	RS	2011	4b, 4d, 4a or 4c	25	(S)	33	(S)		(R)	32	(S)	20	(IR)	32	(S)	30	(S)	33	(S)	30	(S)	35	(S)	25	(S)	40	(S)
125	bovine hide	RS	2011	4b, 4d, 4a or 4c	25	(S)	35	(S)		(R)	30	(S)	12	(R)	30	(S)	25	(S)	33	(S)	27	(S)	32	(S)	22	(S)	37	(S)
126	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	24	(S)	29	(S)		(R)	28	(S)	13	(R)	29	(S)	27	(S)	30	(S)	28	(S)	30	(S)	22	(S)	34	(S)
127	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	22	(S)	35	(S)		(R)	32	(S)		(R)	30	(S)	26	(S)	35	(S)	27	(S)	34	(S)	22	(S)	35	(S)
128	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	23	(S)	35	(S)		(R)	32	(S)		(R)	31	(S)	27	(S)	31	(S)	29	(S)	31	(S)	21	(S)	37	(S)
129	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	21	(S)	30	(S)		(R)	29	(S)	12	(R)	30	(S)	24	(S)	35	(S)	26	(S)	29	(S)	23	(S)	33	(S)
130	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	26	(S)	29	(S)		(R)	30	(S)	13	(R)	30	(S)	28	(S)	38	(S)	30	(S)	34	(S)	22	(S)	35	(S)
131	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	24	(S)	29	(S)		(R)	29	(S)		(R)	31	(S)	26	(S)	37	(S)	28	(S)	31	(S)	23	(S)	37	(S)
132	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	25	(S)	37	(S)		(R)	29	(S)	11	(R)	30	(S)	25	(S)	37	(S)	28	(S)	31	(S)	21	(S)	33	(S)
133	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	23	(S)	36	(S)	12	(IR)	33	(S)	17	(IR)	31	(S)	28	(S)	32	(S)	30	(S)	30	(S)	20	(S)	42	(S)
134	conductive mat chicken	PR	2013	4b, 4d, 4a or 4c	23	(S)	33	(S)	10	(R)	30	(S)	13	(R)	30	(S)	27	(S)	35	(S)	30	(S)	32	(S)	23	(S)	35	(S)
135	bovine carcass	RS	2011	4b, 4d, 4a or 4c	25	(S)	31	(S)		(R)	30	(S)		(R)	33	(S)	26	(S)	36	(S)	28	(S)	31	(S)	26	(S)	33	(S)
136	bovine carcass	RS	2011	4b, 4d, 4a or 4c	26	(S)	29	(S)		(R)	29	(S)	11	(R)	32	(S)	31	(S)	40	(S)	26	(S)	30	(S)	24	(S)	35	(S)
137	bovine carcass	RS	2011	4b, 4d, 4a or 4c	27	(S)	30	(S)		(R)	34	(S)	12	(R)	32	(S)	27	(S)	36	(S)	29	(S)	29	(S)	24	(S)	35	(S)

Brazilian states: DF: Distrito Federal, ES: Espírito Santo, MG: Minas Gerais, MT: Mato Grosso, PB: Paraíba, PE: Pernambuco, PR: Paraná, RJ: Rio de Janeiro, RS: Rio Grande do Sul, SC: Santa Catarina, SP: São Paulo. Antibiotics: gentamicin (CN); ampicillin (AMP); oxacillin (OX); penicillin (P); clindamycin (DA); erythromycin (E); chloranphenicol (C); sulphamethoxazole/ trimethoprim (SXT); rifampicin (RD); tetracycline (TE); vancomycin (VA); and imipenem (IPM).

CHAPTER 4 - Molecular serogrouping of *Listeria monocytogenes* from Brazil using PCR

Anderson Carlos Camargo, Deyse Christina Vallim, Ernesto Hofer, Luís Augusto Nero

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Title page

Running title: *L. monocytogenes* serotypes distribution and serogrouping

Research Note

Molecular Serogrouping of *Listeria monocytogenes* from Brazil Using PCR

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Keywords: *Listeria monocytogenes*, conventional serology, serogrouping, recommendations

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ABSTRACT

We assessed the serotypes distribution of *Listeria monocytogenes* isolates from clinical, beef and environment samples using two PCR-based protocols for serogrouping. A panel of 134 isolates (clinical samples = 22, beef cuts = 79, beef processing environment = 33) was subjected to conventional serology and identified as serotypes 1/2a (n = 12), 1/2b (n = 21), 1/2c (n = 71) and 4b (n = 30); isolates from clinical samples were predominantly 4b, and the most prevalent serotype among cuts and environment was 1/2c. The protocol described by Doumith et al. in 2004 presented contradictory results for seven 1/2a isolates, which presented positive results for *lmo1118* and determined the profile IIc (serotypes 1/2c and 3c); in addition, fifteen 4b isolates amplified the target *lmo0737*, presenting the atypical profile IVb-v1. The results of protocol described by Borucki and Call in 2003 presented full agreement with the conventional serology. We propose recommendations for using this multiplex PCR by adding one pair of the reported primers to the panel to reduce total effort by one reaction while maintaining specificity. We present additional recommendations to improve the efficiency and reproducibility of this serogrouping assay.

INTRODUCTION

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes infections in animals and humans. This pathogen is widespread in the environment and survives in a wide range of conditions while being found in many different food products *(8)*. Infections occur through the consumption of contaminated foods, and ready-to-eat foods are often described as products with a high risk of transmission *(25, 26)*. Despite the low morbidity, this intracellular bacterium presents 20% to 30% mortality and 95% hospitalization rates. Neonates, pregnant women, the elderly, and immunosuppressed individuals are particularly at risk *(10, 13)*.

A variety of methods have been used to differentiate *L. monocytogenes* strains and predict their virulence potential *(16)*. The classical serotyping previously described by Seeliger and Höhne *(22)* is routinely used to identify potentially pathogenic strains. This method is based on variation in the somatic (O) and flagellar (H) antigens, allowing the identification of 13 different serotypes; among them, serotypes 1/2a, 1/2b and 4b are responsible for more than 95% of listeriosis cases *(25)*. This method, however, is expensive, labor-intensive, time-consuming and demands high-quality antisera *(2, 6)*.

Several molecular assays have been developed to identify the main *L. monocytogenes* serogroups associated with listeriosis *(2, 6, 12, 24, 27, 30)*. Some of these methods have been used extensively because they are relatively simple to perform, are relatively rapid and once the infrastructure is in place, assay expense is limited. Multiplex PCR described by Doumith *et al. (6)* is a popular protocol for presumptive differentiation of *L. monocytogenes* strains, grouping them into four different serogroups *(7, 12, 23, 27-29)*. Borucki and Call *(2)* developed a similar assay *(2, 5, 24)*, but it has been used less frequently.

Herein we assessed the serotypes distribution of *L. monocytogenes* isolates from clinical, beef cuts and environment samples from Brazil while evaluating the performance of these two PCR protocols relative to the serotyping gold standard.

MATERIAL AND METHODS

Microorganisms. A panel of 134 isolates identified as *L. monocytogenes* was considered in this study, obtained from the *Listeria* Culture Collection of Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil) and isolated by Camargo *et al. (3)*. Isolates were obtained between 1978 and 2012 from different sources: clinical samples (n = 22), beef

cuts (n = 79) and beef processing environments (n = 33), and they were stored at -20°C in trypticase soya broth (TSB, Oxoid Ltd., Basingstoke, England) supplemented with glycerol at 20% (v/v).

Serotyping. All *L. monocytogenes* isolates were subject to conventional serology using polyclonal somatic and flagellar antisera produced by the Laboratory of Bacterial Zoonoses (FIOCRUZ), as previously described by Seeliger and Höhne *(22)*.

Molecular analysis. Isolated colonies from each isolate were transferred to TSB (Oxoid), incubated at 35°C for 24 h, and the obtained cultures subjected to DNA extraction and purification using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). This DNA was subjected to multiplex PCR, as previously described by Doumith *et al. (6)*. The size of expected PCR included 691 bp for *lmo*0737, 906 bp for *lmo*1118, 597 bp for ORF2110, 471 bp for ORF2819 and 370 bp for *prs*. The extracted DNA was also subjected to the Borucki and Call *(2)* PCR assay. Expected PCR products included 214 bp for D1, 140 bp for D2, 538 bp for FlaA and 483 bp for GLT.

In addition, a modified PCR protocol was developed based on the Borucki and Call *(2)* assay. In this case extracted DNA was subjected to a multiplex PCR composed of 12.5 µL of GoTaq Green Master Mix (Promega), 2 µL of DNA, 40 µM of each of the following primers: D1 (F, R), D2 (F, R) and GLT (F, R), and ultrapure PCR water (Promega) to a final volume of 25 µL. Amplification conditions were the same as those described by Borucki and Call *(2)* for D1, D2 and GLT primers. A separate PCR was conducted with the FlaA primers (Borucki and Call *(2)*.

L. monocytogenes strains Scott A (serotype 4b), ATCC 7644 (serotype 1/2c), and ATCC 15313 (serotype 1/2a) included as positive controls for all assays. Atypical results obtained by Doumith method were confirmed independently by the Laboratory of Bacterial Zoonoses (FIOCRUZ) using the same protocols except reference strains CDC F4555 (4b) and CDC F6254 (1/2c) were used as positive controls for these reactions.

RESULTS AND DISCUSSION

Based on conventional serology, the majority of clinical isolates were serotype 4b (68.2%) and 1/2a (22.7%) (Table 1). For beef cuts serotype 1/2c was detected most frequently (50.6%), but other serotypes were also detected [1/2b (24%), 4b (17.7%) and 1/2a (7.6%)]. According to Orsi *et al. (20)*, serotypes from lineage I (1/2b, 3b, 3c, 4b) can be isolated from various sources, being overrepresented among human isolates, while serotypes from lineage II (1/2a, 1/2c, 3a) are overrepresented among foods, as well as

environmental sources. For example, Cartwright *et al. (4)* reported that clinical isolates in the U.S. were composed of 60.7% 4b and 33.1% 1/2a. Martín *et al. (17)* reported a high prevalence of serotype 1/2c (38.4%) for meat products while serotype 1/2a prevailed among food contact surfaces (45.5%). According to Ochiai *et al. (19)*, serotype 1/2c is most commonly isolated from pork meat in Japan while Moreno *et al. (18)* reported a high prevalence of serotype 1/2c and 4b in pork processing environment and pork meat in São Paulo state, Brazil. The handling of beef and pork in the same establishments may explain the high environmental contamination by serotype 1/2c among beef products.

Table 1. Serotypes of the 134 *L. monocytogenes* isolates used in the present study according to their sources.

Source	Sample type	No. (%) of isolates by serotype				
		1/2a	1/2b	1/2c	4b	Total
Clinical samples[a]	-	5 (22.7)	2 (9.1)	-	15 (68.2)	22
Beef cuts	-	6 (7.6)	19 (24.1)	40 (50.6)	14 (17.7)	79
Beef processing environment	Carcass	1 (14.3)		5 (71.4)	1 (14.3)	7
	Equipment			11 (100)		11
	Employee hands			15 (100)		15
Total		12	21	71	30	134

[a] one isolate from clinical sample of sheep brain, and other isolates from human clinical samples.

The Doumith *et al. (6)* protocol correctly classified 1/2b, 1/2c and 4b isolates into their corresponding serogroups with 100% accuracy (Table 2). Accuracy was problematic for 1/2a isolates for which only 42% were correctly serogrouped while the remainder were misclassified (Table 2). An additional refinement to the Doumith assay, as described by Leclercq *et al. (14)* did not distinguish the 4b isolates (serogroup IVb) as being different from an additional IVb-v1 grouping. The isolates that presented atypical results (seven isolated from 1/2a serotype, and 15 isolates from 4b serotype) were subjected to independent PCR testing at the Laboratory of Bacterial Zoonoses (FIOCRUZ) and all results were confirmed by using the two tested protocols. The Borucki and Call *(2)* correctly classified all isolates by the proposed divisions and serogroups (Table 2). The proposed modifications to the Borucki and Call *(2)* assay produced identical results to the original assay (data not shown).

Table 2. Summary of PCR results after amplification protocols according Doumith, et al. (2004) and Borucki and Call (2003) of *Listeria monocytogenes* isolates previously identified according their serotypes by conventional serological tests (Seeliger and Höhne, 1979).

Serotype	n	Doumith, et al. (2004)							Borucki and Call (2003)					
		lmo1118	*lmo0737*	ORF2110	ORF2819	*prs*	profile (serotypes)	n	D1	D2	*fla*A	GLT	division (serotypes)	n
1/2a	12	-	+	-	-	+	IIa (1/2a, 3a)	5	-	+	+	-	II (1/2a, 3a)	12
		+	+	-	-	+	IIc (1/2c, 3c)[a]	7						
1/2b	21	-	-	-	+	+	IIb (1/2b, 3b, 7)	21	+	-	-	+	I (1/2b, 3b)	21
1/2c	71	+	+	-	-	+	IIc (1/2c, 3c)	71	-	+	-	-	II (1/2c, 3c)	71
4b	30	-	-	+	+	+	IVb (4b, 4d, 4e)	15	+	-	-	-	I (4b, 4d)	30
		-	+	+	+	+	IVb-v1 (4b)[b]	15						

[a]Doumith et al., 2004 and Kérouanton et al., 2010

[b] Leclercq et al., 2011

Serogrouping proposed by Doumith *et al. (6)* differentiates *L. monocytogenes* into four molecular serogroups: IIa corresponded to serotypes 1/2a and 3a; IIc to 1/2c and 3c; IIb to 1/2b, 3b and 7; IVb to 4b, 4d and 4e. Nowadays, this is the main protocol considered for serogrouping *L. monocytogenes* isolates obtained from food and clinical samples. A number of the strains from serotype 4b can have an atypical amplification profile, being named as IVb-v1 *(9, 14)* (also observed for 15 isolates in the present study; Table 2). Other studies described strains with this atypical IVb-v1 profile from food processing plants, foods and clinical samples *(9, 14, 15, 21)*. Lee *et al. (15)* demonstrated that IVb-v1 strains presented a specific gene cassette, comprising the genes *lmo0734* to *lmo0739* (6.3 kb) that are common to 1/2a and 3a, 1/2c and 3c strains, suggesting a horizontal gene transfer to serotype 4b (lineage I), generating the atypical profile IVb-v1.

Moreover, we identified seven strains from serotype 1/2a that presented positive results to *lmo1118*, consistent with their inclusion in profile IIc (related to serotypes 1/2c and 3c) (Table 2). Similar results were observed by Doumith *et al. (6)* for the EGDe strain, and Kérouanton *et al. (12)* reported a similar observation for isolates from food processing sources and foods. Bécavin *et al. (1)* compared the genomes of widely used serotype 1/2a strains 10403S, EGD, and EGD-e, and showed that the pathogenic EGDe is more closely genetically related to serotype 1/2c. Consequently, it is possible that many potentially pathogenic strains from serotype 1/2a can be incorrectly classified as serotype 1/2c; the latter being pathogenic but not common among clinical isolates. This discordance can be resolved after an additional PCR based on the amplification of *flaA* in these atypical strains *(12)*.

The serogrouping method proposed by Borucki and Call *(2)* categorizes *L. monocytogenes* isolates into similar groups, as proposed by Doumith *et al. (6)*, but takes into consideration the major phylogenetic divisions within the species: Division I includes serotypes 1/2b, 3b, 4b, 4d and 4e, and Division II includes serotypes 1/2a, 1/2c, 3a and 3c. A third division was also described (III), including serotypes 4a and 4c (less common serotypes from clinical and food samples), can be identified by adding MAMA-C primers in the protocol *(11)*. We did not include these primers in our study because this lineage is not usually identified among isolates from human and animal clinical cases *(20)*. Additional PCR is required for complete identification of possible serotypes in each Division *(2, 24)*.

Our recommended changes to the Borucki and Call *(2)* provided identical results when compared to the original protocol. The original protocol required three PCR reactions (one multiplex and two simplex) for complete serogrouping whereas our

recommended modification requires one less reaction (one multiplex and one simples). With this improvement in efficiency and the 100% correspondence with conventional serotyping, this improved protocol may serve practitioner's needs better than the protocol described by Doumith *et al. (6)*.

