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Genetic Diversity of *Listeria monocytogenes* in Fruits and Vegetables: Environmental Persistence and Antimicrobial Resistance

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Abstract: Background/Objectives: *Listeria monocytogenes* is a foodborne pathogen that can infect humans and animals, causing non-invasive gastrointestinal listeriosis or invasive listeriosis. The objectives of this study were to determine: the genetic diversity of *L. monocytogenes*; the genes associated with the resistance to antibiotics, benzalkonium chloride (BC), and $CdCl_2$, and biofilm formation. Methods: Seventeen strains of *L. monocytogenes* isolated fresh fruits and vegetables were selected for this study. Genetic diversity and the genes associated with antibiotic resistance were determined by PCR amplification. Susceptibility to antibiotics were determined using the agar diffusion method. Resistance to BC and $CdCl_2$ was determined using the minimum inhibitory concentration method. Capacity for biofilm formation was evaluated using the crystal violet staining method. Results: This study demonstrates that the isolates of *L. monocytogenes* belong to serotypes 1/2a (29.4%) and 1/2b (70.5%). Potential hypervirulent strains containing 70.5% *Listeria* pathogenicity island 1 (LIPI-1-2), 17.6% (LIPI-1-3), and 11.7% (LIPI-1-4) were also identified. Antibiotic susceptibility tests show that *L. monocytogenes* exhibit six different multiresistant patterns with a MAR index of ≥ 0.46 (70.5%); besides, the gen *Ide, tetM,* and *msrA* were detected in 52.9%, 29.4%, and 17.6%, respectively, associated with efflux pump Lde, tetracycline and ciprofloxacin resistance. Phenotypic test shows that 58.8% cadmium-resistant *L. monocytogenes* have a co-resistance to BC of 23.5%. Subsequently, all strains of *L. monocytogenes* exhibited moderate biofilm. Conclusions: This study contributes to the persistence and genetic diversity of strains *L. monocytogenes* isolated fresh fruits and vegetables; in addition, resistance to $CdCl_2$ and correlation with co-resistance to BC disinfectant used in the food industry.

Keywords: *Listeria monocytogenes*; virulence factors; antimicrobial resistance; benzalkonium chloride; biofilms

1. Introduction

Listeria monocytogenes is a facultative intracellular pathogen widely distributed in nature and causes non-invasive gastrointestinal listeriosis or invasive listeriosis. Clinical manifestations of invasive listeriosis are septicemia, encephalitis, endocarditis, meningitis, abortions, and fetal death, or non-invasive gastrointestinal listeriosis may be asyntomatic or have flu-like symptoms or a febrile gastroenteritis syndrome [1,2]. Invasive listeriosis affects high-risk groups, including populations such as adults (> 65 years), pregmant women newborns, immunocompromised people and patients with cancer or diabetes [3,4]. The Centers for Disease Control and Prevention (CDC) estimates that there are approximately 1600 cases of listeriosis and 260 deaths annually [3]. In the European Union

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(EU), invasive listeriosis in humans is (2500 cases annually), but it is the most serious cause of foodborne disease, with a high rate of hospitalization and death, besides, the zoonosis caused by this pathogen in 2022 increased by 15.9% compared to 2021 with, 2738 cases [4]. *L. monocytogenes* can be spread through the agricultural environments, such as soil and irrigation water these sources may be contaminate fresh produce including fruits and vegetables at various stages of production and processing [5–7], creating a public health problem with economic losses for the food industry.

In fact, the Interagency Food Safety Analytics Collaboration (IFSAC) reports that 76% of foodborne *L. monocytogene*s illnesses (2016-2020) in the United States were associated with three categories in particular with dairy products (37.1%), fruits (24.8%), and vegetable row crops (14.1%) [8]. Recalls, Market Withdrawals, & Safety Alerts in the United States reports recalls in 2023 of fruits and vegetables due to the presence of this pathogen in organic green kiwifruit, organic frozen pineapple, and frozen fruit blend containing organic frozen pineapple, kale, spinach, collard green products, mung bean sprouts [9]. Furthermore, Food Standards Australia-New Zealand (FSANZ) in the period from 2019 to 2023 reports 83 food recalls associated with contamination by foodborne pathogens where *L. monocytogenes* represents 36% (30 recalls) of these recalls followed by *Salmonella* spp. (33%; 27 recalls) and *Escherichia coli* (22%; 18 recalls) the recalls include several food categories such as fruits and vegetables, dairy products, meats and processed meats, etc [10].

