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Research Article

Intracellular Adaptation of *Listeria monocytogenes* and *Listeria ivanovii* Isolated from Fresh Produce in Côte d'Ivoire: An *in Vitro* Infection Study Using JEG3 Human Cells

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Abstract

Listeria monocytogenes (*L. monocytogenes*) is a pathogen that frequently contaminates foods, and is the cause of listeriosis worldwide. Although *L. monocytogenes* is the primary species associated with human listeriosis, *Listeria ivanovii* (*L. ivanovii*) traditionally considered pathogenic mainly to ruminants has also been implicated in rare but severe infections in immunocompromised humans. The objectives of this study were to examine the ability of *L. monocytogenes* and *L. ivanovii* strains isolated from fresh vegetables and market garden produce in Abidjan, Côte d'Ivoire, to enter and multiply in JEG3 cells, to disseminate and to form LisCVs persistence vacuoles. After identification by 16sDNAr gene sequencing and serogrouping by PCR of *Listeria* strains isolated from fresh vegetables and market garden produce, three strains were identified as belonging to the species *L. monocytogenes* (2 strains, L208 and L238) and *L. ivanovii* (1 strain, L135). In vitro infection was carried out using JEG3 trophoblastic cells, with three reference strains (*L. monocytogenes* EGDe serotype 1/2a, *Listeria monocytogenes* CLIP80459 serotype 4b and *L. ivanovii* ATCC19119) serving as controls. The results showed that all the *Listeria* strains tested had similar characteristics in terms of their ability to penetrate, multiply and form LisCV, and higher cytotoxicity in *L. monocytogenes* species than in *L. ivanovii* in general. *L. monocytogenes* strains L208 and L238 showed similar invasion rates to *L. monocytogenes* CLIP80459.

Keywords

L. monocytogenes, *L. ivanovii*, Infection, JEG3 Cells, Côte d'Ivoire

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1. Introduction

Listeria is a bacterial genus in the *Listeria*ceae family, whose generally known pathogenic species are *L. monocytogenes* and *L. ivanovii* [1]. Although there have been no reported cases of major human listeriosis outbreaks in Africa, *Listeria* species have already been isolated from foods such as carrot, lettuce, cucumber, tomato and coaker in southwestern Nigeria [2, 3]. *Listeria monocytogenes* is a species with great genetic diversity. It has been classified into four distinct genetic lineages (I, II, III and IV) on the basis of phylogenetic studies. Each lineage is composed of certain serotypes. Lineage I comprises (1/2b, 3b, 4b, 4d, 4e, and 7), lineage II (1/2a, 1/2c, 3a, and 3c), lineage III (4b, 1/2a, 4a, and 4c), and lineage IV serotypes (4a, 4c) [4]. The phenotypic characteristics of *Listeria* during long-term infection of JEG3 trophoblastic cells have been described with several laboratory strains, EGDe and 10403S [5], and an epidemic strain (CLIP63713) [6]. The process of host cell infection by *L. monocytogenes* involves several virulence factors. The ActA protein is essential for actin polymerization and intracytoplasmic movement of *L. monocytogenes* and the PlcA and PlcB proteins are involved in lysis of the double-membrane vacuole formed during cell-to-cell propagation [7, 8]. The *hly* gene encodes an extracellular listeriolysin O (LLO) which plays a role in host cell infection by *L. monocytogenes*. The InlA protein is involved in *L. monocytogenes* invasion of intestinal epithelial cells by expressing the E-cadherin receptor. The InlB protein induces hepatocyte invasion via the c-Met receptor [8]. PrfA is the positive regulatory factor for the hly, plcA, mpl, actA and plcB genes. It regulates the expression of genes required for cell invasion (inlA and inlB) and intracellular proliferation (hpt) [8]. For strains rarely associated with clinical situations, some have a diminished ability to infect cells in culture. The processes leading to diminished virulence are not yet fully understood. However, some strains have a gene for InlA that is altered by mutation, generating premature stop codons and producing a truncated internalin that is not bound to peptidoglycan and therefore unable to invade epithelial cells [6, 9]. These results suggest that this type of mutation in inlA may explain, in part, why certain strains are more frequently isolated from food, or from the environment, than from clinical cases. Epidemiological data support this hypothesis [9-12]. In recent years, the discovery of Listeria-containing vacuoles (LisCVs) has added a new layer of complexity to our understanding of *Listeria*'s intracellular lifecycle. LisCVs are vacuolar compartments formed during long-term infection of epithelial or trophoblastic cells, in which *L. monocytogenes* can persist in a viable but non-replicating state [15]. The ability to form LisCVs is increasingly recognized as a potential persistence strategy, particularly relevant to chronic infection models and intracellular survival [15]. Understanding LisCV dynamics could also help distinguish between hypervirulent strains and those adapted to environmental persistence or food contamination [20]. Some information regarding the presence of *Listeria* in C ôte d'Ivoire has been reported, and it is important to verify the ability of *Listeria* isolates present in fresh vegetables to infect and penetrate human cells. Based on this observation, the objectives of this study were (i) to examine the ability of the different strains to enter and then multiply in JEG3 cells, (ii) the ability to disseminate and (iii) to show the ability to form LisCVs persistence vacuoles.