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CHAPTER 5 - Invasion and spread ability of *Listeria monocytogenes* in Caco-2 cells are dictated by serotype specific traits

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Title page

Invasion and spread ability of *Listeria monocytogenes* in Caco-2 cells are dictated by serotype specific traits

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ABSTRACT

Listeriosis is a foodborne disease caused by the Gram-positive bacteria *Listeria monocytogenes*, an intracellular pathogen that modulates its intracellular survival via vacuolar escape and cytosolic replication. In the present study, we examined the ability of 59 *L. monocytogenes* strains recovered over the last four decades in Brazil (from beef, clinical and environmental sources) to invade, replicate and spread in a human intestinal epithelial cell line (Caco-2). The strains varied widely in their intracellular doubling times, and there was no clear relationship between serotype or source. Premature stop codons (PMSCs) were common in *inlA* gene of serotype 1/2c (lineage II) strains from beef and environment, and significantly affected their potential to invade Caco-2 cells when compared to other serotypes ($p < 0.005$). There were also significant differences between serotypes for cell-to-cell spread: 1/2a strains were generally impaired in their spreading ability, while most serotype 1/2b exhibited increased spreading ability ($p < 0.0001$). In addition, we identified three serotype 4b strains that spread nearly twice as much as the reference strain 10403s. The work presented is consistent with the invasive potential and cell spread of *L. monocytogenes* tracks more strongly with serotype than source (beef vs. environment vs. clinical), although the most invasive strains were primarily isolated from beef. Additionally, we have identified isolates that could provide novel insight into infectivity of *L. monocytogenes* that may not be revealed by studying common reference strains.

KEYWORDS: *Listeria monocytogenes*, Caco-2, serotype, invasion, spreading ability

Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, low-GC-content (39%) bacterium that is the etiological agent of listeriosis. Listeriosis is typically characterized as a self-limiting gastroenteritis in immunocompetent adults. In more vulnerable populations, however, *L. monocytogenes* can become invasive causing to spontaneous abortions or stillbirths, sepsis, meningitis, encephalitis, and even death [1]. Mortality rates from listeriosis range from 20% to 30% [2]. *L. monocytogenes* is divided into 13 different serotypes that are grouped into four phylogenetic lineages (I, II, III, and IV) [3, 4]. Serotypes 1/2a (lineage II), 1/2b and 4b (lineage I) are responsible for more than 95% of confirmed cases of listeriosis. Serotype 4b is prevalent worldwide, while serotype 1/2a has become more common in Northern Europe [2, 5].

Although all *L. monocytogenes* strains are considered virulent by regulatory bodies, genetic diversity in genes needed for infectivity presumably contributes to differential pathogenicity. For example, the interaction between internalin A (InlA) and the E-cadherin receptor on human epithelial cells is required for invasive listeriosis. Strains with mutations that create premature stop codon in *inlA* attenuate *L. monocytogenes* [6-8]. Upon invasion of the host cell, *L. monocytogenes* employs listeriolysin O (LLO; *hly*) to escape the phagosome [9, 10]. Once in the cytosol, *L. monocytogenes* express *actA* that enables the bacterium to appropriate host F-actin to power its own motility [11, 12] that, in turn, permits cell-to-cell dissemination. Strains deficient in *hly* or *actA* are profoundly attenuated because they cannot escape the primary infected cell. Studies detailing the intracellular virulence phenotypes of natural *L. monocytogenes* isolates are important because having the ability to differentiate virulent from less virulent strains based on serotype and virulence gene sequences could potentially mitigate unnecessary recalls of foodstuffs, and reveal further insight into how this important human pathogen efficiently hijacks host cells.

In this study, we used cell-culture (Caco-2) experiments to characterize the invasion, replication and cell-to-cell spread of 59 *L. monocytogenes* strains recovered over the last four decades in Brazil. Four common serotypes were included (1/2a, 1/2b, 1/2c and 4b) from beef, clinical or environmental sources. We did not observe any differences in intracellular doubling times when comparing the source or serotype of the strains. Serotype 1/2c strains were significantly impaired in Caco-2 invasion while most serotype 1/2b and 4b from beef exhibited invasive phenotypes. We evaluated the distribution of premature stop codons (PMSCs) in the *inlA* gene and found that PMSCs

are common in serotype 1/2c (lineage II) from environment and food, but were not present in any of the 22 strains from clinical cases or those from lineage I. There were also significant differences in cell-to-cell spread between the serotypes, where serotype 1/2a strains were generally impaired in their spreading ability and serotype 1/2b were found to have increased spread ability. Further analysis are needed to demonstrate whether mutations found in the *actA* gene of each serotype may be affecting the cell-to-to cell spread ability.

Results

Intracellular growth of L. monocytogenes in Caco-2 cells

We assessed the intracellular growth of 59 *L. monocytogenes* strains from beef, clinical or environmental sources (**Table 1 and Table S1**) in Caco-2 cells using a gentamicin protection assay. At 2 and 8 hours post-infection (hpi), the Caco-2 cells were lysed and then the bacteria CFUs were enumerated. For all experiments we used the reference strain 10403S (wild-type) as control. As expected, *L. monocytogenes* Δ*hly* exhibited a significant growth defect, likely due to an inability to access to the cytosol (**Fig. 1A**). Additionally, the non-*monocytogenes* spp. *L. welshimeri*, *L. innocua* and *L. seeligeri*, which lack the traits required for infection, were unable to grow inside of Caco-2 cells (**Fig. 1B**). The doubling times of Δ*hly* and the other *Listeria* sp. increased by > 2-fold in Caco-2 cells (**Fig. 1C**), whereas the doubling time for *L. monocytogenes* Δ*actA* and Δ*inlA*Δ*actA* strains was similar to wild-type 10403S. This was consistent with expectations because InlA only affects entry and ActA deficiency only begins to impair growth later during infection (>12 h).

Table 1. *Listeria monocytogenes* strains recovered from Brazil and respective lineages and serotypes.

Source	Total	Lineage I		Lineage II	
		1/2b	4b	1/2a	1/2c
Clinical	22	2	15	5	-
Environment	12	-	4	3	5
Raw beef	23	7	6	5	5
Cooked meat	2	-	2	-	-
Total	59	9	27	13	10

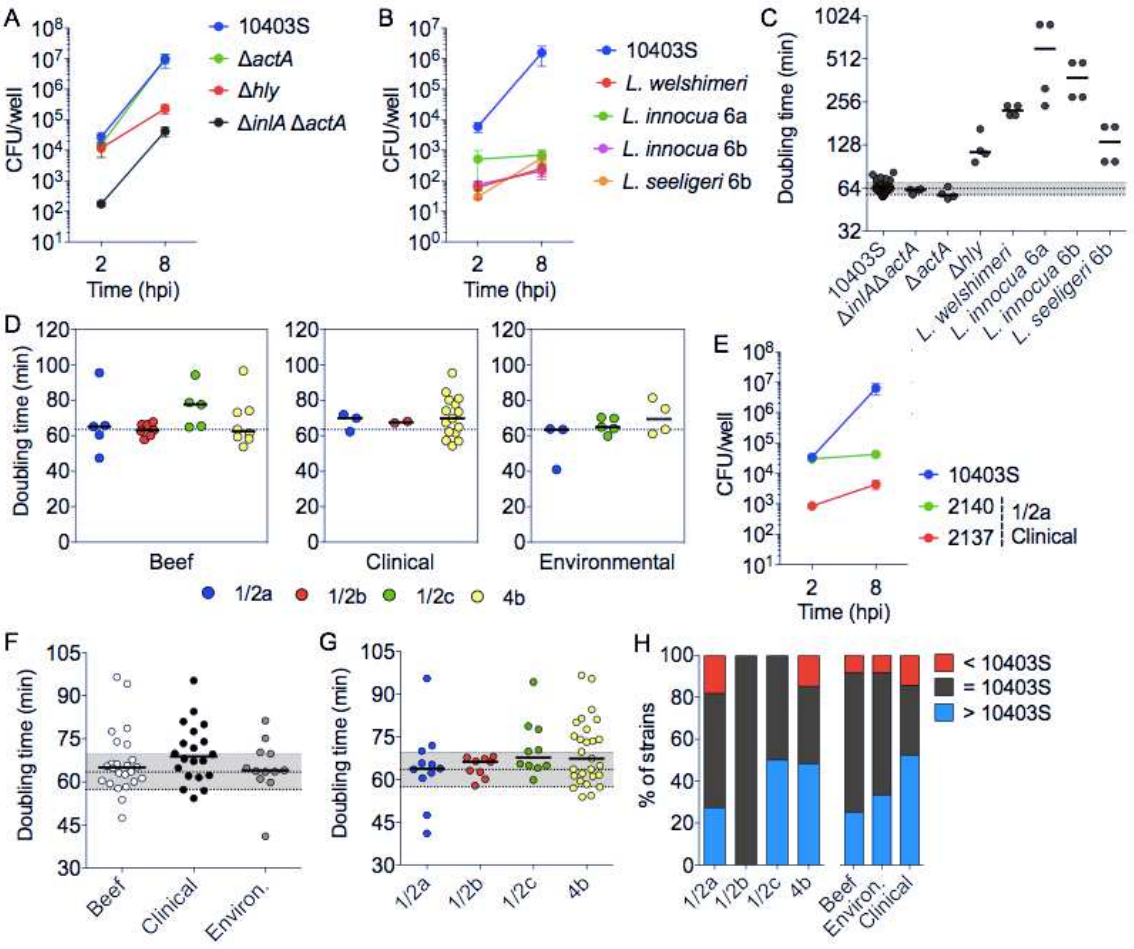

Figure 1. Intracellular growth of *L. monocytogenes* isolates in Caco-2 cells. Caco-2 cells were infected with the indicated *L. monocytogenes* strains (A) or *Listeria* spp (B) and CFUs were enumerated at 2 and 8 hours post-infection (hpi). (C) Intracellular doubling times of the indicated control strains inside Caco-2 cells. (D) Intracellular doubling times of 59 *L. monocytogenes* isolates plotted according to serotype and source (D), source only (F), or serotype only (G). (E) Growth curves of selected serotype 1/2a strains. (H) Percent distribution of the strains exhibiting increased (>70 min, blue), equivalent (grey), or reduced (<57 min, red) intracellular doubling times as compared to the 10403S *L. monocytogenes* lab strain. Error bars represent mean ± SD of 2 biological replicates performed in technical duplicate, and where all data points are shown medians are indicated by horizontal bars. (D, F, G) Each data point represents the mean of 2 biological replicates performed in technical duplicate.

When we calculated the doubling times of the 59 *L. monocytogenes* strains, we did not observe any statistically significant differences when the strains were grouped by serotype or source (**Fig. 1D-G**). There were 2 strains both serotype 1/2a, 2137 (clinical, CSF of newborn) and 2140 (clinical, placenta), that exhibited a huge growth defect andnot grow in Caco-2 cells, respectively (**Fig. 1E**). To ensure that these or any growth differences observed in Caco-2 cells were not due to general slow growth defects, we assessed the BHI broth growth of all of the strains. There were five strains (2138, 3728, 3864, 2140 and 1078) that grew slightly slower in BHI and 3 strains (3738, 3712, and

2137) that grew significantly slower (**Fig. S1A**). Although 2140 grew slightly slower in BHI, it was significantly impaired for growth in Caco-2 cells and was indeed the most attenuated strain of all the 59 strains tested (**Fig. 1E and Fig. S1B**).

There were only 2 strains, 3864 (beef, serotype 1/2a) and 2137, that had equivalently impaired growth in BHI and Caco-2 cells, and therefore these strains were not included in the general doubling time analyses. As expected, the *L. monocytogenes* mutant strains (Δ*hly*, Δ*actA* and Δ*inlA*Δ*actA*) and *Listeria* spp. showed no difference in BHI growth versus 10403S wild-type (**Fig. S1C**). We also did not observe any trends between serotype or source with respect to the distribution of the percent of strains that exhibited increased (> 10403S), similar (= 10430S), or decreased (< 10403S) doubling times (**Fig. 1H and Fig. S1D**). Therefore, the variability in intracellular replication seen amongst these 59 strains is likely to be determined by strain-specific factors rather than by serotype or source.

Invasion of L. monocytogenes into Caco-2 cells

To assess the invasion ability of the strains, we compared the CFUs within Caco-2 cells at 2 hpi. Invasion defects were confirmed in the controls Δ*inlA*Δ*actA* and non-*monocytogenes Listeria* spp. (**Fig. 2A**). Unlike intracellular replication, there were significant differences in invasion ability between serotypes. Serotype 1/2c strains were significantly impaired for invasion, independent of the source (beef versus environment) (**Fig. 2B-D and Table S1**). While 50% of serotype 1/2c strains had increased doubling times in Caco-2 cells (**Fig. 1H**), none of these strains exhibiting decreased doubling times (**Fig. 1H**). Thus, this serotype appears to be generally attenuated for both invasion and growth. Interestingly, although we previous observed that all serotype 1/2b strains were within the standard deviation of doubling time of 10403S (**Fig. 1G**), they vary significantly in their ability to invade, with 50% these strains exhibiting increased invasion versus 10403S (**Fig. 2C and 2E**). Of all the serotypes examined, serotype 4b strains exhibited the highest variability in both intracellular growth (**Fig. 1G and 1H**) as well as invasion (**Fig. 2C**).

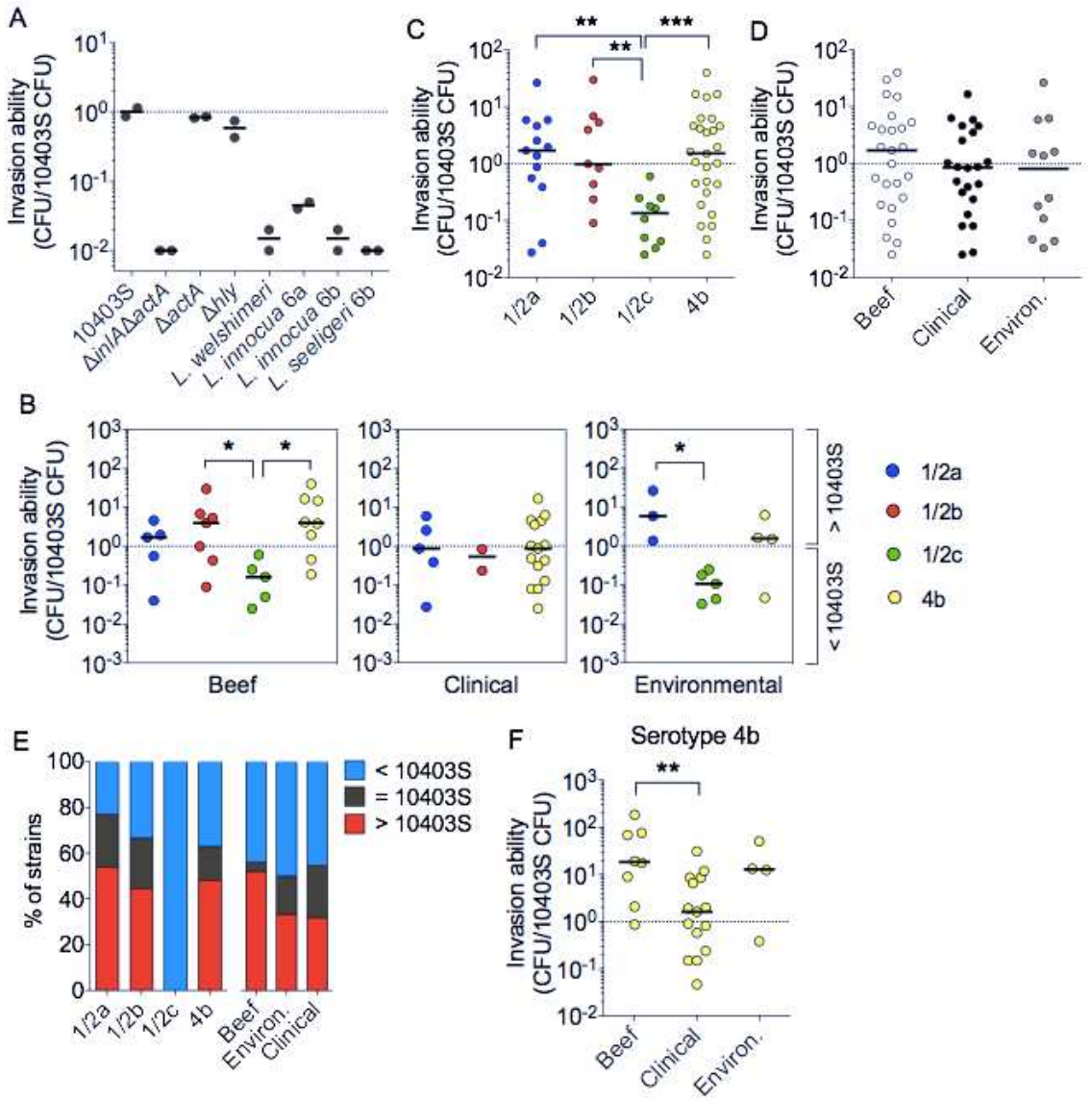

Figure 2. Invasion of *L. monocytogenes* isolates into Caco-2 cells. (A) Invasion ability plotted as the CFU of the indicated strains at 2 hpi divided by the CFU of 10403S. Invasion ability of 59 *L. monocytogenes* isolates plotted according to serotype and source (B), serotype only (C) or source only (D). (E) Percent distribution of the strains exhibiting defective invasion (<0.7, blue), equivalent invasion (grey), or hyper-invasion (>1.5, red) compared to 10403S. (F) Comparison of invasion of serotype 4b strains sorted by source. All data points are shown and medians are indicated by horizontal bars. (B-D, F) Each data point represents the mean of 2 biological replicates performed in technical duplicate. *p < 0.05; **p < 0.005; ***p = 0.0007, Mann-Whitney U test.