Additionally, the prevalence of resistance of *L. monocytogenes* isolates in food and environment has been associated with the use of antibiotics in medicine, veterinary medicine, and agricultural production systems, with some practices such as soils treated with manure, growth promoters, and misuse of therapeutic treatments for veterinary purposes [11–13]. The increase in multi-resistance pathogens is a significant public health problem; besides, the situation becomes increasingly severe as these pathogens spread globally, and acquire new resistance mechanisms, and therefore, there are no alternative therapies for their control [14,15]. In fact, the World Health Organization estimates that bacterial resistance will cause 10 million deaths by 2025 [16]. Moreover, the persistence de *L. monocytogenes* is linked (*i*) to resistance to antimicrobials or sanitizing treatments; (*ii*) the ability of cells to form biofilm on equipment or in the environmental surroundings; (*iii*) the presence of strains to survive in various food preservation conditions or environmental stresses; or (*iv*) the inability to remove cells from niches onto the food environment [17–20]. Therefore, the objectives of the present study were to determine: *i*) the genetic diversity of *L. monocytogenes* in fresh fruits and vegetables; *ii*) the genes associated with resistance antibiotics and multidrug-resistant strains of *L. monocytogenes*; and *iii*) the resistance to benzalkonium chloride (BC), cadmium chloride ($CdCl_2$) and formation of biofilm.

2. Results

2.1. Genomic of L. monocytogenes Sublineages and Virulence Genes

The isolates of *L. monocytogenes* belong to phylogenetic group I.1 (29.4%; serotype 1/2a) and II.2 (70.5%; serotype 1/2b). Additionally, pathogenicity islands were detected in *L. monocytogenes*, including LIPI-1 and LIPI-2 (100%), LIPI-3 (29.4%) and LIPI-4 (11.7%). The *prfA* and *actA* genes were detected in 100% of the isolates. Among the isolates, the following pathogenicity islands were found in significant prevalence: 70.5% (LIPI-1+ LIPI-2), 17.6% (LIPI-1+ LIPI-2+ LIPI-3), and 11.7% (LIPI-1+ LIPI-2+ LIPI-3+LIPI-4) (Table 1).

2.2. Antimicrobial Resistance Gene Profiling

Sixteen isolates were screened for the ciprofloxacin resistance gene *Ide* (52.9%). Of these, 41.1% showed phenotypic intermediate resistance and 11.7% resistance against ciprofloxacin. The tetracycline resistance gene *tetM* was detected in 29.4%, whereas five isolates showed phenotypic resistance. Macrolide resistance gen *msrA* was detected in 17.6%. However, the macrolide resistance gene *ermA* and the chloramphenicol resistance gene *cat* were not detected (Table 1).

Table 1. Genetic analysis and antibiotic resistance of *L. monocytogenes* isolates.