2. Materials and Methods

2.1. Cell Lines and Bacterial Strains

The human cell line and strains of *L. monocytogenes* and *L. ivanovii* used in this study are listed in Table 1, The serogroups of strains L208 and L238 were determined by PCR targeting the *ORF2819* gene specific to genetic lineage I and the *lmo0737* gene specific to genetic lineage II (Table 2).

Table 1. *Study cell and control strain.*

Cell lineage	Specifications	Origin
JEG3	Human epithelial cells derived from placental carcinoma	ATCC HBT-36
Bacterial strains	Specifications	Genetic lineage and CC
L. monocytogenes EGDe	Reference strain (serotype 1/2a)	Lineage II, CC9 [21]
L. monocytogenes CLIP80459	Reference strain (serotype 4b)	Lineage I, CC4 [22]
L. ivanovii ATCC19119	Reference strain (serotype 5)	

CC: clonal complex.

Table 2. Strains used for long-term infection of JEG3 cells.

Strains	Species	Genetic lineage	Serogroups	Origin	Strain accession number
L135	*L. ivanovii*	nd	nd	lettuce	PQ473711
L208	*L. monocytogenes*	I	(1/2b, 3b, 4b, 4d, 4e et 7)	lettuce	PQ473712
L238	*L. monocytogenes*	I	(1/2b, 3b, 4b, 4d, 4e et 7)	lettuce	PQ473714

nd: not determined

2.2. Infection of JEG3 Cells

Briefly, 2 to 4 days pre-infection, JEG3 cells were seeded in 24-well plates at a concentration of around 7.10^3 to 5.10^4 cells/mL, in order to achieve 90-100% confluence on the day of infection. The day before infection, the bacteria were cultured in liquid BHI medium and incubated overnight at 37 ℃ with agitation. On the day of infection, the cells were washed and then infected with 1 mL of bacterial suspension diluted 1/400000 iem. a multiplicity of infection, MOI ~ 0.01. The plates were centrifuged at 300 g for 2 min to synchronise bacterial entry into the cells. After 1 h of infection, complete medium containing gentamicin 25 µg/mL was added to the cells to eliminate extracellular bacteria. After 6 h of infection, the infected cells were lysed with cold water and the lysates were plated on BHI agar to check the state of bacterial entry into the cells. After 72 h of infection, the cells were either lysed with cold water (in the same way as at 2 h p.i.), for bacterial counts on BHI agar, or fixed with 4% paraformaldehyde (in 1X PBS) for immunofluorescence experiments. In the latter case, the cells are then permeabilised with PBS-Triton ×100 (0.4%) and labelled with antibodies against *Listeria* and LAMP1, as well as fluorescent phalloidin (to label F-actin) and Hoechst (to label DNA). The labelled cells were analysed by epifluorescence microscopy, at low magnification (20x) to quantify strain dissemination sites, and at high magnification (63x or 100x) to visualise the formation of persistence vacuoles. Each experimental condition was performed in duplicate to ensure reproducibility of the data.