Although there was large variation in invasion ability within and across the sources, we found that 50% of the beef isolates were more invasive than 10403S versus just 30% of environment and clinical isolates (**Fig. 2E**). Serotype 4b strains isolated from beef were significantly more invasive ($p < 0.005$) than 4b strains isolated from clinical sources (**Fig. 2F and S2A**). We also looked at the strains within serotype or sources when invasion and intracellular doubling times were plotted together. There were no broad trends evident between sources, but we did observe that serotype 1/2c strains were all

similar in their trends towards reduced invasion and increased doubling times (**Fig. S2B and S2C**).

Detection of premature stop codons in inlA of L. monocytogenes

Five (41.7%) strains from environment and five (21.7%) from raw beef harbor three different premature stop codons (PMSCs) in *inlA*. Interestingly, we did not detect PMSCs in any of the clinical strains (**Table 2**). Nearly all (9/10) serotype 1/2c strains carry PMSCs (**Table 3**), which likely explains the significant invasion defect we observed for this serotype (**Fig. 2C**). We found several 1/2a and 1/2c strains with mutations leading to amino acid changes, including strains from lineage II expressing amino acids commonly observed from lineage I (**Fig. 3A)**. We also identified two strains from serotype 4b with 9-nucleotide deletions (2167 and 3192, from clinical and beef, respectively), resulting in a 3-amino acid residue deletion (**Fig. 3A**).

Table 2. *Listeria monocytogenes* with *inlA* mutations resulting in premature stop codons (PMSCs) or 9 nucleotide deletion resulting in 3 amino acid residue deletion.

inlA genotype	Clinical strains (%)	Environmental	Raw beef	Cooked meat
With PMSCs	-	5 (41.7)	5 (21.7)	-
type 6	-	-	1 (4.3)	-
type 11	-	1 (8.3)	1 (4.3)	-
type 19	-	4 (33.3)	3 (13)	-
Without PMSCs[1]	22 (100)	7 (58.3)	18 (78.2)	2 (100)
With 3 amino acid residue deletion	1 (4.5)	-	1 (4.3)	-

[1] Including the two strains with 3 amino acid residue deletion.

Table 3. Details of each premature stop codons (PMSCs) identified in *Listeria monocytogenes inlA* genotypes.

AA position	PMSC type	Mutant allelic type	Wild-type allelic type	Strains	Serotype	Source	Multiple state	Previous described by:
492	6	TAG	CAG	3864	1/2a	Raw beef	no	Nightingale 2008; Ragon et al. 2008, Van Stelten et al. 2010, Kanki et al. 2015
685	11	TAG	TGG	45[1], 232[2]	1/2c	Environment and raw beef	no	Rousseaux et al. 2004; Ragon et al. 2008, Kovacevic et al. 203, Kanki et al. 2015
326	19	TAA	GAA	19[2], 133[2], 139[2], 227[2], 508[1], 525[1], 3726[1]	1/2c	Environment and raw beef	yes	Wu et al. 2016

[1] raw beef

[2] environment

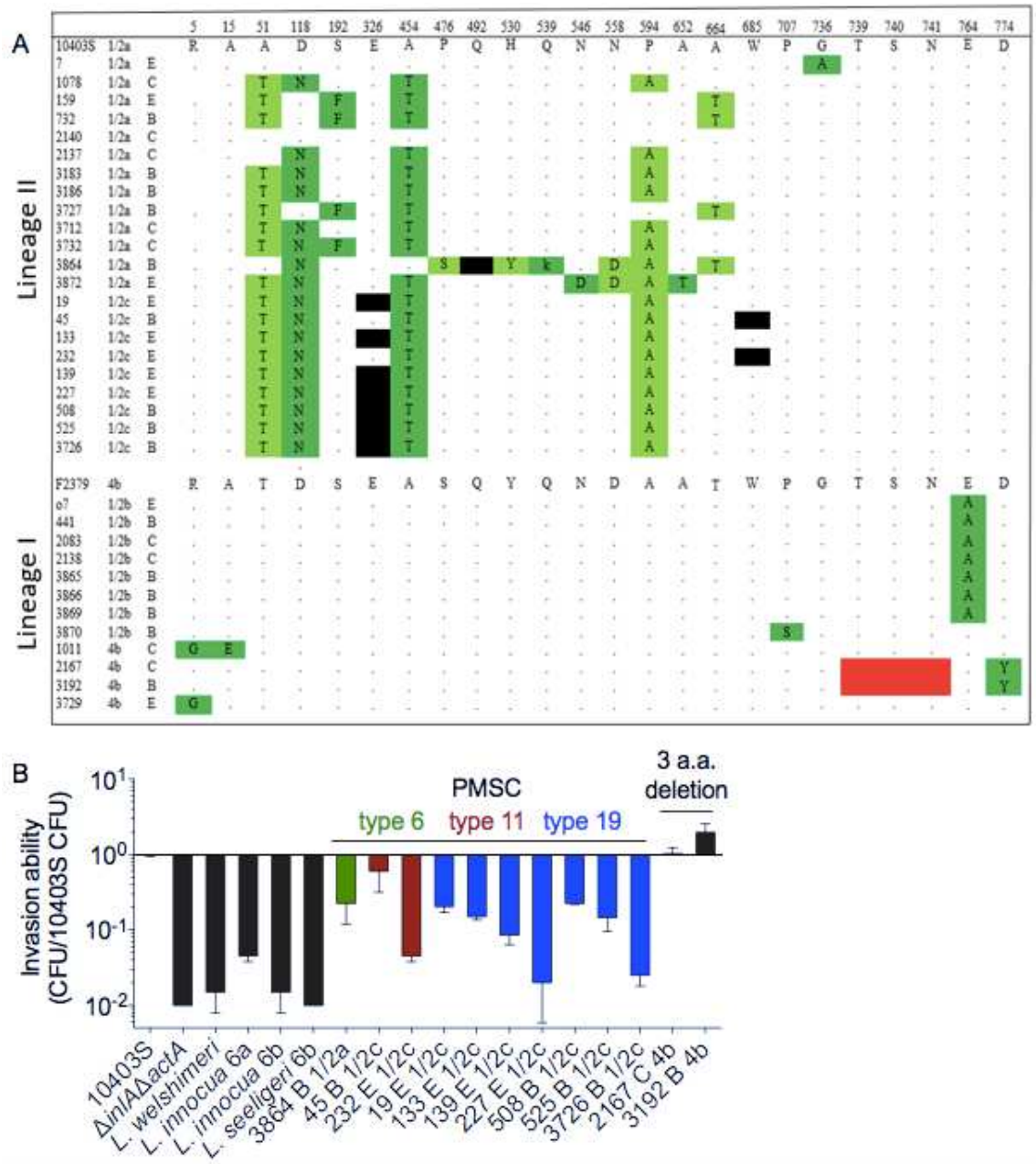

Figure 3. Sequence analysis of internalin A across *L. monocytogenes* isolates. (A) Alignment of the internalin A protein sequences from Lineage I and II strains showing amino acid variation versus the 10403S reference lab strain (green boxes), deletions (red boxes), or introduction of premature stop codons (black boxes). (B) Invasion ability plotted as the CFUs of the indicated strains at 2 hpi divided by the CFUs of 10403S. Strains carrying a premature stop codon (PMSC) type 6 (green), type 11 (maroon) or type 19 (blue) are indicated.

Correlating the results from the sequence analysis with the invasion phenotypes, it was clear that the PMSCs, particularly type 19, had an impact on invasion while the 3-amino acid deletion did not (**Fig. 3B**). There was no correlation between invasion and growth defects, as most of the strains grew normally once inside of Caco-2 cells (**Fig. S3**). We also identified strains that exhibited attenuated invasion despite encoding a full-length InlA, suggesting other mechanisms can impact invasion aside from the *inlA* coding sequence (data not shown).

Cell-to-cell spread of L. monocytogenes isolates in Caco-2 cells

We characterized the cell-to-cell spread phenotypes of the 59 strains by measuring the area of bacterial spread by microscopy within a monolayer of Caco-2 cells after 24 h. We observed significant differences in areas of foci across the serotypes, with serotype 1/2a strains exhibiting impaired spread and serotype 1/2b strains exhibiting increased cell-to-cell spread ability (**Fig. 4A, 4B, Fig. S4 and Table S1**). There was no correlation between cell-to-cell spread and the source of the strains (**Fig. 4C and 4D**). There were 3 interesting clinical strains (1015, 2944, and 3723) from serotype 4b that formed the largest foci and significantly deviated from the standard foci areas of their serotype (**Fig. 4A-B**).

Both LLO, encoded by *hly*, and ActA are important virulence determinants of cell-to-cell spread. We noted striking similarities between strains that were unable to spread (3864 beef 1/2a, 2140 clinical 1/2a, 2137 clinical 1/2a, and 3726 beef 1/2c) and *L. monocytogenes* Δ*hly* and Δ*actA* (**Fig. 4F-H**). All of these strains had increased doubling times, which is consistent with a defect in vacuolar escape as Δ*hly* but not Δ*actA*, exhibited an increased doubling time (**Fig. 1**). However, 2137 and 2140 were significantly more impaired for growth than Δ*hly*, therefore a defect in phagosomal escape is not fully sufficient to explain their growth defects.

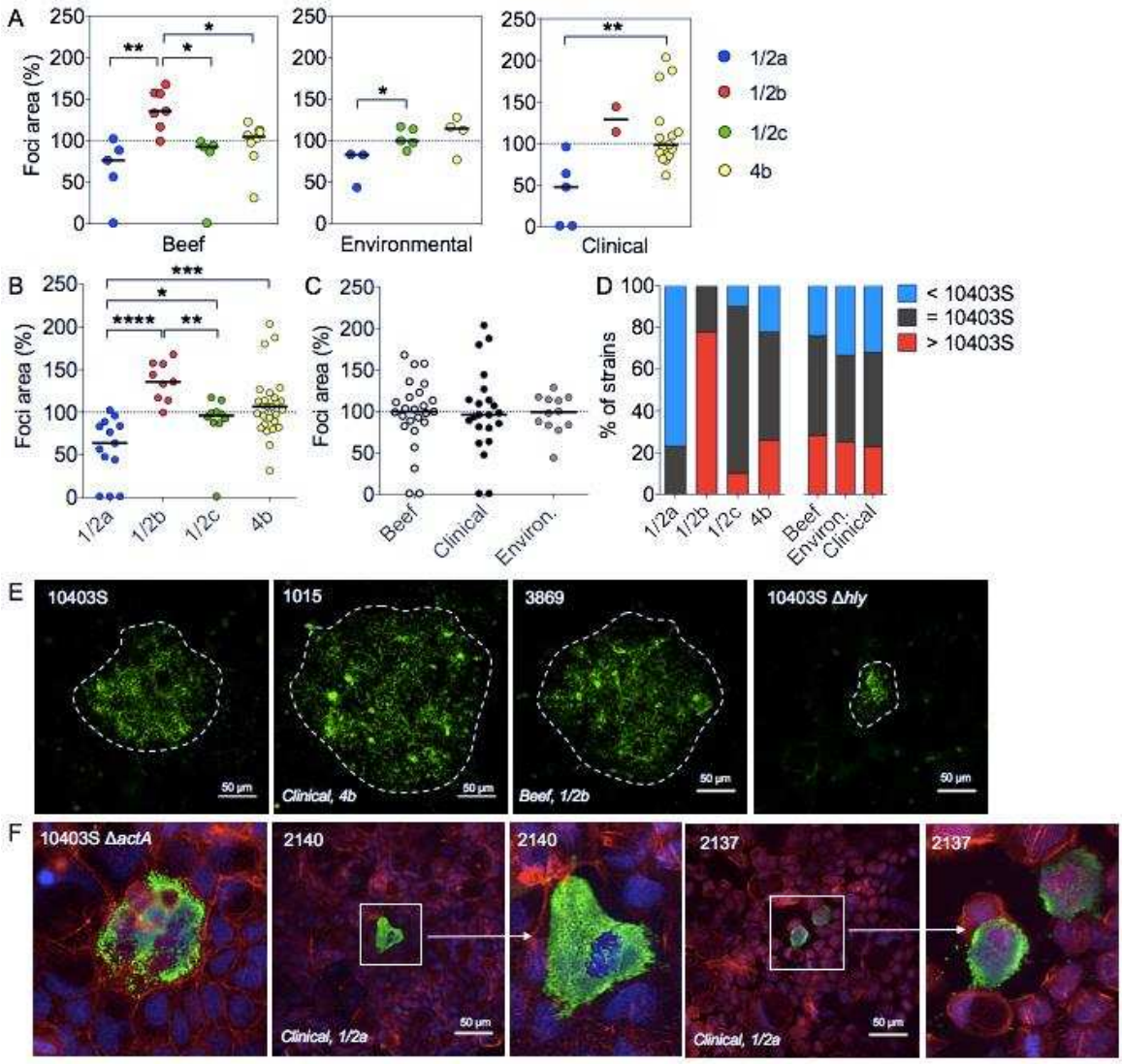

Figure 4. Cell-to-cell spread of *L. monocytogenes* isolates in Caco-2 cells. Quantification of focus areas of 59 *L. monocytogenes* isolates plotted according to serotype and source (A), serotype only (B), or source only (C). Data are presented as the percent focus size compared to 10403S. (D) Percent distribution of strains exhibiting smaller foci (<85%, blue), equivalently sized foci (grey), or larger foci (>115%, red) as compared to the 10403S lab strain. (E, F) Images of *L. monocytogenes* (green) as they spread through Caco-2 cells (black area) for 24 h. Dotted line indicates the edge of the focus area. Scale bar = 50 µm. (A-C) Each data point represents the mean focus area (n = 32 ± 12). *p < 0.05; **p < 0.009; ***p = 0.0002, ****p < 0.0001, Mann-Whitney U test.

Detection of SNPs leading to non-synonymous mutations and deletion of 105 bp in actA gene

The ActA protein is composed by multiple functional domains and has a crucial role in bacterial actin-based motility, as well as mediate escape from autophagic recognition. Among the strains from lineage II (except 3872), only one strain (3864) from serotype 1/2a exhibited SNPs leading to non-synonymous mutations in *actA* **(Table 4)**. Interestingly, *actA* of strain 3872 (1/2a) showed high identity with serotype 1/2b, and

because this we placed 3872 with serotype 1/2b to extract SNPs. Different from strains of lineage II, various SNPs leading to non-synonymous mutations were identified among lineage I strains **(Table 4)**. Considering the results obtained in cell spread experiment, the mutations identified in 1/2b strains may contribute to increase cell-to-cell spread, demanding further analysis to confirm this hypothesis. Moreover, all strains from serotypes 1/2b, 1/2c, and 4b exhibited a 105 bp deletion after the base 792 (aa 260). Curiously, serotype 1/2a strains (that presented the complete *actA* sequence) exhibited lower cell-to-cell spread ability in Caco-2 when compared to other serotypes (**Fig. 4B**).