Strain no.	Source	Genetic determinants of virulence	Phylogenetic groups	Serotype	$CdCl_2$	CPF	Antimicrobial resistance genes	Antibiotic Resistance Pattern	MAR index
Lm-11	Vegetable	LIPI-1 + LIPI-2	II.2	1/2b	R	S	---	PE-CF-AM-CFX-DC	0.38
Lm-14	Vegetable	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC	0.38
Lm-13	Vegetable	LIPI-1+ LIPI-2	II.2	1/2b	R	S	*msrA + tetM*	PE-CF-AM-CFX-DC-TE	0.46
Lm-17	Vegetable	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC	0.38
Lm-18	Vegetable	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-42	Fruit	LIPI-1+ LIPI-2	II.2	1/2b	S	S	*tetM*	PE-CF-AM-CFX-DC-CLM-TE	0.53
Lm-43	Fruit	LIPI-1+ LIPI-2	II.2	1/2b	S	S	---	PE-CF-AM-CFX-DC-TE	0.46
Lm-68	Fruit	LIPI-1 + LIPI-2	II.2	1/2b	S	I	*Ide + msrA*	PE-CF-AM-CFX-DC	0.38
Lm-136	Fruit	LIPI-1 + LIPI-2	II.2	1/2b	S	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-133	Fruit	LIPI-1 + LIPI-2	II.2	1/2b	S	S	---	PE-CF-AM-CFX-DC-CLM	0.46
Lm-147	Fruit	LIPI-1 + LIPI-2	II.2	1/2b	S	R	*Ide + tetM*	PE-CF-AM-CFX-DC-CPF-CLM-TE	0.61
Lm-138	Fruit	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-19	Vegetable	LIPI-1+ LIPI-2 + LIPI-3	I.1	1/2a	S	S	*tetM*	PE-CF-AM-CFX-DC-TE	0.46
Lm-24	Vegetable	LIPI-1+ LIPI-2 + LIPI-3	I.1	1/2a	R	S	---	PE-CF-AM-CFX-DC-CLM	0.46
Lm-15	Vegetable	LIPI-1+LIPI-2 + LIPI-3	I.1	1/2a	R	S	*msrA*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-27	Vegetable	LIPI-1 + LIPI-2 + LIPI-3+ LIPI-4	I.1	1/2a	R	R	*Ide + tetM*	PE-CF-AM-CFX-DC-CPF-CLM-TE	0.61
Lm-41	Vegetable	LIPI-1 + LIPI-2+ LIPI-3+ LIPI-4	I.1	1/2a	R	I	*Ide*	AM-CFX-DC	0.23

LIPI-1: Isolates harboring virulence genes (*prfA, hly, plcA, plcB, mpl, actA)*; LIPI-2: Isolates harboring virulence genes (*inlA, inlB, inlC, inlJ*); LIPI-3: Isolates harboring virulence genes (*llsA, llsG, llsH, llsX, llsB, llsY, llsD, and llsP*); LIPI-4: Isolates harboring virulence genes (*licC, licB, licA*, and *glva*); I, Intermediate resistance to ciprofloxacin; R, resistance to ciprofloxacin or $CdCl_2$; S, Susceptible to ciprofloxacin or $CdCl_2$; CPF, ciprofloxacin; AM, ampicillin; CLM, clindamycin; CF, cephalothin; CFX, cefotaxime; CL, chloramphenicol; GE, gentamicin; E, erythromycin; TE, tetracycline; VA, vancomycin; SXT, trimethoprim-sulfamethoxazole; PE, penicillin; DC, dicloxacillin.

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2.3. Antimicrobials, Sanitizing, Cadmium, and Biofilm

L. monocytogenes was found to be susceptible to ciprofloxacin, tetracycline, vancomycin, chloramphenicol, gentamicin, erythromycin, and trimethoprim-sulfamethoxazole (47-100%) according to Table 2. However, the isolates were resistant to 1st and 3rd generation β-lactam (penicillin, ampicillin, dicloxacillin, cephalothin and cefotaxime) (94.1-100%), clindamycin (52.9%), tetracycline (29.4%), and ciprofloxacin (11.7%). Among the tested *L. monocytogenes* isolates, six different multiresistant patterns were observed, with the most frequently being PE-CF-AM-CFX-DC (23.5%) and PE-CF-AM-CFX-DC-CLM (35.2%). The MAR index for *L. monocytogenes* isolates ranged from 0.23 to 0.61, with 70.5% of them presenting a MAR index of ≥ 0.46 (Table 1). Additionally, the clindamycin and ciprofloxacin showed intermediate resistance of 41.1% (Table 2). In addition, MIC of 76.4% of isolates laid between 0.7 and 3.1 μg/mL BC, while the criteria for resistance of BC is CMI ≥ 6 μg/mL, which is at least twice that of the MIC for the predominant number of *L. monocytogenes* strains (MIC = 3.1 μg/mL) (Table 3). MIC of 41.1% of isolates were to < 70 μg/mL $CdCl_2$ and 58.8% cadmiun-resistance *L. monocytogenes* (MIC ≥ 70 μg/mL). Finally, all *L. monocytogenes* were classified as carrying a moderate biofilm (Table 3).