2.3. Charge Calculation

The number N (germs/mL) expressed in CFU/mL was determined using the following formula:

$$N = \frac{\Sigma C}{d \cdot V(n_1 + 0{,}1 n_2)}$$

N: Number of colony-forming units (CFU) per mL of initial product N

ΣC: Sum of colonies in the plates of two successive dilutions considered

V: Volume of the inoculum

d: Factor of the first dilution considered

n1: Number of boxes considered at the first dilution

n2: Number of boxes considered at the second dilution

The Turkey test followed by two-factor analysis of variance was performed using R software version 4.4.2. The graphs were created using Excel software version 2016.

3. Results

3.1. Invasive Capacity of Strains After 6 Hours of Infection

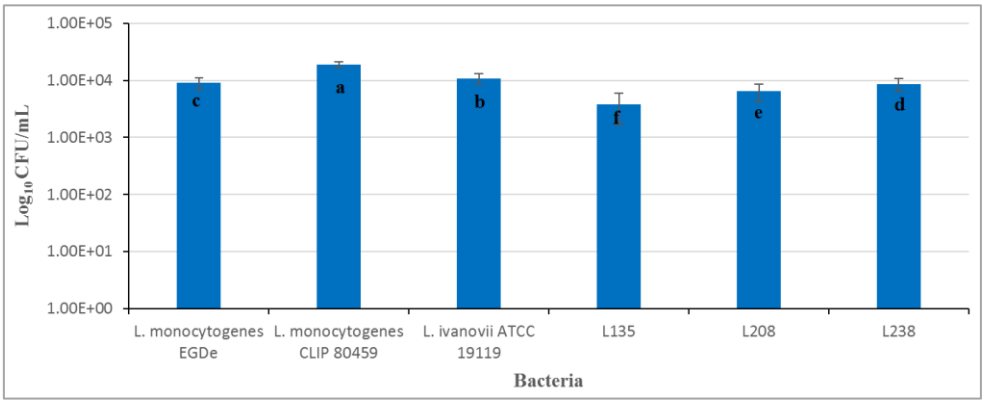


Figure 1. Inoculum (in Log_{10}CFU/mL) of the different bacterial strains tested.

The ability of the strains to penetrate JEG3 cells was studied taking into account a comparable MOI of approximately 1 bacterium per 100 cells, i.e. MOI ~ 0.01. Inocula were counted on BHI agar (Figure 1). Loads ranged from 10^3 to 10^4 CFU/mL.

After 6 hours of infection, the cells were lysed and the intracellular bacteria counted on BHI agar plates. The percentage entry of each strain was determined by dividing the number of intracellular bacteria by the number of bacteria in the inoculum. The results are shown in Figure 2. The percentages varied between 41.26 and 45.25% for *L. ivanovii* strains, and between 46.6 and 52.48% for *L. monocytogenes* strains (Figure 2).

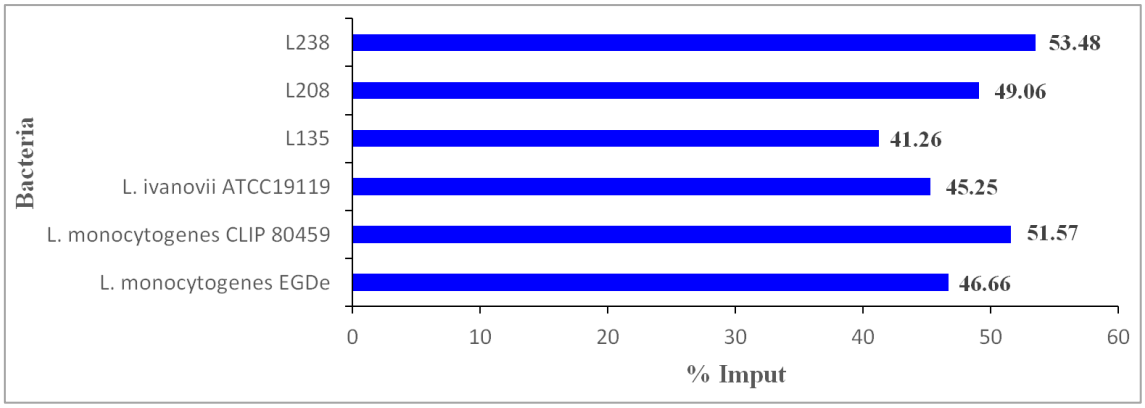


Figure 2. *Invasiveness of bacterial strains.*

3.2. Infectivity of Strains After 72 Hours of Infection

After 72 h of infection in the JEG3 cell monolayer, the number of intracellular bacteria was quantified. Infections were also performed at an MOI of 0.1. At 72 h post infection (p.i). the cells were lysed and the lysates spread on BHI agar to determine the number of intracellular bacteria. The results are shown in Figure 3.