Hemolytic and phospholipase C activity

LLO and phosphatidylinositol-specific phospholipase C (PI-PLC) are virulence determinants only present in *L. monocytogenes* and *L. ivanovii*. Hemolytic and PI-PLC activity of some strains unable to invade and spread, as well as some strains that spread significantly more were assessed on blood agar and Agar Listeria according to Ottaviani and Agosti (ALOA). The results showed that avirulent, non-spreading strains (2137 and 2140) exhibited hemolysis defect and no hemolysis on horse blood agar, respectivelyv (**Table 5 and Fig. 5A**) and completely lacked hemolysis activity on bovine blood agar (**Fig. 5B**), consistent with the absence of LLO activity. While non-invasive strains that carry PMSCs type 6 (3864) and type 19 (3726) exhibited normal hemolysis on horse and sheep blood agar, little hemolysis on bovine blood agar, and presented normal PLC activity (**Fig. 5B**).

Strain 2137 displayed uncommon small colonies on ALOA and low PI-PLC activity, while the most attenuated strain (2140) exhibited absence of PLC activity (**Fig. 5C**). Strains that were average in spreading ability or spread significantly more exhibited normal hemolytic and PI-PLC activity **(Table 5)**. Taken together, these results are in agreement with what we observed in Caco-2 cells for intracellular growth and cell-

Table 4: SNPs in *actA* leading to non-synonymous mutations across the 59 *Listeria monocytogenes.*

Lineage	Serotype	Nucle. Posi.	Wild	Mutant	AA Position	Wild	Mutant	Isolates
II	1/2a[1]	1210	C	A	404	L	M	3864
		1259	A	C	420	E	A	3864
	1/2c[2]			none				
I	1/2b[3]	336	T	G	112	D	E	3872[5], 2083, 2138, 7, 441, 3865, 3869, 3870
		371	A	C	124	D	A	3870
		1044	C	G	348	I	M	3872[5], 2083, 2138, 7, 441, 3865, 3869
		1265	C	G	422	T	S	2083, 2138, 7, 441, 3865, 3869
		1276	C	T	426	P	S	3872[5], 2083, 2138, 7, 441, 3865, 3869
		1598	G	A	533	G	E	3872[5], 2083, 2138, 7, 441, 3865, 3869, 3870
	4b[4]	265	C	G	89	L	V	1015
		336	T	G	112	D	E	629, 2167, 2168, 2930, 3723, 3734, 3739, 233, 1282, 3729, 74, 3192, 3724, 3863
		1040	T	C	347	L	P	629, 2167, 2168, 2930, 3723, 3734, 3739, 233, 1282, 3729, 74, 3192, 3724, 3863
		1044	G	C	348	M	I	2168, 2930, 3734, 1282, 74
		1265	G	C	422	S	T	2167, 2168, 2930, 3723, 3734, 3739, 233, 1282, 3729, 74, 3192, 3724, 3863
		1276	T	C	426	S	P	2167, 2168, 2930, 3723, 3734, 3739, 233, 1282, 3729, 74, 3192, 3724, 3863
		1460	C	A	487	A	E	2167,3723, 3739, 233, 3729, 3192, 3724, 3863
		1524	T	A	508	D	E	3863

[1] SPNs extracted using *actA* sequence of strains 10403s and EGD-e as reference. [2] SPNs extracted using *actA* sequence of LM41 (1/2c) as reference.[3] SPNs extracted using *actA* sequence of LM11 (1/2b) as reference. [4] SPNs extracted using *actA* sequence of F2365 (4b) as reference. [5] serotype 1/2a

Table 5. Details of virulence phenotypes exhibited by selected *Listeria monocytogenes* in Caco-2 cells, hemolysis activity, phospholipase activity and morphology.

ID	Source	Serotype	Doubling time (min)	Foci area (%)[1]	Hemolysis		PLC activity (ALOA)[2]
					Horse	Bovine	
L. innocua	Raw beef	6a			-	-	-
L. monocytogenes							
ATCC 15313 (avirulent)	Rabbit	1/2a			-	-	++++
ScottA	Clinical (human)	4b			+++	++	+++
2137	CSF of newborn	1/2a	211,94	1,00	+	-	+
2140	Placenta	1/2a	1264,03	1,00	-	-	-
3712	CSF	1/2a	62,34	63,72	+++	++	+++
3864	Raw beef	1/2a	95,40	1,00	+++	++	+++
732	Raw beef	1/2a	60,38	102,43	+++	++	+++
159	Meat grinder	1/2a	63,47	83,79	+++	++	+++
3726	Raw beef	1/2c	94,22	1,00	+++	++	+++
7	Raw beef	1/2b	63,09	99,63	+++	++	+++
441	Raw beef	1/2b	66,32	157,61	+++	++	+++
1015	Blood (kidney transplantation)	4b	57,27	187,83	+++	++	+++
2930	Blood (newborn sepsis)	4b	95,32	85,36	+++	++	+++
2944	CSF	4b	56,95	203,57	+++	++	+++
3723	CSF	4b	62,14	180,23	+++	++	+++
3738	Sheep brain	4b	73,41	61,56	+++	++	+++
3724	Raw beef	4b	73,11	110,79	+++	++	+++

[1]The spread ability of WT *L. monocytogenes* was calculated in compassion with 10403S, and expressed as percentage (foci area of 10403S was considered 100%), [2]Agar Listeria according to Ottaviani and Agosti (ALOA), – negative, ++++ enhanced activity, +++ normal activity, ++ moderated activity, + little activity.

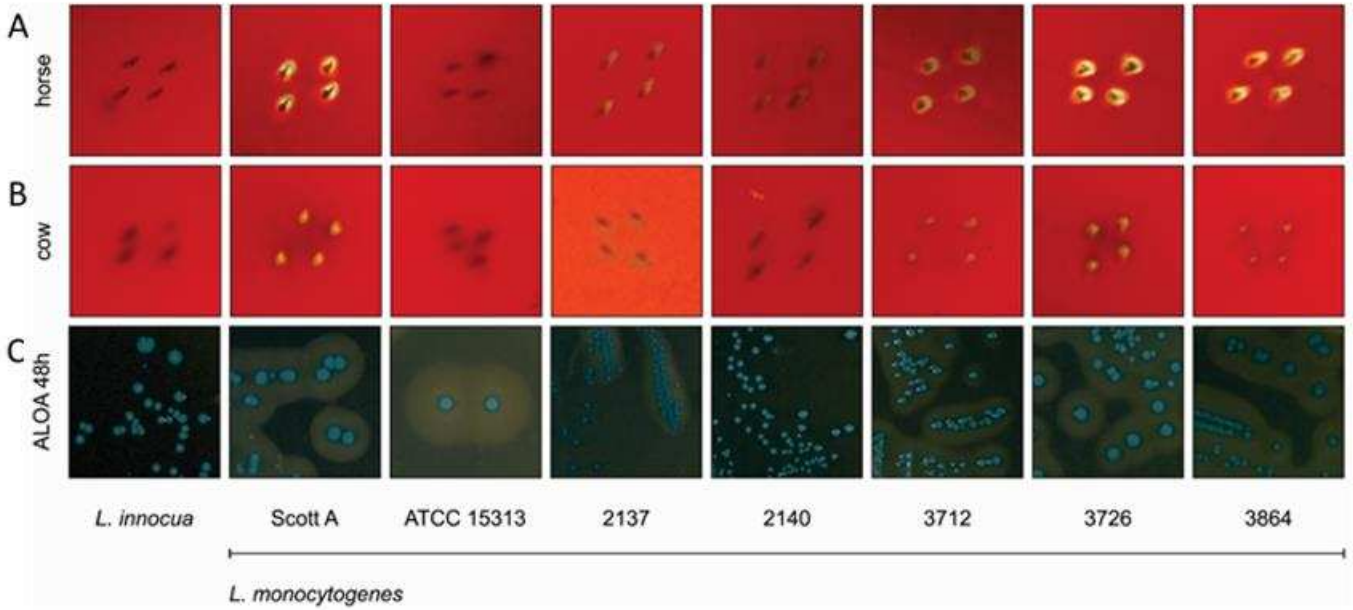

Figure 5. Evaluation of *L. monocytogenes* isolates for hemolytic and phospholipase C activity. Hemolytic activity of *L. innocua* or the indicated *L. monocytogenes* strains in horse (A), cow (B) blood agar plates. (C) Phosphatidylinositol-specific phospholipase C activity on Agar Listeria according to Ottaviani and Agosti (ALOA).

to-cell spread (**Fig. 1E and Fig. 4F-H**). Further genomic analyses are needed to determine where the mutations conferring these nonvirulent phenotypes are located.

Discussion

L. monocytogenes has a range of virulence factors in order to cross the intestinal, placental, and blood-brain barriers, resulting in dissemination to the fetus and central nervous system [13, 14]. The majority of *L. monocytogenes* strains studied here did not present significant differences in the intracellular doubling times in Caco-2 cells, with only 7% of strains exhibiting increased replication compared to the 10403S lab strain (**Fig 1A-H)**, which has never being linked to caused a confirmed laboratory-acquired infection in people. These data suggest that the virulence of natural *L. monocytogenes* isolates is more likely to be influenced by factors affecting *L. monocytogenes* life cycle rather than intracellular growth, and as such assays other than intracellular growth should be employed for future characterizations.

We also determined that the invasiveness and cell-to-cell spreading of *L. monocytogens* strains in Caco-2 cells can be distinguished based on serotype and not by the strain origin (beef vs. environment vs. clinical) (**Fig 2A-F**). The invasion defects seen with serotype 1/2c isolates are likely due to the presence of PMSCs that we identified in the virulence factors such as InlA are commonly observed in strains from environment and food, but not among those from clinical cases [8]. So far, 21 PMSCs have been described in the *inlA* gene, including three (types 19, 20, and 21) recently identified [15, 16]. We found that all 1/2c strains carrying PMSCs were hampered in their ability to invade Caco-2 cells, similar to what we observed for other, non-pathogenic *Listeria* species or Δ*inlA*Δ*actA*.

There are few reports of PMSCs in *inlA* among clinical strains [8, 17, 18], including type 6 and 11, which we detected only in strains from beef and the environment (**Fig 3A-B**). PMSCs in *inlA* were not identified among our 22 clinical strains or among lineage I strains recovered from Brazil, supporting the idea that they are not common among clinical strains. We also identified two serotype 4b strains with 9-nucleotide deletions (2167 and 3192) that exhibited normal invasion, which is consistent with previous observations [19]. Interestingly, we found that serotype 4b strains from beef were among the most invasive of all of the strains tested. It is unclear what governs increased invasion into Caco-2 cells, whether these strains have more InlA on their surface or if some other

factor is involved. Nevertheless, these data indicate that 4b contaminants on beef may represent a real risk for epithelial invasion.

We identified two serotype 1/2a strains, 2137 and 2140, isolated from the CSF of a child newborn and placenta, respectively, which were markedly attenuated for intracellular growth and cell-to-cell spread (**Fig. 1E and Table S1**). These strains lacked hemolytic activity and 2140 was further deficient in PLC activity (**Fig. 5A-C**). The intracellular growth defect of these strains was more pronounced than *L. monocytogenes* 10403S deficient in LLO, therefore these strains may have other mutations affecting their growth ability in Caco-2 cells. It is curious that a strain lacking LLO and PLC activity and which grows so poorly in host cells could be isolated from the placenta. Genomic analyses of this strain would likely serve to enlighten the basis of its survival inside the host and whether mutations at LIPI-1 are leading to its avirulence in Caco-2 cells.

An important mechanism of immune evasion by *L. monocytogenes* is its ability to spread cell-to-cell using actin-based motility. This virulence attribute allows *L. monocytogenes* to remain hidden within epithelial cells where it evades targeting by complement, antimicrobial peptides, and neutrophils. The results of *actA* sequencing revealed that serotypes 1/2b, 1/2c, and 4b have a 105 bp deletion: possessing three instead of four proline-rich region in actA protein. Moriishi, Terao, Koura and Inoue [20] suggested that incidence of two aleles of *actA* gene (with and without the 105 bp) are similar among isolates recovered from humans, suggesting that the number of the proline-rich repeat units in ActA protein may not drastically affect the virulence of *L. monocytogenes*. Similar as had been observed by Roberts and Wiedmann [21]. The present study demonstrated that 105 bp are conserved only among all *actA* sequences of 1/2a strains, which is in agreement with other studies that suggested which 105 bp deletion is more linked to lineage I strains and often detected among clinical [22-26], demonstrating that the mutation does not affect the functionality of the protein. Perhaps, it may confer even an advantage, as most serotype 1/2a strains studied here (that have the complete *actA* sequence) exhibited significand defects during cell-to-cell spread in Caco-2 (**Figure 4B**), demanding to further analysis to confirm this hypothesis, as well to confirm if the mutations in actA of 1/2b are related with increase cell-to-cell spread ability.

Serotype-level analyses revealed that most 4b strains from beef are invasive (**Fig. 2F**) while 1/2b strains spread more in Caco-2 (**Fig. B**). Perhaps not coincidentally, these serotypes are most often associated with human disease. Among these serotypes, the strains isolated from beef presented the most extreme virulence attributes amongst the strains tested in this study. Regardless of their origins, the appearance of these phenotypes

in vitro using Caco-2 cells provides a tractable model for better understanding the factors that increase invasion and spread amongst natural isolates. Novel mechanisms of increased invasion and spread are unlikely to be revealed by the common lab *L. monocytogenes* strains EGD and 10403S that are both serotype 1/2a. In addition to the serotype-specific and strain-specific phenotypes uncovered in this work, these data provide a valuable reference for *Listeria* researchers as we have thoroughly detailed and validated the virulence attributes of 59 *L. monocytogenes* isolates which could be used to uncover novel mechanism of pathogenesis.

Materials and Methods

Bacterial strains

A total of 59 *L. monocytogenes* strains from serotypes 1/2a (n=13), 1/2b (n=9), 1/2c (n=10), and 4b (n=27) were included in this study, most of them from CLIST collection, FioCruz (**Table S1**). Twenty two were from clinical cases, 12 from food-processing environment (referred to as "environmental" strains), and 25 from beef samples (Table 1). *L. welshimeri, L. innocua, L. seeligeri,* and *L. monocytogenes* 10403S (wild-type) were used as a control for infection assays, as well the mutant strains Δ*inlA/actA*, Δ*actA*, and Δ*hly* (all in the 10403S background). *L. innocua*, *L. monocytogenes* ScotA ans ATCC 15313 were used as control for hemolysis and phospholipase C activity assays. Pure cultures were maintained at −80°C in brain heart infusion broth (BHI, Oxoid Ltd., Basingstoke, England) in the presence of 20% (v/v) glycerol (Merck, Whitehouse Station, NJ, USA), and recovered in BHI agar prior use.

Cell culture

Human colon carcinoma enterocyte-like epithelial cells (Caco-2) were grown at 37ºC in controlled atmosphere of 5% CO_2 in Dulbecco modified Eagle medium (GIBCO), supplemented with 20% heat inactivated fetal bovine serum (Thermo Scientific HyClone), 1% L-glutamine, and 1% sodium pyruvate (Thermo Fisher Scientific). Cells were passaged every 2-3 days and maintained at <70% confluency.

Intracellular CFU enumeration

The *L. monocytogenes* strains were grown in Brain Heart Infusion (BHI) media at 30°C overnight without shaking to stationary phase (OD_{600}, 1.1-1.3). 1 mL of the overnight culture was washed 3 times and resuspended in 1 ml of 1X PBS. Caco-2 cells were

infected as previously described [27] with an MOI of 2. Briefly, growth curves were performed in 12-well plates with 0.5 x 10^6 Caco-2 cells seeded in 1 mL the before infection. At the time of infection, the cell monolayers were washed with 1X PBS and infected in complete medium containing 0.1% FBS for 1 h at 37°C. After that, the media was removed, cells were washed twice with 1X PBS, and medium supplemented with 50 µg/mL of gentamicin was added to kill extracellular bacteria. At 2 h and 8 h post-infection, the cells were washed twice with 1X PBS, lysed in 300 µL of cold MilliQ water, and plated for enumeration. For every infection set, the reference strain 10403 was included.

inlA sequencing

DNA extraction of 59 *L. monocytogenes* strains was performed using the Wizard Genomic DNA Purification kit (Promega). The 2,400 bp *inlA* was amplified following recommendations previous described by Ragon, Wirth, Hollandt, Lavenir, Lecuit, Le Monnier and Brisse [28]. The forward primer was 5'-CGGATGCAGGAGAAAATCC-3' and reverse was 5'-CTTTCACACTATCCTCTCC-3'. The obtained sequences were analyzed using MEGA software version 6.