Table 2. Antimicrobial susceptibility test of *L. monocytogenes*.

Antimicrobial Class According to the WHO		Antibiotic[1]	No. (%) of *L. monocytogenes*		
			Resistant	Susceptible	Intermediate
Highly important	Phenicols	CL		100	
	Cephalosporines (1st generation)	CF	94.1		5.8
	Licosamide	CLM	52.9	5.8	41.1
	Sulphonamide	STX		100	
	Cyclic peptides	TE	29.4	70.5	
Critically important	Macrolide	E		94.1	5.8
	Aminoglycoside	GE		94.1	5.8
	Fluoroquinolone	CPF	11.7	47	41.1
	Cephalosporines (3rd generation)	CFX	100		
	Glycopeptide	VA		100	
	β-Lactam	DC	100		
		AM	100		
		PE	94.1	5.8	

[1] AM, ampicillin; CLM, clindamycin; CF, cephalothin; CFX, cefotaxime; CPF, ciprofloxacin; CL, chloramphenicol; GE, gentamicin; E, erythromycin; TE, tetracycline; VA, vancomycin; SXT, trimethoprim-sulfamethoxazole; PE, penicillin; DC, dicloxacillin.

Table 3. Minimum inhibitory concentration values of BC and $CdCl_2$ in *L. monocytogenes* strains in relation with biofilm formation.

Strain no.	Phylogenetic groups	Serotype	MIC (μg/mL)	BC	$CdCl_2$	Biofilm formation (Microtiter plate assays)
Lm-11	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-14	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-13	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-17	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-18	II.2	1/2b	3.1	□	■	Moderate biofilm
Lm-27	I.1	1/2a	3.1	□	■	Moderate biofilm
Lm-42	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-43	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-68	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-133	II.2	1/2b	3.1	□	□	Moderate biofilm

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Lm-136	II.2	1/2b	3.1	☐	☐	Moderate biofilm
Lm-138	II.2	1/2b	1.5	☐	■	Moderate biofilm
Lm-41	I.1	1/2a	0.7	☐	■	Moderate biofilm
Lm-147	II.2	1/2b	0.7	☐	☐	Moderate biofilm
Lm-19	I.1	1/2a	0.7	☐	☐	Moderate biofilm
Lm-24	I.1	1/2a	1.5	☐	■	Moderate biofilm
Lm-15	I.1	1/2a	1.5	☐	■	Moderate biofilm

Black squares indicate the resistance to BC (CMI ≥ 6 μg/mL,) or $CdCl_2$ (CMI ≥ 70 μg/mL).

3. Discussion

Listeria monocytogenes is a foodborne pathogen that causes invasive or non-invasive listeriosis in humans; the severity of the pathogenesis is associated with several factors, such as risk group and hazard characterization. In the present study, strains *L. monocytogenes* isolated from fresh fruits and vegetables belonged to serotypes 1/2a (29.4%) and 1/2b (70.5%). Several authors [5,7,21,22] have reported similar prevalence percentages for serotypes 1/2a – 3a (33-65%) and 1/2b-3b-7 (50-79.6%); but Maćkiw *et al.* [22] and Chen *et al.* [23] identified less prevalence for serotypes 1/2a-3a (10.8%) and 1/2b (2%) of isolated *L. monocytogenes* from ready-to-eat (RTE) foods, fruits, fresh and frozen vegetable. Likewise, Kayode & Okoh [7] does not detect serotype 1/2a but 1/2b (79.61%) and 4b (8.7%) of *L. monocytogenes* in fruits and vegetables samples. Indeed, serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for 95% of human listeriosis cases and have been frequently isolated from food products and patients [5,24,25]. This suggests that the diversity of *L. monocytogenes* serotype prevalence may be related to the geographic region of the country, monitoring procedures, and methodologies for detecting of *L. monocytogenes* in various food categories (fruits, vegetable row crops, dairy, pork, chicken, beef, etc.), specific characteristic of the food (fresh or frozen) or sources of contamination that interact during food production and distribution.