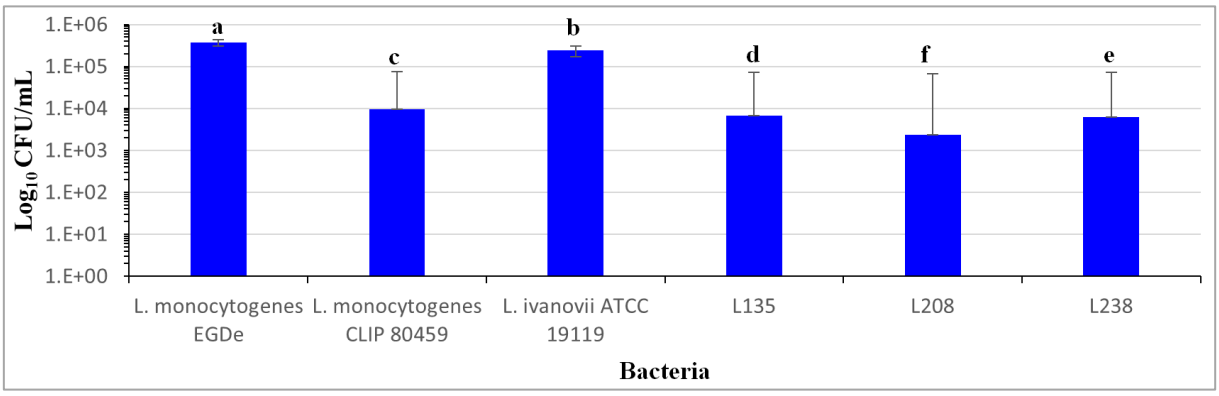


Figure 3. *Long-term infection test (3 days). Log_{10}CFU/mL at 72h p.i.*

3.3. Microscopic Study of Bacterial Dissemination

The ability of the strains of interest to propagate from cell to cell within the cell monolayer was studied. After 72 hours of infection, the infected cells were immunofluorescently labelled and photographed at low magnification, using a 20x objective. This enabled the infection foci to be observed (Figure 4). The foci of infection were larger in the *L. monocytogenes* strains than in the *L. ivanovii* strains overall. Strains L208 and L238 appear to behave like the reference *L. monocytogenes* CLIP80459 (serotype 4b) which is a strain known to be more invasive than strain EGDe.