Microscopy and image analysis

To measure Listeria cell spreading, 0.75 x 10^6 cells/mL Caco-2 cells were plated on collagen-coated glass coverslips (Corning BioCoat) within 6-well plates and infected with a range of MOIs (0.01 – 100) that depended on the invasiveness of the strains – to ensure no overlap between the foci and to ensure sufficient recovery of foci. After 20 h the cells were fixed in 3.5% formaldehyde (Thermo Fisher Scientific) and blocked in Tris-buffered saline with 1% BSA (Sigma). Staining was performed using Listeria O Antiserum (BD Difco 223021) and phalloidin (F-actin probe) (Thermo Fisher Scientific). The coverslips were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). A Nikon Eclipse E600 microscope with a QImaging Retigia EX CCD Camera was used for imaging. Determination of focus areas were done using ImageJ software.

Deep sequencing, assembly, and SNPs extraction of actA gene

A total of 40 *L. monocytogenes* isolates representatives of virulence diversity observed in cells were selected for deep sequencing. The genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Germany), following the manufacturer's instructions. Listeria genomes were sequenced using Illumina MiSeq platform (Illumina,

San Diego, CA, USA) and reads assembled using *de novo*. The alignment of *actA* was performed using as reference sequences from each serotype: 10403S and EGD-e for serotype 1/2a (NC_017544.1 and AL591824.1), LM41 for serotype 1/2c (NZ_MJBT00000000.1), LM11 for serotype 1/2b (CP019624.1), and F2365 for serotype 4b (NC_002973.6). SNPs were extracted based on *actA* alignment using the software MAUVE.

Hemolysis and phospholipase C activity

The hemolytic activity was determined as recommended by ISO 11290-1 [29, 30]. The strains that exhibited avirulent phenotypes (2137, 2140, 3864 and 3726), as well some strains that exhibited spread significant more or average spreading, were recovered on agar BHI prior use (24 h at 37°C) and inoculated on 8% blood agar (Isofar, RJ, Brazil) at 37°C for 24 h (the agar plates were made using blood of horse, sheep and bovine). The phospholipase C activity was evaluated on Agar Listeria according to Ottaviani and Agosti (ALOA) as recommended by ISO 11290-1.

Statistical Analyses

Data were analyzed using Prism 6 software. Statistical significance is indicated in the figure legends.

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Author contributions

A.C.C., A.P.M., D.R.C., S.S., J.J.W., and L.A.N. designed the research; A.C.C. and A.P.M. performed the experiments, analyzed the data, and wrote the paper; J.A and N.H performed the sequencing and genomic assembly; D.R.C., S.S., J.J.W., and L.A.N revised the paper.

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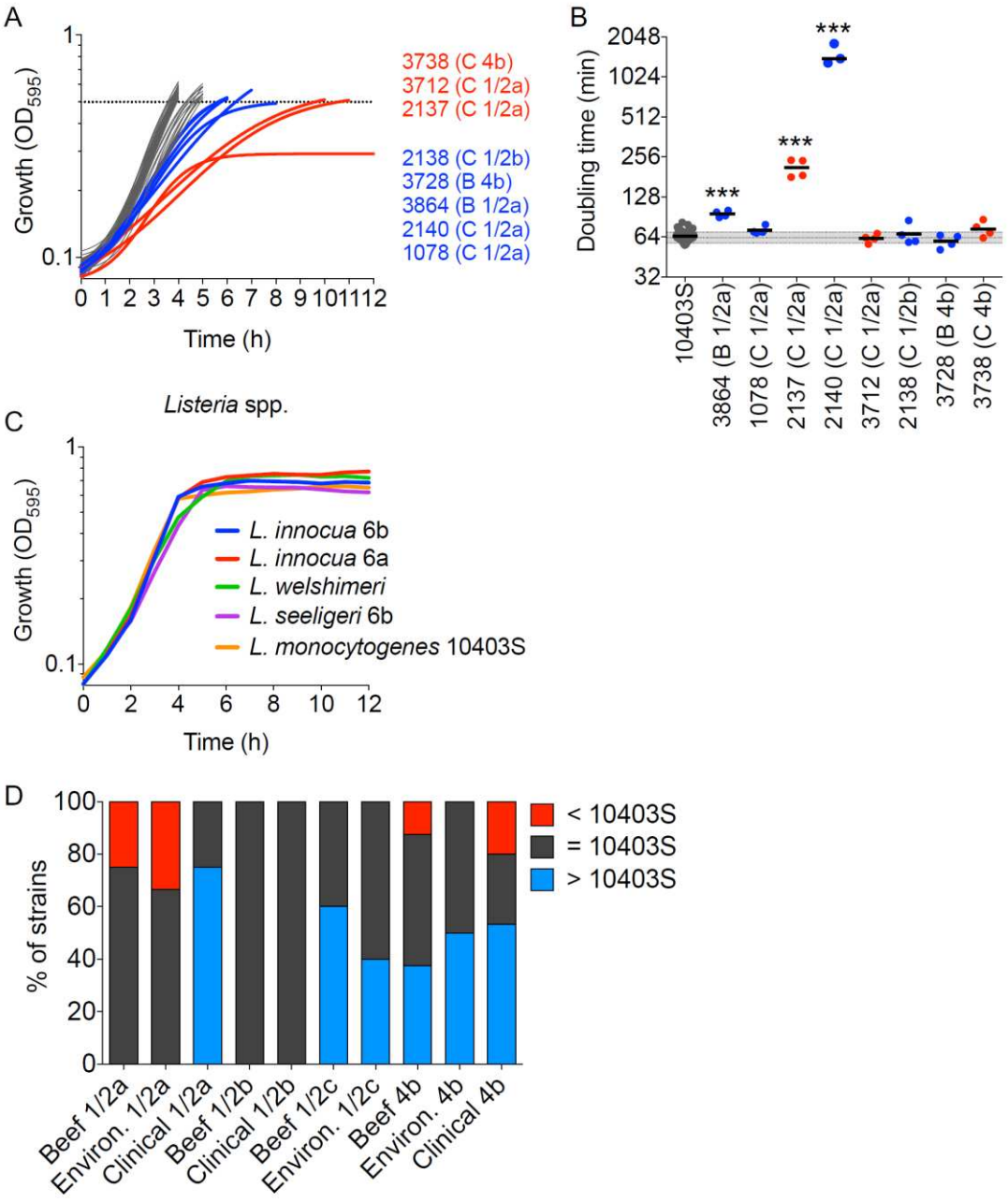
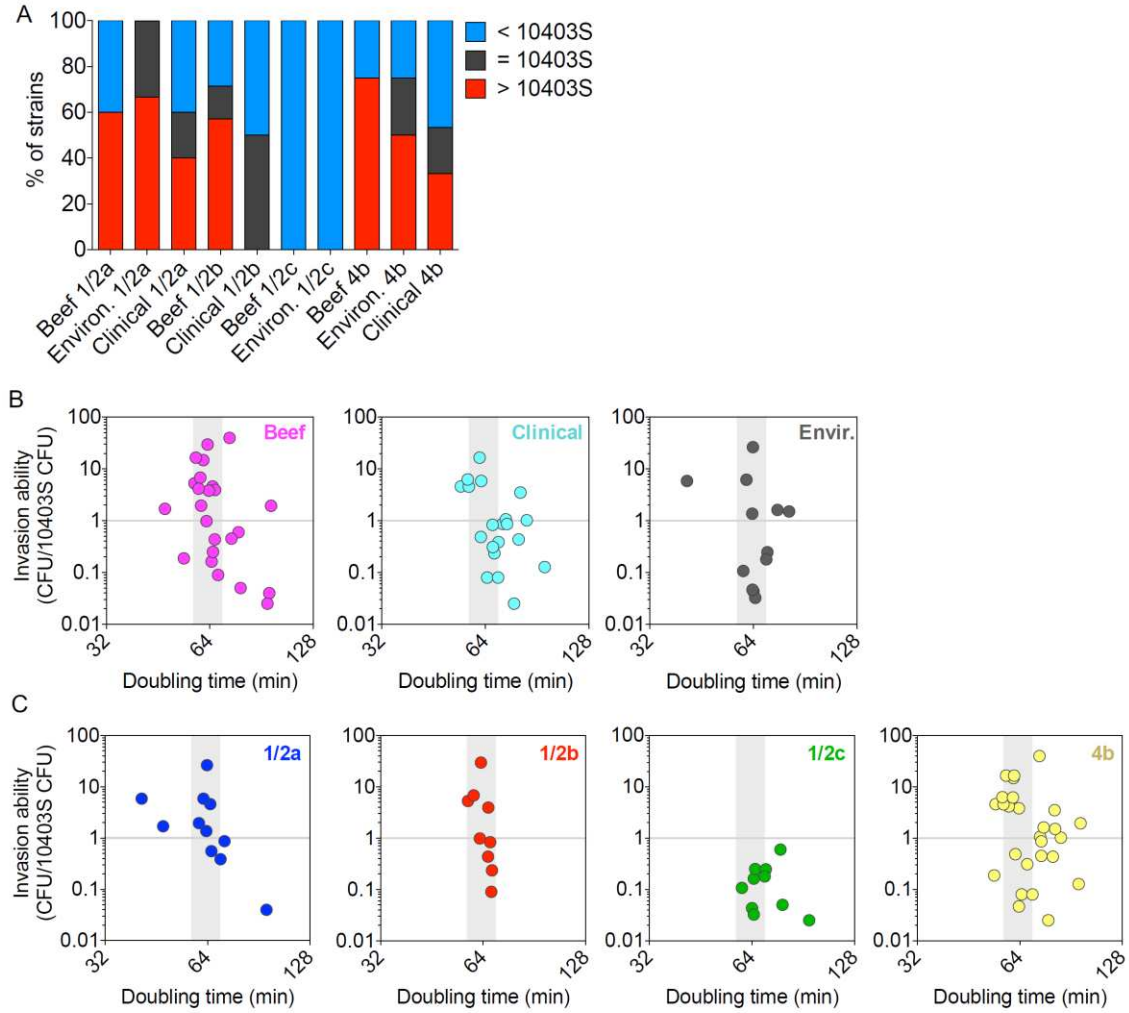
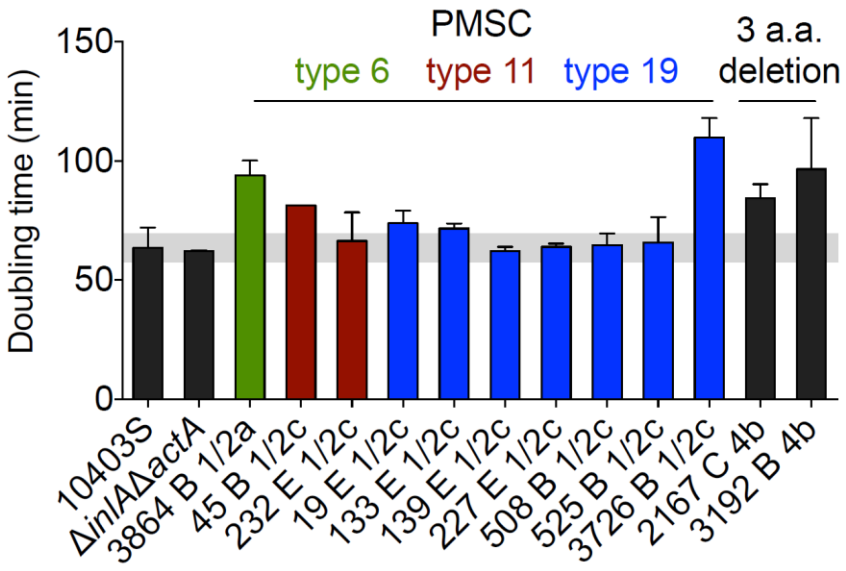


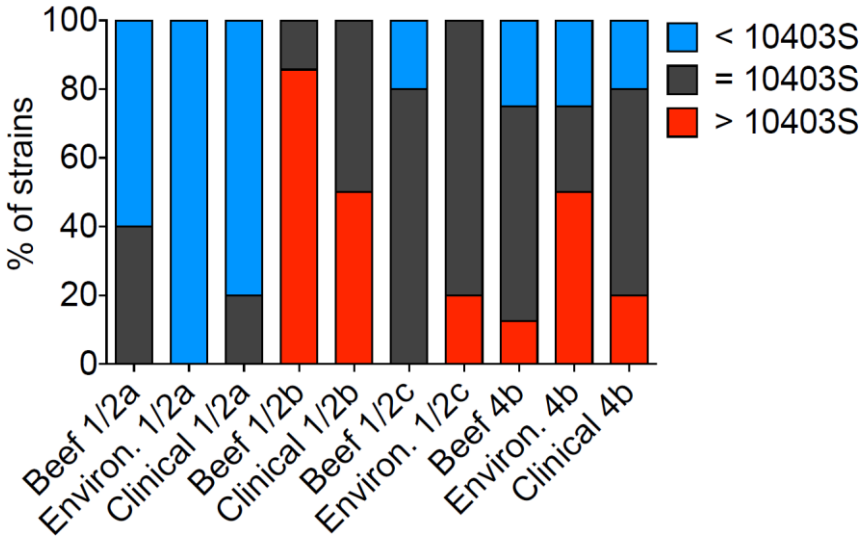
Supplementary Figure 1. Growth of *L. monocytogenes* isolates in BHI broth. (A) Absorbance 595 values of cultures grown statically at 37c and shaken 30 sec prior to hourly reads. (B) Intracellular doubling times of the indicated *L. monocytogenes* isolates inside Caco-2 cells. (C) Same as in (A) but data are showing other *Listeria* spp. (non-*monocytogenes*) as well as control strains used in the study. (D) Percent distribution of the strains exhibiting increased (blue), equivalent (grey), or reduced (red) intracellular doubling times as compared to the 10403S *L. monocytogenes* lab strain. Data are similar to those shown in Figure 1H except here they are segregated by both serotype and source. (A, C) Data represent the means of 3 biological replicates for each strain. (B, D) Data are from 2 biological replicates performed in technical duplicate, and medians are indicated by horizontal bars. ***p < 0.0001, ordinary one-way ANOVA.



Supplementary Figure 2. Invasion of *L. monocytogenes* into Caco-2 cells. (A) Percent distribution of 59 *L. monocytogenes* isolates exhibiting defective invasion (blue), equivalent invasion (grey), or hyper-invasion (red) as compared to the 10403S *L. monocytogenes* lab strain. Data are similar to those shown in Figure 2E except here they are segregated by both serotype and source. XY plots comparing invasion ability (Y axis) with intracellular doubling times (X axis) in Caco-2 cells segregated according to source (B) or serotype (C). Data represent the mean from 2 biological replicates performed in technical duplicate.



Supplementary Figure 3. Doubling times of *inlA* mutant strains. Intracellular doubling times of *L. monocytogenes* isolates carrying premature stop codon (PMSC) type 6 (green), type 11 (maroon) or 19 (blue). Each bar represents the mean ± SD from 2 biological replicates performed in technical duplicate.



Supplementary Figure 4. Cell-to-cell spread of *L. monocytogenes* isolates in Caco-2 cells. Percent distribution of 59 *L. monocytogenes* isolates exhibiting smaller foci (blue), equivalently sized foci (grey), or larger foci (red) as compared to the 10403S lab strain. Data are similar to those shown in Figure 4D except here they are segregated by both serotype and source. Data represent the mean from 2 biological replicates performed in technical duplicate.

Supplementary Table 1.