This investigation detected the presence of genes harboring *L. monocytogenes* pathogenicity islands (LIPIs) to assess the potential risk that *L. monocytogenes* may pose to public health. All strains of *L. monocytogenes* isolated from fresh fruits and vegetables were found to harbor the genetic elements of *Listeria* Pathogenicity Island 1 (LIPI-1; *prfA, hly, plcA, plcB, mpl,* and *actA*), which encode virulence factors that promote the growth and spread of *L. monocytogene*s. Once inside the host cell, the phagocytic vacuole is lysed by listeriolysin O (LLO) encoded by the *hly* gene, which is a pore-forming toxin that mediates the lysis of bacterial cells in the host cytoplasm and enhances its cytolytic action through phosphatidylinositol-PLC and phosphatidylcholine-PLC that mediate pathogen escape from single and double membrane-bound vacuoles. ActA plays a role in facilitating the motility of the bacterial cell to the host cell's cytoplasm, and the actin cytoskeleton is hijacked to favor cell-to-cell spread [26–28]. The *prfA* gen was identified in this study, which encodes the PrfA regulatory protein that controls the expression of the pathogenicity determinants of *L. monocytogenes* [29]. Our results are similar to those reported by several researchers [5,7,30,31]; have shown that *prfA* (100%), *mpl* (92-100%), *plcA* (92-100%), *plcB* (100%), *hly* (100%), and *actA* (84-100%) were detected in *L. monocytogenes* isolated from fruits and fresh and frozen vegetable, and agricultural environments such as irrigation water and agricultural soil. The genes *inlA*, *inlB*, *inlC*, and *inlJ* harbor the *Listeria* Pathogenicity Island 2 (LIPI-2) were detected in all the *L. monocytogenes* strains in this study. This is in agreement with several investigations where the genes *inlA* (74.1-100%), *inlB* (81.5-100%), *inlC* (70.6-100%), and, *inlJ* (66.7-100%) were detected, which encode a set of internalins that play a role in the adhesion, invasion de *L. monocytogenes* cells to the host cell and dissemination of *L. monocytogenes* [5,7,22,29–31]. InlA adheres to and invades intestinal epithelial cells that express the E-cadherina receptor, thereby facilitating the crossing of the intestinal; besides, the InlC and InlJ involved in post-intestinal dissemination of *L. monocytogenes* infection [6,22,32].

Other islands detected in this study were *Listeria* Pathogenicity Island-3 (LIPI-3) in 29.4% and *Listeria* Pathogenicity Island-4 (LIPI-4) in 11.7%. These genes harboring LIPI-3 encode Listeriolysin S (LLS), a bacteriocin with hemolytic and cytotoxic factors that contributes to polymorphonuclear neutrophils survival or alteration of the gut microbiota [22,30,33], and LIPI-4 is involved in the

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infection of the host's neuronal and placental tissues; besides, it confers hyper-virulence strains [34,35].

Additionally, all *L. monocytogenes* isolates exhibited resistance to penicillin, ampicillin, dicloxacillin, cephalothin, cefotaxime, clindamycin, tetracycline and ciprofloxacin being the most frequently encountered. Several studies have reported varying prevalences of antimicrobial resistance to penicillin (2.5-100%), ampicillin (50-100%), gentamicin (20-40%), STX (30%), erythromycin (23.5-100%), tetracycline (90-100%), chloramphenicol (20-70%), cefotaxime (80-100%), clindamycin (57.5-100%), cephalothin (50-100%), and ciplofloxacin (35.2-40%) in *Listeria* spp. and *L. monocytogenes* isolates from different categories of food and food processing environments [31,36–38]. Phenotypic intermediate resistance to clindamycin (41%), ciprofloxacin (41%), erythromycin (5.8%) and gentamicin (5.8%) *L. monocytogenes* isolates in this study is in agreement with other investigators [37–39] where reported prevalence of intermediate resistance to clindamycin (30%), intermediate to ciprofloxacin (5-64.7%), intermediate gentamicin (2.5%), intermediate tetracyclin (2-5.8%) in *L. monocytogenes.* Over-prescription of antibiotics in clinical practice, use of antibiotics in animal production, inadequate veterinary treatment to prevent animal disease, and migration and accumulation of residues of veterinary antibiotics in agricultural soils and irrigation water may contribute to the prevalence of resistance and heterogeneity of antimicrobial resistance patterns observed in *L. monocytogenes* isolates [11,13,38,40].