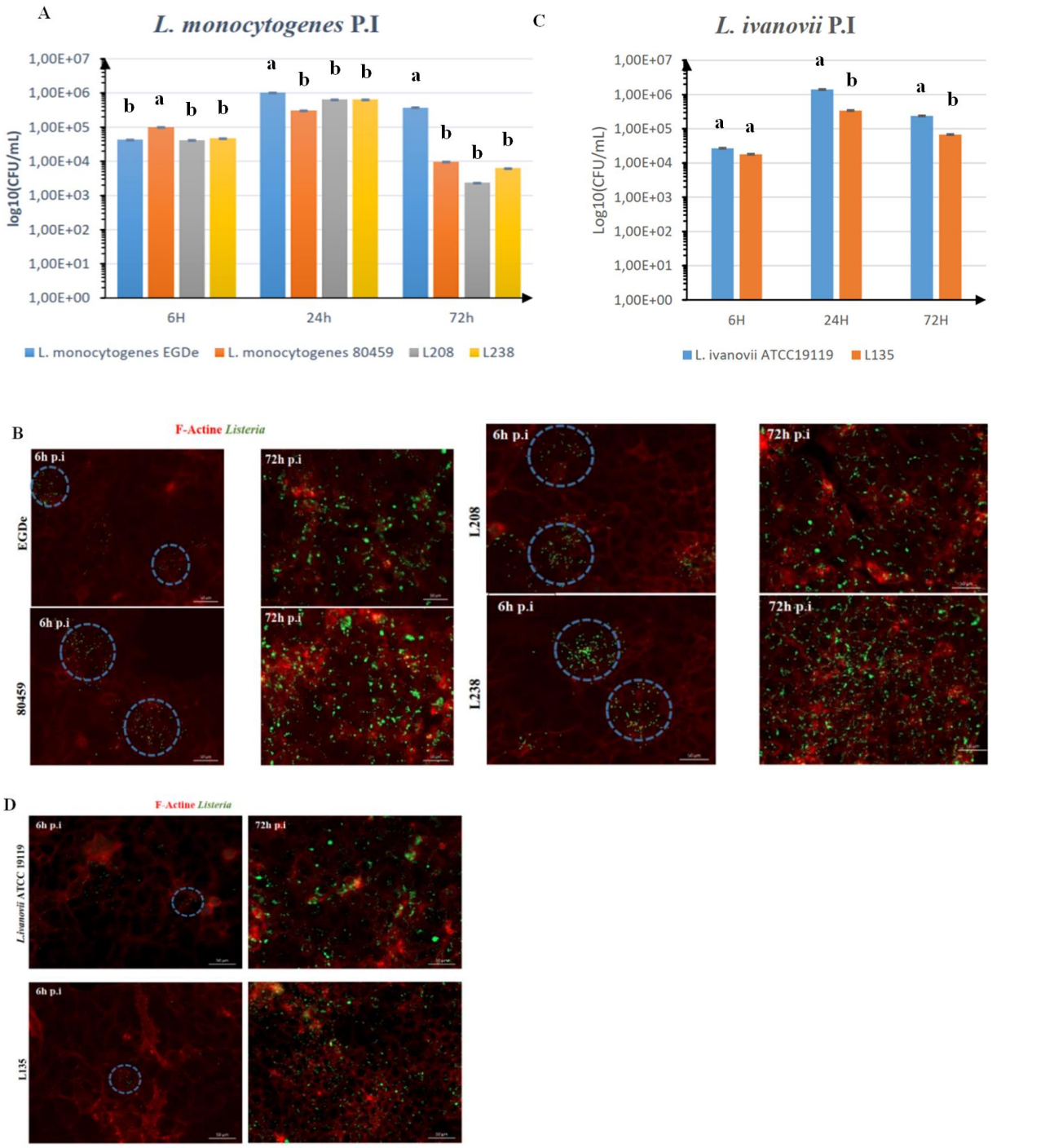


Figure 4. *Bacterial dissemination. A-D. JEG3 cells were infected with L. monocytogenes (EGDe, CLIP80459, L208 and L238) and L. ivanovii (ATCC 19119 and L135) (MOI 0, 1; without 30 min exposure to gentamicin 25 µg/mL). A: Number of bacteria (L. monocytogenes) in the extracellular medium (by CFU conts). B:* Micrographs of cells infected *with L. monocytogenes* for 6 or 72 h and visualized with the objective 20X. Images are overlays of Listeria (green) and F-actin (red) signals. Circles highlight an infection focus at 6 h p.i. Bar: 50 µm. C: *Number of bacteria (L. ivanovii) in the extracellular medium (by CFU conts). D:* Micrographs of cells infected *with L. ivanovii* for 6 or 72 h and visualized with the objective 20X. Images are overlays of Listeria (green) and F-actin (red) signals. Circles highlight an infection focus at 6 h p.i. Bar: 50 µm.

3.4. Cytotoxicity of Strains

Strains of *L. monocytogenes* serotype 4b (CLIP80459) are known to be more cytotoxic and invasive than *L. monocytogenes* serotype 1/2a (EGDe). Uninfected cells have smaller, tightly packed nuclei. We can see that cells infected with strains CLIP80459, L208 and L238 have slightly larger and more widely spaced nuclei (due to detachment) compared with EGDe (Figure 5). Strains L208 and L238 behave like the

reference strain *L. monocytogenes* CLIP80459 serotype 4b, in addition to both belonging to genetic lineage I.