ID	State	Year	Source	Serotype	Serogrouping by PCR		*inlA* PMSC	Caco-2		
					Borucki and Call 2003	Doumith et al. 2004		Invasion (CFU/CFU 10403S) AVG	Doubling times (min) AVG	Foci area (% 10403S) AVG
10403S wild-type				1/2a				1,00	63,50	100,00
10403S Δ*hlyA*				1/2a				0,68	122,41	1,00
10403S Δ*actA*				1/2a				0,83	58,32	1,00
10403S Δ*inlA* Δ*actA*				1/2a				0,01	61,15	1,00
L. innocua 6a	MG	2010	environment (dirty knife)	6a				0,04	583,21	1,00
L. innocua 6b	MG	2011	raw beef (refrigerated)	6b				0,01	378,17	1,00
L. welshimeri	MG	2009	environmental (bovine carcass)	--				0,01	223,36	1,00
L. seeligeri	MG	2012	environment (dirty table)	--				0,01	134,40	1,00
CLIST 1078	SP	1986	clinical (blood of kidney transplantation)	1/2a	1/2a ou 3a	1/2c ou 3c		0,87	71,76	96,00
CLIST 2137	PR	1983	clinical (CSF of newborn)	1/2a	1/2a ou 3a	1/2c ou 3c		0,03	211,94	1,00
CLIST 2140	PE	1978	clinical (placenta)	1/2a	1/2a ou 3a	1/2a ou 3a		2,54	1264,03	1,00
CLIST 3712	SP	1978	clinical (CSF)	1/2a	1/2a ou 3a	1/2c ou 3c		5,89	62,34	63,72
CLIST 3732	DF	1989	clinical (CSF)	1/2a	1/2a ou 3a	1/2c ou 3c		0,39	69,93	47,52
L07	RS	2010	environmental (bovine carcass)	1/2a	1/2a ou 3a	1/2a ou 3a		26,50	63,80	83,19
159	PR	2005	environmental (grinder)	1/2a	1/2a ou 3a	1/2c ou 3c		1,38	63,47	83,79
CLIST 3872	SC	2004	environment (swine carcass)	1/2a	1/2a ou 3a	1/2a ou 3a		5,87	40,97	44,09
CLIST 732	MT	2009	raw beef (refrigerated)	1/2a	1/2a ou 3a	1/2a ou 3a		1,96	60,38	102,43
CLIST 3183	SP	2006	raw beef (frozen)	1/2a	1/2a ou 3a	1/2c ou 3c		0,56	65,69	88,68
CLIST 3186	SP	2006	raw beef (frozen)	1/2a	1/2a ou 3a	1/2c ou 3c		4,60	65,23	76,63
CLIST 3727	SP	2003	raw beef (frozen)	1/2a	1/2a ou 3a	1/2a ou 3a		1,71	47,44	56,54
CLIST 3864	RS	2002	raw beef (refrigerated)	1/2a	1/2a ou 3a	1/2a ou 3a	type 6	0,04	95,40	1,00
CLIST 2083	RJ	2008	clinical (blood, patient with lymphoma)	1/2b	1/2b ou 3b	1/2b, 3b or 7		0,24	67,97	113,85

ID	State	Year	Source	Serotype	Serogrouping by PCR		*inlA* PMSC	Caco-2		
					Borucki and Call 2003	Doumith et al. 2004		Invasion (CFU/CFU 10403S) AVG	Doubling times (min) AVG	Foci area (% 10403S) AVG
CLIST 2138	SP	1982	clinical (CSF, meningitis)	1/2b	1/2b ou 3b	1/2b, 3b or 7		0,84	67,21	144,04
7	MG	2009	raw beef (refrigerated)	1/2b	1/2b ou 3b	1/2b, 3b or 7		29,67	63,09	99,63
CLIST 441	MT	2010	raw beef (refrigerated)	1/2b	1/2b ou 3b	1/2b, 3b or 7		3,95	66,32	157,61
CLIST 3063	MT	2007	raw beef (refrigerated)	1/2b	1/2b ou 3b	1/2b, 3b or 7		0,44	66,24	135,77
CLIST 3865	SP	2002	raw beef (seasoned)	1/2b	1/2b ou 3b	1/2b, 3b or 7		5,31	57,84	116,84
CLIST 3866	SP	2002	raw beef (seasoned)	1/2b	1/2b ou 3b	1/2b, 3b or 7		6,80	60,15	133,31
CLIST 3869	RJ	2004	raw beef (refrigerated)	1/2b	1/2b ou 3b	1/2b, 3b or 7		0,99	62,60	156,80
CLIST 3870	MT	2004	raw beef (frozen)	1/2b	1/2b ou 3b	1/2b, 3b or 7		0,09	67,71	167,96
19	MG	2009	environment (meat handlers)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,25	70,29	87,97
133	RS	2010	environment (knife)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,18	69,81	114,28
139	MG	2010	environment (dirty table)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,11	59,84	97,86
227	RS	2010	environment (table)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,03	64,84	100,31
232	MG	2010	environment (clean table)	1/2c	1/2c or 3c	1/2c or 3c	type 11	0,04	64,01	117,25
45	MG	2010	raw beef (refrigerated)	1/2c	1/2c or 3c	1/2c or 3c	type 11	0,60	77,60	94,44
508	MG	2012	raw beef (refrigerated)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,25	65,44	92,51
525	MG	2012	raw beef (refrigerated)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,16	64,81	86,90
581	MG	2012	raw beef (refrigerated)	1/2c	1/2c or 3c	1/2c or 3c		0,05	78,69	98,87
CLIST 3726	SP	2003	raw beef (frozen)	1/2c	1/2c or 3c	1/2c or 3c	type 19	0,03	94,22	1,00
247	RJ	2007	clinical (blood of stillbirth)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,87	73,98	81,42
CLIST 629	ES	2010	clinical (CSF)	4b	4b, 4d, 4a, or 4c	IVb-v1		16,60	61,64	79,92
CLIST 1011	SP	1985	clinical (blood of kidney transplantation)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		4,60	54,26	106,73
CLIST 1015	SP	1985	clinical (blood of kidney transplantation)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		4,56	57,27	187,83
CLIST 2167	SP	1997	clinical (CSF)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		1,02	84,46	126,81
CLIST 2168	RJ	2007	clinical (CSF of of stillbirth)	4b	4b, 4d, 4a, or 4c	IVb-v1		0,08	64,79	109,28

ID	State	Year	Source	Serotype	Serogrouping by PCR		*inlA* PMSC	Caco-2		
					Borucki and Call 2003	Doumith et al. 2004		Invasion (CFU/CFU 10403S) AVG	Doubling times (min) AVG	Foci area (% 10403S) AVG
CLIST 2930	RJ	2004	clinical (blood newborn sepsis)	4b	4b, 4d, 4a, or 4c	IVb-v1		0,13	95,32	85,36
CLIST 2944	SP	1997	clinical (CSF)	4b	4b, 4d, 4a, or 4c	IVb-v1		6,30	56,95	203,57
CLIST 3723	RJ	1990	clinical (CSF)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,49	62,14	180,23
CLIST 3733	DF	1989	clinical (CSF)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,44	79,96	93,18
CLIST 3734	PE	1989	clinical (CSF)	4b	4b, 4d, 4a, or 4c	IVb-v1		0,03	77,64	98,17
CLIST 3735	SP	1985	clinical (blood of kidney transplantation)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,31	67,32	95,78
CLIST 3737	SP	1980	clinical (CSF, meningitis)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,08	69,69	89,06
CLIST 3738	RJ	1987	clinical (sheep Brain)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		1,07	73,41	61,56
CLIST 3739	PR	2000	clinical (CSF)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		3,52	80,99	113,55
212	MG	2012	environment (plastic box)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		1,51	81,34	112,94
233	MG	2012	environment (grinder)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		1,62	75,25	128,65
1282	SP	2013	environment (mat chicken)	4b	4b, 4d, 4a, or 4c	IVb-v1		6,21	61,12	116,86
CLIST 3729	RS	1991	environmental (animal)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,08	63,55	77,49
74	MG	2010	raw beef (refrigerated)	4b	4b, 4d, 4a, or 4c	IVb-v1		3,82	63,61	103,19
CLIST 3192	SP	2006	raw beef (refrigerated)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		1,95	96,52	82,33
CLIST 3724	RJ	2003	raw beef (milled)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		0,45	73,96	122,68
CLIST 3728	RJ	2003	raw beef (refrigerated)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		40,00	73,11	110,79
CLIST 3863	RJ	2000	raw beef (refrigerated)	4b	4b, 4d, 4a, or 4c	4b, 4d, 4e		4,17	59,41	106,63
CLIST 3868	MT	2003	raw beef (frozen)	4b	4b, 4d, 4a, or 4c	IVb-v1		0,19	53,75	31,48
CLIST 3721	SP	2004	cooked meat	4b	4b, 4d, 4a, or 4c	IVb-v1		14,83	61,27	97,79
CLIST 3731	MT	2001	cooked meat (frozen)	4b	4b, 4d, 4a, or 4c	IVb-v1		16,55	58,34	113,12

CHAPTER 6 - Wild-type *Listeria monocytogenes* from Brazil induces pyroptosis and production of type I IFN through STING dependent and cGAS dependent or independent pathways

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Title page

Wild-type *Listeria monocytogenes* from Brazil induces pyroptosis and production of type I IFN through STING dependent and cGAS dependent or independent pathways

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Abstract

The Gram-positive bacteria *Listeria monocytogenes* is a foodborne pathogen able to multiply in a variety of host cells after escape of the phagocytic vacuole and gain the cytosol, despite of host cell defense responses. Upon infection, *L. monocytogenes* is detected by multiple pattern recognition receptors (PRRs); ligand recognitions can trigger production of type I interferons (IFN) and activate the inflammasome. Here we have examined the ability of *L. monocytogenes* isolates obtained in Brazil to multiply into macrophages, to induce cell death, and to activate type I IFN production. No trends were observed regarding intracellular doubling times in iBMDM by comparing sources and serotypes, indicating that the intracellular growth is dictated by genotype characteristics of each strain. Clinical (629, 1011, 2167, 3723) and raw beef (3727, 7, 3865) isolates induced pyroptosis through the activation of caspase-1; clinical isolate 629 (serotype 4b, atypical IVb-v1 by molecular serogrouping) also induced nearly the double of type I IFN production in comparison with reference strain 10403S, being STING dependent and partially cGAS dependent. Similar, isolates 3727 (1/2a), 7 (1/2b) and 508 (1/2c) induced higher production of type I IFN by a STING dependent pathway, being partially cGAS dependent (3727, 508) and fully cGAS independent (7). This work demonstrated that wild-type *L. monocytogenes* from Brazil induced pyroptosis and type I IFN production in macrophages through the activation of STING and cGAS pathways.

Key words: *Listeria monocytogenes*, pyroptosis, type I IFN, STING, cGAS

Introduction

In the last three decades, the Gram-positive pathogen *Listeria monocytogenes* has been broadly used as model organism for host-pathogen interaction studies due to its ability to enter and to multiply in a variety of host cells, including non-phagocytic (epithelial and endothelial cells) and macrophages [1, 2]. Systematic analysis based on epidemiological data, virulence phenotypes characterization *in vitro* and *in vivo*, and most recently whole-genome sequencing (WGS) have been contributed to better understand the *L. monocytogenes* virulence cycle and the host innate and acquired immune signaling pathways in response to infection [3-5].

After the phagocytosis by macrophages, *L. monocytogenes* secretes listeriolysin O (encoded by the *hly* gene), a protein that plays a major role to disrupt the vacuolar membrane, allowing the pathogen to escape and to replicate into the host cell cytoplasm [6]. As response, the host cells display strategies to limit bacteria replication by different innate immune signaling pathways. *L. monocytogenes* pathogen-associated molecular patterns (PAMPs) released during infection allow the activation of innate immunity through a sequence of sensing pathways. Cytosolic receptors, like cGAS/STING, DDX41 and AIM2, can trigger the type I interferon (IFN) production and the inflammasome activation after detection of PAMPs [5, 7-9].

Ligand recognition of *L. monocytogenes* by a nod-like receptor (NLR) or by a cytosolic DNA sensor can lead to activation of human or mouse caspase-1 or mouse caspase-11, resulting in the activation of the inflammasome complex, cleavage of pro-IL-1β and pro-IL-18 into their active forms, and cell death by pyroptosis, which would work in favor of host by eliminating the bacteria from intracellular niche [8, 10-12]. The major host cytosolic sensor is the stimulator of IFN genes (STING), an ER-localized transmembrane protein activated by cyclic dinucleotides (CDNs) produced and released by bacteria or by endogenous CDNs [7]. Most recently, the cyclic-GMP-AMP (cGAMP) synthase (cGAS) was identified as another important cytosolic DNA sensor which synthesizes cGAMP, binding and activating STING to induce type I IFN [13].

Most of the studies that focus on characterization of *L. monocytogenes* virulence traits, including activation of innate immune signaling pathways, are restricted to few reference strains such as 10403S, EGD and EGD-e, which are all from serotype 1/2a [14]. Here we performed *in vitro* assays to demonstrate the virulence potential and innate signaling pathways induced by 59 wild type *L. monocytogenes* recovered from beef, environment, and clinical sources in Brazil. The results highlight the identification of wild

type strains that induce pyroptosis and type I IFN production in macrophages through the activation of STING and cGAS pathways, demanding further genomic analysis and other experimental approaches to uncover the mechanisms conferring the most extreme detected phenotypes.

Results

Intracellular growth in iBMDM

We assessed the intracellular doubling times of 59 *L. monocytogenes* strains obtained from beef, clinical and environmental sources (**Table S1**) in iBMDM cells by CFU enumeration at 2 and 8 hours post-infection (hpi). As expected, *L. monocytogenes* Δ*hly* exhibited a major growth defect due to the inability to access the cell cytosol, as well as the non-*monocytogenes* spp. *L. welshimeri*, *L. innocua* and *L. seeligeri*, while the doubling times for *L. monocytogenes* Δ*actA* and Δ*inlA*Δ*actA* were similar to wild type 10403S (data not showed).

No significant differences were observed in doubling times when we grouped the strains by sources and serotypes (**Fig. 1A**), source only (**Fig. 1B**) or serotype only (**Fig. 1C**) ($P > 0.05$). Two strains from serotype 1/2a, 2137 (clinical, CSF of newborn) and 2140 (clinical, placenta) exhibited the most attenuated virulence phenotypes (**Fig. S1**), as they did not grew in iBMDM cells. Strain 2137 also grew significantly slower in BHI and 2140 grew slightly slower (data not showed). The results indicated that the variability in intracellular replication seen amongst the strains is likely to be determined more by strain-specific factors rather than by serotype or source.

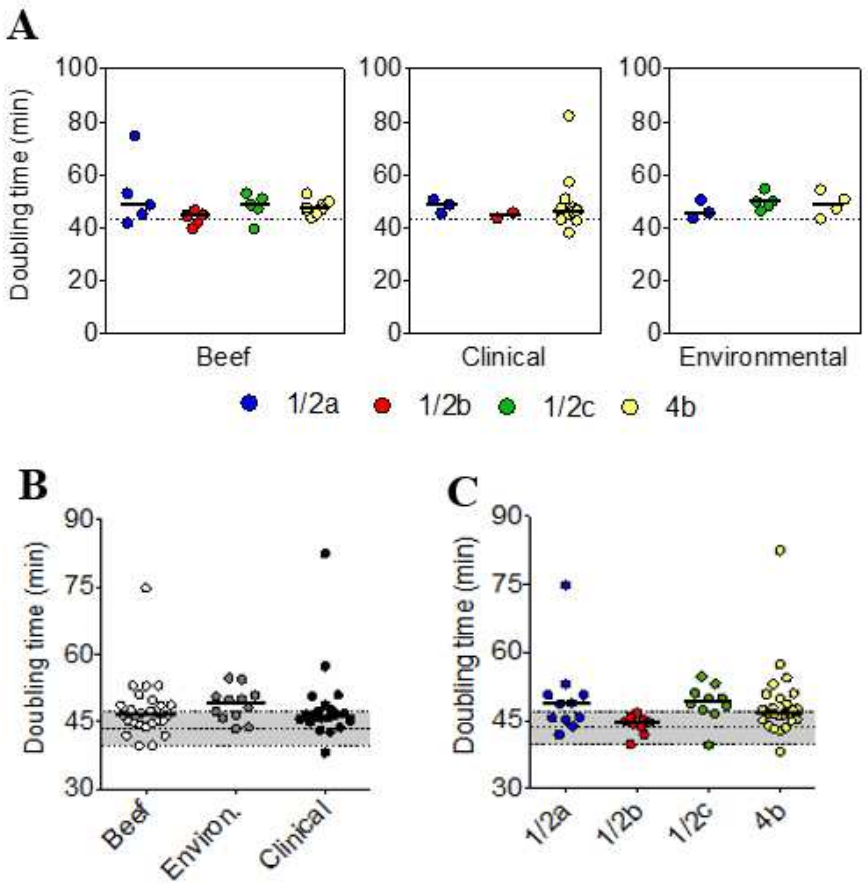

Figure 1. Intracellular doubling times of 57 *L. monocytogenes* isolates in iBMDM cells, plotted according to serotype and source (A), source only (B), or serotype only (C). The dotted line indicates the doubling time of the reference strain 10403S. Two clinical strains from serotype 1/2a (2137 and 2140) which did not grow in iBMDM were not included in the analysis. Each data point represents the mean of 2 biological replicates performed in technical duplicate. No statistical difference between sources or serotypes was observed using ordinary one-way ANOVA.