This study demonstrates that *L. monocytogenes* exhibit six different multiresistant patterns with a MAR index of ≥ 0.46 (70.5%), indicating a higher risk source exposed to antibiotics. A MAR index of ≥ 0.2 suggests intensive use of antibiotics in the region and a high risk of promoting antibiotic resistance [41,42]. Iwu & Okoh [31] and Maurice Bilung *et al.* [36] reported similar MAR index (0.31-0.85) for multidrug-resistant *Listeria* spp. and MAR index (0.2-1) for *L. monocytogenes* isolates from irrigation water and agricultural soil, suggesting a higher risk source that is constantly exposed to antibiotics, which are used to prevent or treat animal disease and promote animal growth. Agricultural activities such as the use of fertilizers containing antibiotic residues increase resistance or the presence of antibiotic-resistant strains in the soil or the use of water for plant irrigation [12,43].

Furthermore, *L. monocytogenes* has been found to develop resistance to several antibiotics, tetracycline, ciprofloxacin, erythromycin, clindamycin, penicillin and ampicillin, through the acquisition of genetic elements such as conjugative transposons, and self-transferable and mobilizable plasmids [13,28,44]. Therefore, the detection of *Ide*, *tetM*, and *msrA* in the *L. monocytogenes* isolates in our study may be related to resistance mechanisms such as efflux pump Lde and the transposon Tn916 harboring the *tetM* confers to resistance to ciprofloxacin and tetracycline [44,45]. In accordance with the detection of *Ide, tetM*, and *msrA* in *Listeria* spp. and *L. monocytogenes* isolates from slaughtering and processing environments, food and clinical [7,15,39,46]. Although we did not detected the presence of *cat* and *erm*A genes among our isolates, they have already been detected in *L. monocytogenes*; *cat* (100%) and *ermA* (16.9%) *Listeria* spp. in food and processing environments [15,46]. In fact, these mechanisms of resistance affect the treatment of human listeriosis, drugs such as (*i*) first-line ampicillin or penicillin G in combination with an aminoclycosid (gentamicin); (*ii*) second-line trimethoprim in combination with sulfonamide, such as sulfamethoxazole-co-trimoxazole, as well as erythromycin, tetracycline, and vancomycin [28,45].

This investigation suggests that the high prevalence of resistance and intermediate resistance in the isolates of *L. monocytogenes* may be due to an inadequate use of antimicrobial agents in veterinary medicine, the extensive use animal foodstuff, agricultural production systems or to the intrinsic resistance of *L. monocytogenes* against cephalosporins and fluoroquinolones, which is associated with the lack or low affinity of the enzyme that catalyzes the final step of cell wall synthesis [28,45]. However, the prevalence of antibiotic resistance reported in different countries is influenced by the health policies related to comprehensive antimicrobial management and the determination of antimicrobial breakpoints specific to veterinary medicine in the methods for antimicrobial susceptibility testing of bacterial pathogens of animal origin and zoonotic bacteria that can affect humans [39,47].

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Moreover, *L. monocytogenes* has demonstrated resistance to non-essential toxic metals, including arsenic and cadmium [48]. In this study, 58.8% of the *L. monocytogenes* isolates were resistant to $CdCl_2$ (MIC ≥ 70 μg/mL); this was a particularly common occurrence among isolates of serotype 1/2a and 1/2b. Similar findings have been reported by other researchers [30,49–51] regarding the prevalence of cadmium resistance (63-90%) in serotypes 1/2a and 1/2b of *L. monocytogenes* isolated from food and environment. The presence of heavy metal residues in the environment is related the industrial sector (anthropogenic sources) such as agricultural practices such as using of phosphate fertilizers, which represent a significant source of cadmium input to agricultural soil, water and food onto chain food [52]; this way increases the survival potential of *L. monocytogenes* and the acquisition of mobile genetic elements of heavy metal resistance-determinants in diverse environmental niches. Likewise, Zhang *et al.* [51] argue that cadmium exerts long-term selective pressure, allowing *L. monocytogenes* to produce tolerance.