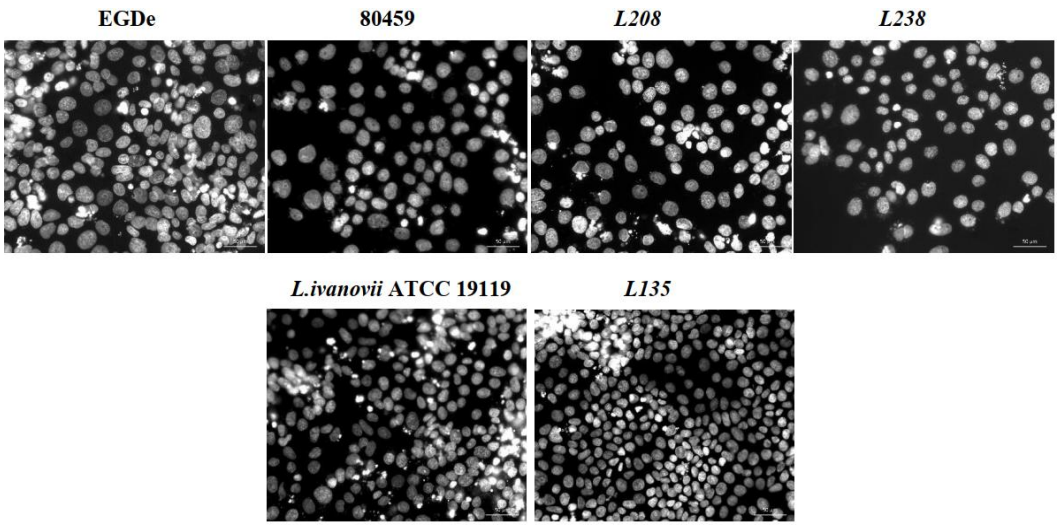
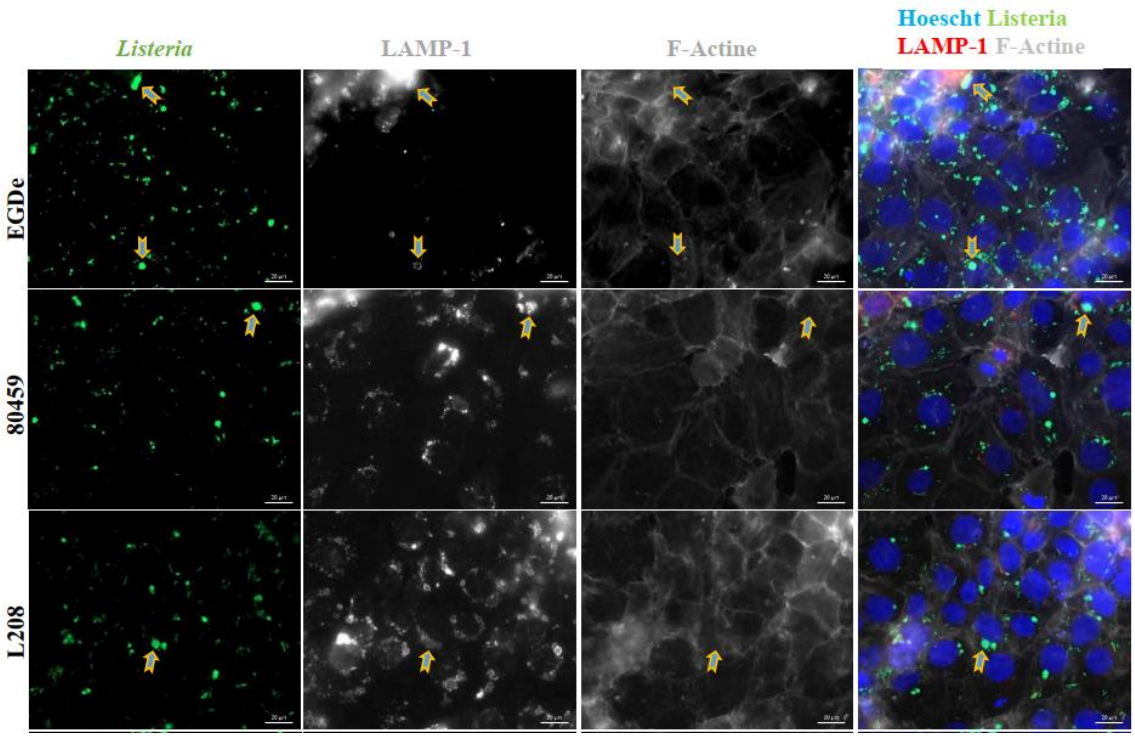