Wild type L. monocytogenes *inducing pyroptosis*

Cell death induced by the 59 *L. monocytogenes* was indirect measured by the amount of lactate dehydrogenase (LDH) released by iBMDM into supernatant 8 hours after infection, using a colorimetric reporter substrate. As expected, 10403S induced very low levels of cell death. Among the wild type strains, statistical difference ($P < 0.05$) were observed only among isolates from environmental sources, when comparing 1/2a and 4b strains ($P < 0.05$) **(Fig. 2A).** No trends emerged when we grouped all strains by source only (**Fig. 2B**) or serotype only (**Fig. 2C**). Strains 3727 (1/2a from beef), 7, 3865 (1/2b from beef), and 629, 1011, 2167, 3723 (4b from clinical) induced the highest levels of cell death in comparison with the reference strain 10403S (**Table S1**), suggesting that they trigger pyroptosis. To confirm this hypothesis, we performed infections in wild type and Casp1$^{-/-}$ iBMDM primed overnight with Pam3CSK4. Cell death induced by all strains was caspase 1 dependent (**Fig. 3**), as well as cell death induced by the positive control

10403S-LpFlaA, which express the *Legionella pneumonia* flagellin (**Fig. 3**), confirming that these isolates trigger pyroptosis of iBMDM.

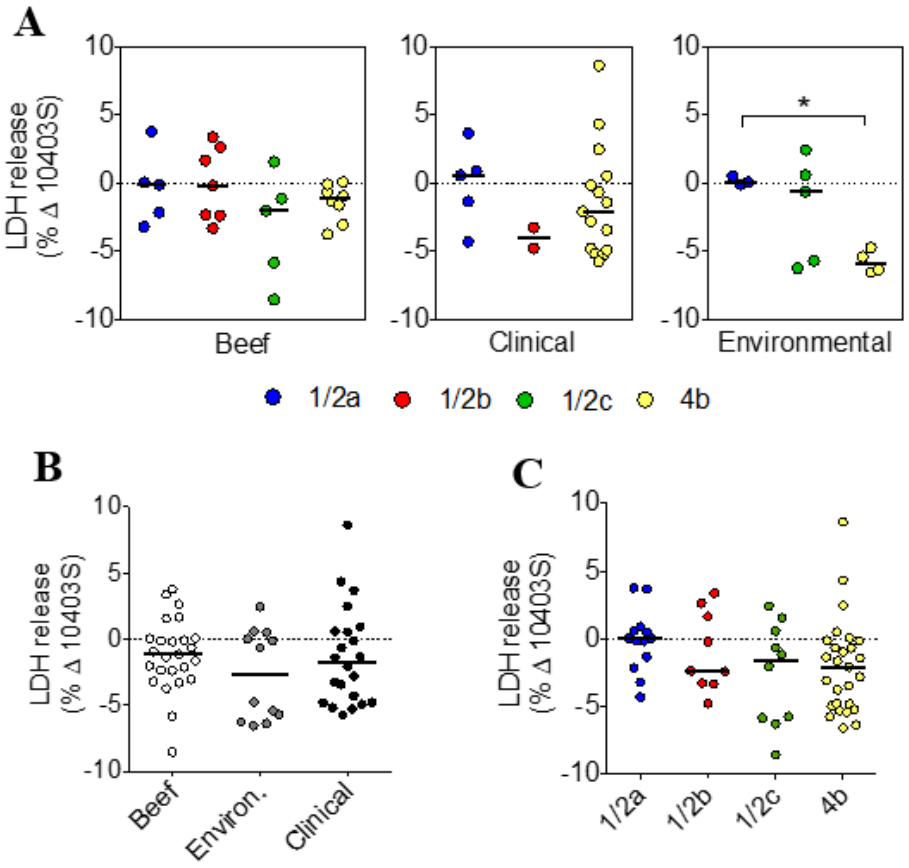

Figure 2. LDH released after infection of iBMDM cells by *L. monocytogenes* isolates. The 59 *L. monocytogenes* isolates are plotted according to serotype and source (A), source only (B), or serotype only (C). Each data point represents the mean of 4 technical replicates from 2 biological duplicate. Statistical difference ($P < 0.05$) was observed in environmental 1/2a versus 4b. Statistical significance was calculated using ordinary one-way ANOVA.

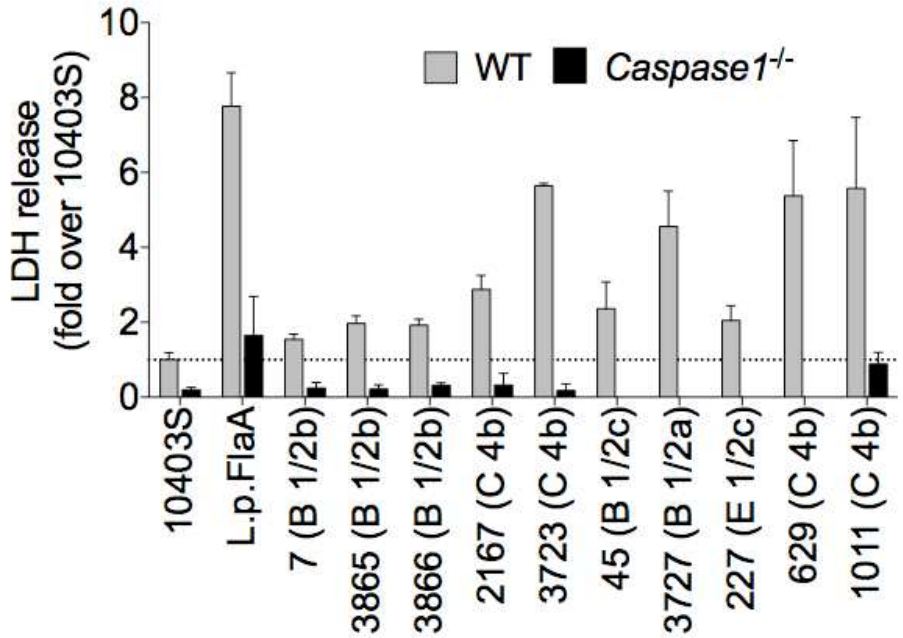

Figure 3. LDH released after infections of WT and Casp1$^{-/-}$ iBMDM primed overnight with Pam3CSK4. Cell death induced by selected strains were caspase 1 dependent, as well as cell death induced by the positive control 10403S LpFlaA, which express the *Legionella Pneumonia* flagellin, confirming that these isolates triggers pyroptosis in iBMDM. Each data point represents the mean of 4 technical replicates from 2 biological duplicates.

The role of cGAS and STING on Type I IFN expression in response to infection

Production of type I IFN can be stimulated by *L. monocytogenes* DNA and CDNs detected in the host cell cytoplasm. We did not detected significant differences ($P > 0.05$) in IFN production induced by *L. monocytogenes* when we grouped the strains by sources and serotypes (**Fig. 4A**), source only (**Fig. 4B**) or serotype only (**Fig. 4C**). However, we identified some wild type *L. monocytogenes* isolates that induced nearly the double of type I IFN production when compared to the reference strain 10403S **(Fig. S2).** Clinical isolate 629 (serotype 4b), which exhibited the atypical IVb-v1 by molecular serogouping, induced nearly the double of type I IFN in comparison with 10403S, being STING dependent and partially cGAS dependent **(Fig. 5)**. Isolates 3727 (1/2a), 7 (1/2b) and 508 (1/2c) also induced higher production of type I IFN by a STING dependent pathway, being partially cGAS dependent (3727, 508) and fully cGAS independent (7) **(Fig. 5)**. There were no significant trends comparing ISRE response and LDH release or growth, but some of the strains that gave the highest ISRE responses also have high cell death.

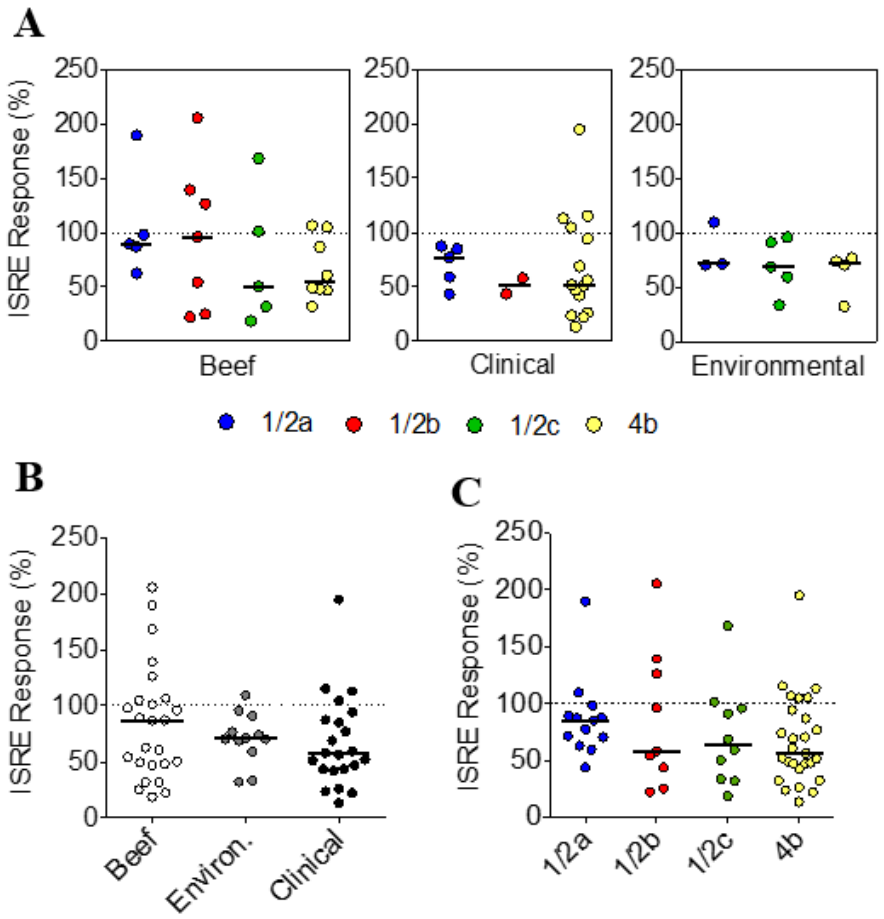

Figure 4. IFN released after infection of iBMDM cells by *L. monocytogenes* isolates. All 59 *L. monocytogenes* isolates plotted according to serotype and source (A), source only (B), or serotype only (C). Each data point represents the mean of 4 technical replicates from 2 biological duplicates. No statistical difference between sources or serotypes was observed using ordinary one-way ANOVA.

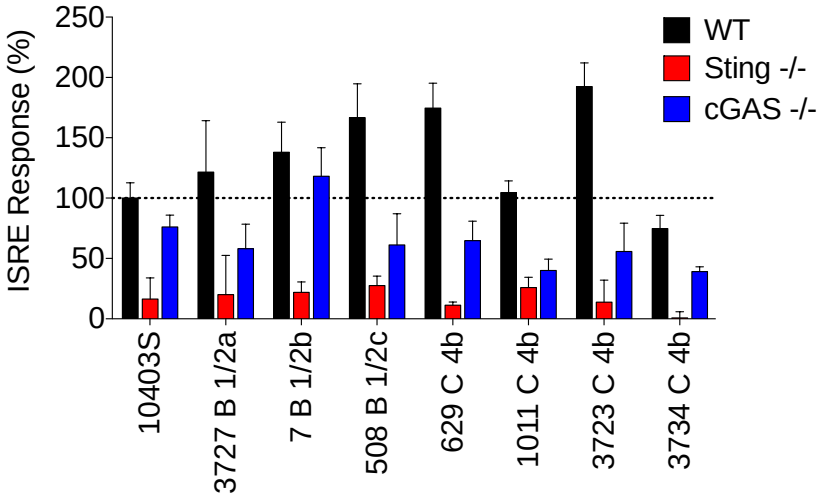

Figure 5. IFN released after infections of WT, STING$^{-/-}$ and, cGAS$^{-/-}$ primary macrophages. IFN production induced by selected strains was STING dependent, being partially cGAS dependent (629, 3727, 508, 3734), most cGAS dependent (1011 and 3723) and fully cGAS independent (10403S and 7). Each data point represents 3 technical replicates from one biological experiment done using primary macrophages, results are indicated as ISRE percentage over 10403S.

Discussion

A variety of proteins are expressed in the host cell cytosol by *L. monocytogenes* during its virulence cycle, and most of the genes required for cytosolic replication and cell-to-cell spread (*hly, ActA, plcA, plcB, mpl* and *prfA*) are placed in the LIPI-1 and are expressed under positive control of the PrfA regulon [15]. No trends regarding *L. monocytogenes* doubling times in iBMDM were revealed in this study by comparing serotypes and sources **(Fig. 1A, B, C)**. The growth inability exhibited by strains 2137 and 2140 **(Table S1)** seems to be influenced by factors affecting *L. monocytogenes* life cycle, and genomic analysis combined with other assays should be employed for further characterization.

Intracellular pathogens are adapted to survive and to replicate within host cells. Otherwise, the host cells display some strategy to limit microbial replication, for example by programmed cell death [12, 16]. Pyroptosis is one such form of cell death that can be triggered by *L. monocytogenes* in a inflammasome dependent activation pathway [8]. Multiple inflammasomes were described so far, and they are composed by a receptor, like a nod-like receptors (NLR), coupled to caspase-1 or mouse caspase-11 [10].

The ligand recognitions of *Listeria* DNA released during bacteriolysis by NLR leads to activation of human or mouse caspase-1 or mouse caspase-11, triggering pyroptosis and activation of pro-inflammatory cytokines IL-1β and IL-18 [8, 11, 12, 16], which has a critical role in innate immunity response against *L. monocytogenes* infection *in vivo* [17]. Macrophages undergoing death supposedly help to eliminate *L. monocytogenes* from the cytosolic niche; however, we found clinical strains (629, 1011, 2167, and 3723) that induced high levels of pyroptosis (**Fig. 3**).

The type I interferons (IFNs) are recognized as central regulators of immune responses against viral and bacterial pathogens. Hansen, Prabakaran, Laustsen, Jørgensen, Rahbæk, Jensen, Nielsen, Leber, Decker, Horan, Jakobsen and Paludan [5] demonstrated that *Listeria* DNA is a master trigger for IFNβ expression in human myeloid cells, being sensed by IFI16 and cGAS to activate the STING pathway. Our results suggest that IFN production by iBMDM and primary macrophages **(Fig. 5 and Fig. S2)** was induced by DNA released during *L. monocytogenes* bacteriolysis or by second messengers, such as cyclic diadenosine monophosphate (ci-di-AMP) produced by *L. monocytogenes* during infection. Second messengers (signaling molecules), secreted by strains that overexpress multidrug efflux pumps (MDRs), activate a cytosolic pathway on innate immunity, being also critical for *L. monocytogenes* growth and infection

establishment [9, 18, 19]. Activation of STING by *L. monocytogenes* c-di-AMP, and consequent production of type I interferon, downregulates cell-mediated immunity resulting in decreased protective immunity in mice [20].

The results showed that there were no trends in relation to doubling times, cell death and IFN induction in macrophages when we compare sources (food, environment, clinical) or serotypes (1/2a, 1/2b, 1/2c, 4b), indicating that the most extreme phenotypes are linked to specific genetic characteristics of each strain. Wild type *L. monocytogenes* that induced pyroptosis and high production of IFN after activation of cytosolic sensors were sequenced, demanding further genomic analysis and additional experiments to uncover new aspects regarding the innate and acquired immune response pathways triggered by *L. monocytogenes*.