On the other hand, QACs are used in the food industry within disinfection processes to control, reduce, and inactivate foodborne pathogens [53–56]; however, the prevalence of resistance to QACs, in particular to BC has been detected in *L. monocytogenes* isolated from food and processing plant environments, and has association to cadmium [57–60]. Our study shows that four *L. monocytogenes* isolates (23.5%) were resistant to BC, and ten isolates (58.8%) were resistant to cadmium with co-resistance to BC and cadmium (23.5%), respectively. Ratani *et al.* [61] showed 14% resistant to BC and 57% to Cd and Xu *et al.* [50] detected 16.7 % resistant to BC and Cd in *L. monocytogenes*. However, the cadmiun-resistant *L. monocytogenes* and BC were not always correlated [49]. Based on the cadmium or BC resistance result, this could be due to the genetic diversity of *L. monocytogenes* strains associated with genetic determinants cadmium resistance such as *cadA*1 (plasmid-transposon Tn5422), *cadA*2 (plasmid pLM80), *cadA3* (at chromosome level of *L. monocytogenes*) and *cadC* [48,62]; BC resistance genes qac*A/B*, *qacC/D*, *qacE*, *qacE1Δ-sul*, *qacF*, *qacG*, *bcrABC*, transposon Tn6188 (containing the *qacH* gene), or *mdrL* (chromosome and plasmid-borne encodes an efflux pump) [50,60,63,64]. In addition to the various breakpoints specific to determine resistance to disinfectants (MIC = 4 - 32 μg/mL), it may interfere with the prevalence of phenomena of resistance BC in *L. monocytogenes* as they are established according to the number of *L. monocytogenes* isolates, origin of strains, medium for susceptibility testing medium, etc. [65].

Additionally, the decrease in the efficiency of QACs is related to (*i*) environmental niches with sites that are difficult to clean and disinfect, the inability to remove cells, (*ii*) the presence of organic matter on food contact surfaces, (*iii*) exposure to sublethal concentrations of QACs on food contact surfaces that allow BC tolerance of *L. monocytogenes*; besides, this is associated with the persistence of *L. monocytogenes* in the food industry and the subsequent adaptation and formation of biofilms [18,66–68]. Our results indicate that all *L. monocytogenes* isolates can form a biofilm, which harbors the genes *inlA*, *prfA*, *plcA*, *hly*, *plcB*, and *actA* associated with biofilm formation. Previous research indicated that *inlA*, *inlL*, *prfA*, *plcA*, *actA*, *Imo0673*, *bapL*, *recO*, *Imo2504*, and *luxS* play a role in the different stages of *L. monocytogenes* biofilms formation [35,46,69]. Likewise, Price *et al.* [70] argue that the presence of LIPI-1 genes, *hly* and *prfA*, are required for biofilm formation by *L. monocytogenes*. However, biofilm formation is a complex and dynamic process that is contingent upon a number of factors, including the availability of nutrients in the environment, the origin and biodiversity of the strain, and the quorum sensing (QS) that have activation and regulation of biofilm-associated genes and virulence factors [19]. Several studies have demonstrated that *L. monocytogenes* forms biofilms exhibit significantly greater resistance to sanitizing and antibiotic compounds than free-floating cells [44,71]. Therefore, it could represent a source of concurrent food contamination, increasing the risk to the consumer and impacting public health, in addition to the economic losses associated with voluntary recalls or damage to equipment within the food industry. Moreover, in this study, *L. monocytogenes* strains isolated from fresh fruits and vegetables can potentially cause severe human infection; however, the severity of the clinical manifestations of *L. monocytogenes* is related to genetic diversity, immune system status, or host comorbidities. Indeed, Castañedas-Ruelas *et al.* [72] argument that the dearth of data concerning the significance of *L. monocytogenes* in Mexico underscores the necessity to sensitize authorities to the characterization of the risks associated with

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food and human exposure to *L. monocytogenes*, thereby facilitating an understanding of the clinical and epidemiological impact of listeriosis in Mexico. Notably, it is essential to incorporate techniques that allow us to determine the biodiversity of *L. monocytogenes.* This enables the identification of clonal complexes (CCs) and sublineages (SLs), enabling a relationship between the origin of isolation and the infection's severity in this investigation's context. For example, SL121 (CC121), SL9 (CC9), and CC8 + CC16 are associated with Lineage II, which has a food origin, and SL1 (CC1), SL2 (CC2), SL4 (CC4), and SL6 (CC6) with clinical cases of lineage I. CC1 and CC4 are related to invasive forms of listeriosis, including maternal-neonatal and CNS infection [34,73].