Figure 5. *JEG-3 cell nuclei in white, 72 h post-infection. DAPI staining of Listeria monocytogenes (EGDe, CLIP80459, L203 and L238) infected and L. ivanovii (ATCC19119 and L135) infected JEG3 cells at 72 h p.i. Bar: 50 µm.*

3.5. Formation of LisCVs Persistence Vacuoles

The ability of the strains to form LisCVs after 72 h of infection (Figure 6) was studied. These vacuoles were labelled with an antibody directed against the lysosomal protein LAMP-1. We observed that after 72 h of infection, the majority of bacteria no longer polymerised actin and were present in vacuoles labelled by LAMP1, regardless of the strain studied. However, qualitatively, the results suggest differences in the number of intra-vacuolar bacteria, the size of the vacuoles and the number of vacuoles per cell. For example, the vacuoles encompassing *L. ivanovii* ATCC19119 and *L. ivanovii* L135 appear to be smaller and contain fewer bacteria than those of *L. monocytogenes* EGDe, CLIP80459, L208 and L238.



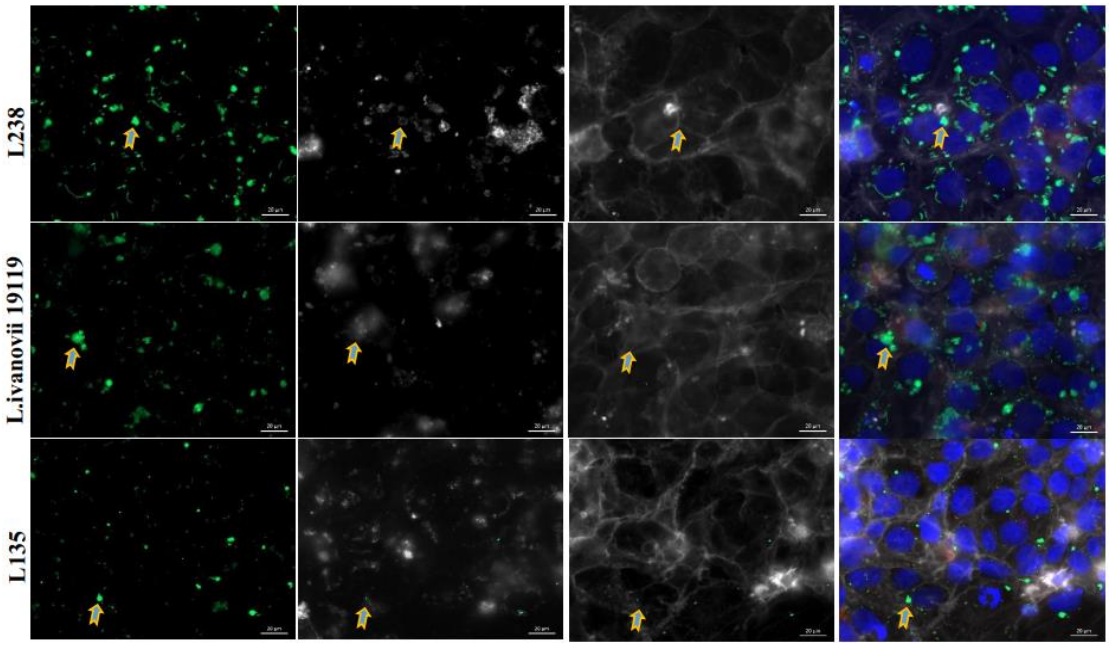


Figure 6. *Formation of LisCVs at 72 h p.i. JEG3 cells were infected for 72 h and labelled by immunofluorescence. JEG3 nuclei are labelled in blue, bacteria in green, LAMP1 in red and filamentous actin in white, The arrows indicate the LisCVs. Scale bar: 20 µm.*