Materials and Methods

Listeria monocytogenes *culture collection*

A collection composed by 59 *L. monocytogenes* strains, from serotypes 1/2a, 1/2b, 1/2c, and 4b, were screened in this study. The strains were obtained in Brazil from beef, food processing environment and clinical sources, most of them from CLIST collection, FioCruz (Table S1). The experiments were conducted using as control *L. welshimeri, L. innocua, L. seeligeri*, reference strain 10403S (wild type) and the mutants Δ*inlA/actA*, Δ*actA*, Δ*hlyA* as controls, as well as 10403S capable to express *L. pneumophila* flagellin (LpFlaA). Pure cultures were maintained at −80°C in brain heart infusion broth (BHI, Oxoid Ltd., Basingstoke, England) in the presence of 20% (v/v) glycerol (Merck, Whitehouse Station, NJ, USA), and recovered in BHI agar prior use.

Cell Culture

Immortalized bone marrow-derived macrophages (iBMDM) and Casp1$^{-/-}$ iBMDM were grown at 37ºC in controlled atmosphere of 5% (v/v) CO_2 using plastic tissue culture flask containing Dulbecco modified Eagle medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Thermo Scientific HyClone), 1% of L-glutamine and 1% sodium pyruvate (Thermo Fisher Scientific), 55 µM 2-mercaptoethanol and macrophage colony-stimulating factor (M-CSF) at final volume of 10%. Primary macrophages at concentration 0.50 x 10^6 cells/mL were transferred to 12 wells plate (1 mL per well) one day before the infection and incubated at 37ºC in controlled atmosphere of 5% (vol/vol) CO_2.

Intracellular enumeration

L. monocytogenes were grown overnight in Brain Heart Infusion (BHI) media at 30°C to stationary phase (OD_{600}, 1.1-1.3). Then, 1 mL of the overnight culture was washed 3 times and suspended in 1 mL of 1X PBS. iBMDM cells were infected as described by Sauer, Witte, Zemansky, Hanson, Lauer and Portnoy [8]. Briefly, growth curves were performed in 12-well plates with 0.75 x 10^6 cells seeded in 1 mL one day before infection. For infections, the cell monolayers were washed with 1X PBS and infected in complete media for 30 minutes at 37°C. After that, the medium was removed, cells were washed twice with 1X PBS, and medium supplemented with 50 µg/mL of gentamicin was added to kill extracellular bacteria. At 2 h and 8 h post-infection, the cells were washed twice with 1X PBS, lysed in 500 µL of cold MilliQ water, followed by decimal dilutions and plated on Lysogenic agar. The duplicates were quantified by enumerating intracellular bacteria. For each infection set, reference strain 10403S and uninfected wells were used as control, and differences normalized in slope.

Cell death assays

Before CFU enumeration at 8h, the supernatants were collected for mensuration of the amount of DHL released by the cells, following recommendations of Sauer, Witte, Zemansky, Hanson, Lauer and Portnoy [8]. Supernatants from controls (uninfected and infected by 10403S) were collected from each set of experiments, as well uninfected cells lysed with Triton 1%. All those wild strains that induced more cell death in WT iBMDM were selected for infections in WT, Casp1$^{-/-}$ iBMDM, pretreated overnight with 100 ng/mL Pam3CSK4 (Invitrogen, San Diego, CA) to confirm that they induces pyroptosis in the infected iBMDM. The absorbance values were measured by a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA), using a wavelength of 490 nm. The lysis values were calculated as percentage of cells lysed over 10403S.

Type I INF production assays

The type I INF secreted by infected macrophage in the supernatant was measured using the reporter cell line interferon stimulated response element (ISRE-L929), as previously described [21, 22]. The ISRE-L929 cells were cultured in ISRE media (DMEM, 2 mM glutamine, 1 mM pyruvate, 10% heat inactivated FBS, and penicillin-streptomycin). ISRE cells were grown using 96-well plates and incubated with 40 µL of supernatant of infected macrophages for 6 h. Then, cells were lysed using a lysis buffer, mixed with

firefly luciferin substrate, and the luminescence measured, using a luminometer (LmaxII384; MDS Analytical Technologies). Type I IFN values were calculated as percentage over 10403S. To determine the trigged IFN response pathways the wild strains that induced high levels of type I IFN were selected for infections in WT, STING$^{-/-}$, and cGAS$^{-/-}$ using primary macrophages. The IFN production was measured as indicated above.

Statistical Analyses

Statistical significance was calculated using ordinary one-way ANOVA. Data were analyzed using Prism 6 software.

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Author contributions: A.C.C., A.P.M., J.J.W., and L.A.N. designed the research; A.C.C. and A.P.M. performed the experiments, analyzed the data, and wrote the paper; J.J.W., and L.A.N revised the paper.

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Supplementary Table 1.

Strain	**Serotype**	**Source**	**LDH release**	**Doubling times (min) AVG**	**IFN production**
10403S	1/2a	Clinical	0.00	43.44	100.00
L. welshimeri	--	Environmental (bovine carcass)	0.00	D.N.G*	26.59
L. innocua 6a	6a	Environment (dirty knife)	-0.77	D.N.G*	28.09
L. innocua 6b	6b	Raw beef (refrigerated)	-1.80	D.N.G*	30.06
L. seeligeri	--	Environment (dirty table)	0.15	D.N.G*	27.23
CLIST 732	1/2a	Beef	-3.21	45.17	89.27
CLIST 3183	1/2a	Beef	-2.14	74.80	87.29
CLIST 3186	1/2a	Beef	-0.14	41.93	98.00
CLIST 3727	1/2a	Beef	3.77	48.72	189.61
CLIST 3864	1/2a	Beef	0.05	53.03	62.74
CLIST 1078	1/2a	Clinical	-4.30	50.62	59.37
CLIST 2137	1/2a	Clinical	3.67	D.N.G.*	43.65
CLIST 2140	1/2a	Clinical	-1.33	D.N.G.*	77.17
CLIST 3712	1/2a	Clinical	0.91	45.57	87.46
CLIST 3732	1/2a	Clinical	0.58	48.83	85.06
159	1/2a	Environment	-0.13	50.64	71.48
CLIST 3872	1/2a	Environment	0.49	45.81	109.53
L07	1/2a	Environment	0.03	43.80	70.46
7	1/2b	Beef	2.63	41.89	205.58
CLIST 441	1/2b	Beef	-2.35	45.06	126.40
CLIST 3063	1/2b	Beef	-2.39	39.79	25.29
CLIST 3865	1/2b	Beef	3.38	44.93	139.22
CLIST 3866	1/2b	Beef	1.65	44.35	96.30
CLIST 3869	1/2b	Beef	-0.20	44.70	54.36
CLIST 3870	1/2b	Beef	-3.32	46.67	22.34
CLIST 2083	1/2b	Clinical	-4.78	45.83	43.69
CLIST 2138	1/2b	Clinical	-3.26	43.73	58.02
45	1/2c	Beef	1.56	39.65	101.31
508	1/2c	Beef	-1.16	47.37	168.21
525	1/2c	Beef	-5.83	51.01	31.91
581	1/2c	Beef	-8.54	53.08	18.76
CLIST 3726	1/2c	Beef	-2.02	48.74	50.31
19	1/2c	Environment	0.58	50.01	68.77
133	1/2c	Environment	-0.65	48.22	95.80
139	1/2c	Environment	-5.73	54.76	59.23
227	1/2c	Environment	2.42	46.49	91.10
232	1/2c	Environment	-6.27	49.97	33.61
74	4b	Beef	-3.75	43.91	47.00
CLIST 3192	4b	Beef	-3.05	48.70	106.57
CLIST 3721	4b	Beef	-1.36	53.04	60.88
CLIST 3724	4b	Beef	-0.65	46.58	105.03
CLIST 3728	4b	Beef	-1.62	46.97	86.90
CLIST 3731	4b	Beef	0.07	47.65	48.16

Strain	Serotype	Source	LDH release	Doubling times (min) AVG	IFN production
CLIST 3863	4b	Beef	-0.10	49.96	49.38
CLIST 3868	4b	Beef	-0.95	45.34	32.15
247	4b	Clinical	-4.80	51.00	94.43
CLIST 629	4b	Clinical	-0.13	38.14	194.92
CLIST 1011	4b	Clinical	2.47	46.07	115.23
CLIST 1015	4b	Clinical	-2.79	45.00	113.12
CLIST 2167	4b	Clinical	8.63	45.14	26.17
CLIST 2168	4b	Clinical	-2.08	42.74	51.20
CLIST 2930	4b	Clinical	-5.20	82.45	56.38
CLIST 2944	4b	Clinical	0.53	46.61	69.15
CLIST 3723	4b	Clinical	4.34	47.73	104.95
CLIST 3733	4b	Clinical	-0.66	57.38	42.18
CLIST 3734	4b	Clinical	-1.41	46.06	13.40
CLIST 3735	4b	Clinical	-5.29	47.50	47.33
CLIST 3737	4b	Clinical	-5.74	46.79	22.25
CLIST 3738	4b	Clinical	-3.44	46.59	23.80
CLIST 3739	4b	Clinical	-4.92	43.19	52.02
212	4b	Environment	-5.42	50.89	73.87
233	4b	Environment	-4.77	47.33	76.71
1282	4b	Environment	-6.57	43.43	70.45
CLIST 3729	4b	Environment	-6.36	54.41	32.23

*D.N.G., did not grow

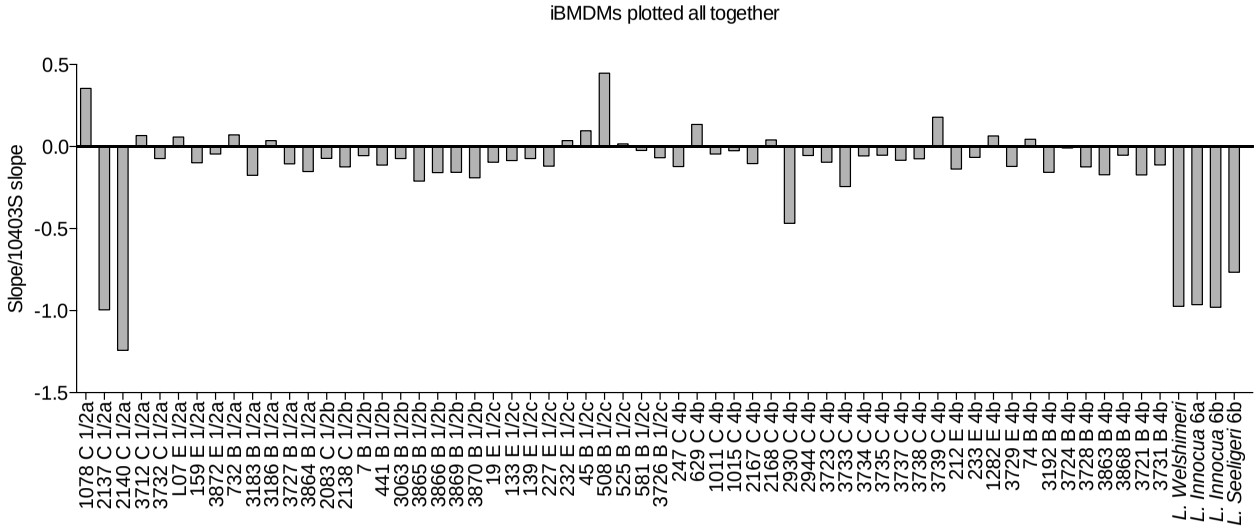

Figure S1. CFU differences normalized in slope [(isolate slope)/(10403S slope)], showing the growth diversity among 59 *L. monocytogenes* in iBMDM.

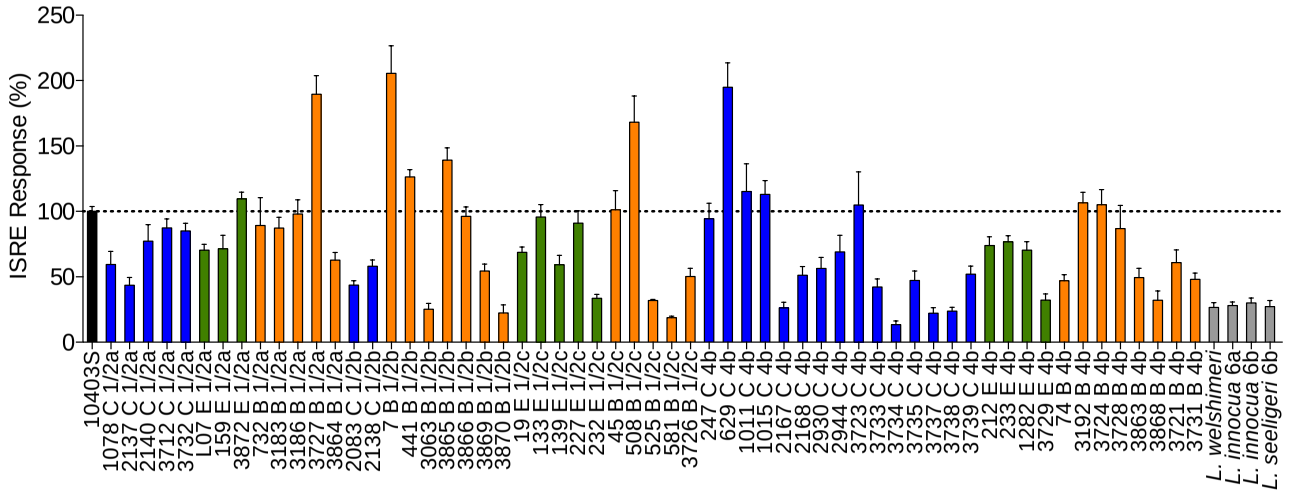

Figure S2. IFN released by WT iBMDM infected with the 59 *L. monocytogenes*, data are indicated as percentage over 10403S. Each data point represents the mean of 4 technical replicates from 2 biological duplicates.

FINAL REMARKS

The present work characterized a collection of *L. monocytogenes* isolates obtained in Brazil from clinical samples, food, and food processing environment. *L. monocytogenes* is recognized as the causative agent of listeriosis, a serious disease that affects several species of mammals. In humans, the invasive disease is more common in risk groups, such as pregnant women, newborns, the elderly, and immunocompromised individuals, causing gastroenteritis, septicemia, abortion and infections in the central nervous system, leading to mortality rates of up to 30%.

Here we showed a high frequency of resistance to clindamycin, oxacillin amongst the *L. monocytogenes* isolates from Brazil, and the presence of virulence genes (Camargo et al., 2015). Molecular serotyping produced conflicting results for seven isolates of serotype 1/2a, which were positive for *lmo1118* and exhibited profile IIc (serotypes 1/2c or 3c). In addition, fifteen isolates from serotype 4b amplified the gene *lmo0737*, and were classified as atypical IVb-v1 (Camargo et al., 2016), an emerging clone involved in several recent outbreaks of listeriosis in the USA (Chen et al., 2017).

The research also identified the diversity in relation to invasion and intracellular doubling times of *L. monocytogenes*. The 4b isolates from beef showed a greater ability to invade the Caco-2 cells when compared to the 4b from clinical origin. Two old strains (both serotype 1/2a), 2137 (CSF of the newborn) and 2140 (placenta) were not able to multiply in Caco-2 cells and macrophages, and were deficient in hemolytic activity and the enzyme phospholipase, suggesting the presence of mutations in virulence genes required for the intracellular cycle. Serotype 1/2a exhibited a defect in cell-to-cell dissemination, and serotype 1/2b presented a greater ability to disseminate in Caco-2 cells. In addition, three isolates of serotype 4b were able to spread in an area approximately twice as large as the reference strain 10403S.

Strains 3727 (1/2a), 7, 3865, 3866 (1/2b), 45, 227 (1/2c) and 629, 1011, 2167, 3723 (4b) induced higher levels of programmed cell death (by pyroptosis) compared to the strain reference 10403S after infection of macrophages. A high production of INF (an inflammatory modulator response) was induced after after infection of macrophages by strains 3727 (1/2a), 7,441, 3865 (1/2b), 508 (1/2c), and 629, 3723 (4b). This inflammatory response was activated after cytoplasmic receptors detect DNA or messenger molecules produced and released by these strains into the cytoplasm of the macrophages.

This research was the first in Brazil to carry out a deep characterization of *L. monocytogenes* isolates. The study showed that isolates from lineage I (recovered from beef) presented risks for consumers. In addition, some strains have been able to elicit a more robust immune response, these strains can be used for other studies in future, to better understand the pathways of immune response in order to discovery alternative forms of diseases treatment. Finally, the sequenced genomes of 40 will help eludidade the virulence phenotypes identified and the mechanisms associated to the virulence traits of *L. monocytogenes* isolates from Brazil.