4. Discussion

The objective of this work was to examine the ability of different strains of *Listeria* isolated from fresh vegetables and market garden produce in Abidjan (Côte d'Ivoire) to enter and then multiply in JEG3 trophoblastic cells, the ability to disseminate and show the capacity to form LisCVs persistence vacuoles. Human listeriosis are caused in 95% of cases by serotypes 1/2a, 1/2b or 4b [13]. In additin, serotype 4b isolates are responsible for about 50% of human outbreak cases [14]. Since 1980, the study of intracellular infection by *L. monocytogenes* has led to major advances in our understanding of the pathogenicity of this bacterium and has made it a paradigm in fundamental and cellular microbiology. *L. monocytogenes* belongs to the category of bacteria capable of multiplying in the cytosol of host cells; it is also one of the best characterised species of these so-called 'cytosolic' bacteria. *L. monocytogenes* emerges as a bacterium with the capacity to nest in vacuoles for long-term persistence. Such intravacuolar parasitism could encourage asymptomatic carriage of this pathogen in humans or animals, making it less sensitive to antibiotics and more difficult to diagnose using routine clinical biology techniques. The cell infections carried out showed that the *L. monocytogenes* L208 and *L. monocytogenes* L238 strains behaved like the reference strain *L. monocytogenes* CLIP 80459 of serotype 4b. Strains of this serotype are most often isolated in sporadic epidemics [15], *L. monocytogenes* serotype 4b are not frequently isolated from food matrix, our results are not similar to the work of Kohler and collaborator in 2015 concerning the origin of strains of this serotype [16]. Similar focis diameters were found in the work of Kortebi in 2018 in laboratory strains such as *L. monocytogenes* 10403S [17]. During intracellular invasion and dissemination of *L. monocytogenes* strains in mammalian cells, after the bacteria enter the host cell and temporarily reside in a primary vacuole, they escape into the cytoplasm, multiply, and induce the expression of the actin polymerization factor ActA. Actin polymerization promotes bacterial motility and cell-to-cell spread via the generation of membrane protrusions from the primary infected cell to neighboring cells. After these protrusions resolve into secondary double-membrane vacuoles, from which the bacteria escape, a new cycle of infection is initiated [18]. By studying the capacity of strains to internalise and disseminate cells, we were able to show that *L. monocytogenes* L208 and *L. monocytogenes* L238 strains are 'hyper-invasive' like *L. monocytogenes* CLIP80459. All the pathogenic *Listeria* strains (*L. monocytogenes* and *L. ivanovii*) tested showed the capacity to form LisCVs (vacuolar phenotype) after 72 h of infection. These results are similar to those obtained by [19]. According to the adapted model of Kortebi, after the active phase of bacterial propagation from cell to cell, bacteria do not re-express ActA after leaving the secondary vacuole, or cease to express ActA after a transient ActA-positive cytosolic phase [15]. ActA-free bacteria multiply in the cytosol and are captured by a process analogous to xenophagy, forming *Listeria*-containing vacuoles (LisCV). In these lysosome-like compartments, subpopulations of bacteria resist stress and degradation and enter a slow/non-replicative state, while those sensitive to stress eventually die. Upon stimuli that are still unidentified, bacteria can exit the vacuoles and restart a new cycle of actin polymerization after ActA re-expression. LisCVs appear in primary human hepatocytes cultured in vitro [15].

5. Conclusions

This study aimed to determine the pathogenicity of *L. monocytogenes* and *L. ivanovii* strains isolated from fresh vegetables in Côte d'Ivoire. Intracellular bacterial counts (L208 and L238) from 24 to 72 h post infection showed mean values statically similar to the reference serotype 4b strain, ranging from 9.5×10^3 to 3×10^5 CFU/mL. The *L. ivanovii* L135 strain showed statistically different mean values to the reference (*L. ivanovii* ATCC 19119) and ranged from 6.8×10^4 to 1.4×10^6 CFU/mL. The *L. monocytogenes* L208 and *L. monocytogenes* L238 strains appear to behave like *L. monocytogenes* CLIP80459, which is a strain known to be more invasive than *L. monocytogenes* EGDe.

Abbreviations

ATCC	American Type Culture Collection
ISO	International Organization for Standardization
CFU	Colony-Forming Units
CLIP	Pasteur Institute Line Collection

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Author Contributions

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Amoikon Simon Tiemélé: Writing – review & editing

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Alloue Mireille Boraud: Writing – review & editing

Pagliuso Alessandro: Writing – review & editing

Milohanic Eliane: Methodology, Supervision, Writing – review & editing

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Conflicts of Interest

The authors declare no conflicts of interest.

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