4. Materials and Methods

4.1. Bacterial Strains

Seventeen strains of *L. monocytogenes* were selected for this study from various fresh fruits and vegetables, including Hass avocados, lettuce, parsley, cilantro, broccoli, and cucumber. The strains were confirmed by PCR using *hly* (Listeriolysin O) and *prs* (Putative phosphoribosyl pyrophosphate synthetase) [24,25]. Stocks were stored in tryptic soy broth (TSB; Becton Dickinson Bioxon, Le Pont de Claix, France) containing 30% glycerol at – 80 ºC.

4.2. Genomic Characterization: Genes Involved in Pathogenicity Islands, Biofilm Formation and Resistance Antibiotics

L. monocytogenes strains were reactivated in TSB with 0.6% yeast extract (TSBYE) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 30 ºC. According to the manufacturer's instructions, genomic DNA was extracted from *L. monocytogenes* using a Bacteria DNA Preparation Kit (Jena Bioscience, Jena, Germany). All *L. monocytogenes* strains were investigated for detection of the genes (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, *actA*, *inlA*, *inlB*, *inlC*, *inlJ*, *llsA*, *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, *llsD*, *llsP*, *licC*, *licB*, *licA*, and *glvA*) that harbored the *L. monocytogenes* pathogenicity islands (LIPI's) by PCR using the protocol of Zhang *et al.* [21]. Subsequently, phylogenetic group of *L. monocytogenes* [I.1 (172a-3a), I.2 (1/2c-3c), II.1 (4b-4d-4e), II.2 (1/2b-3b-7), and III (4a-4c)] and the genes associated with resistance antibiotics [efflux pump Ide (*Ide*), chloramphenicol acetyltransferase (*cat*), macrolide-lincosamide-streptogramin B efflux pump (*msrA*), rRNA adenine-N-6-methyltransferase (*ermA*), and ribosomal protection protein tetM (*tetM*)] were determined using the protocol of Doumith *et al.* [24] and Boháčová *et al.* [39]. After amplification, the products were electrophoresed on 1 % (w/v) agarose gel (UltraPure agarose, Invitrogen, Carlsbad, USA) using SYBR Green (Sigma-Aldrich, St. Louis, MO, USA) and visualized by transillumination under UV light (UVP, DigiDoc-It Darkroom, Upland, CA, USA).

4.3. Phenotypic Characterization for the Persistence of L. monocytogenes

4.3.1. Disinfectant and Heavy Metal Sensitivity

Benzalkonium chloride (BC) (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the sensitivity of *L. monocytogenes* strains to quaternary ammonium compound (QAC) using the protocol of Gray *et al.* [30], with modifications. Briefly, *L. monocytogenes* strains were grown overnight in Mueller Hinton broth (MHB; Becton Dickinson Bioxon, Le Pont de Claix, France) at 30 ºC and diluted to ~10^8 CFU/mL. The BC stock concentration, 100 µL, was added to the microtiter plates (Corning® 96-Well Assay Microplate, Lowell, MA, USA) with concentrations of 100, 50, 25, 12.5, 6.2, 3.1, 1.5, and 0.7 µg/mL. The microtiter plates were then incubated at 30 ºC / 24 h, and growth was monitored by measuring the OD_{560} using a Multiskan FC (Thermo Fisher Scientific, Inc., Madison, WI, USA) to determine the minimum inhibitory concentration (MIC). Each assay was performed in triplicate, with positive and negative controls. Cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA) was used to determine the resistance of *L. monocytogenes* to the heavy metal cadmium. Mueller Hinton Agar (MHA; Becton Dickinson Bioxon, Le Pont de Claix, France) was supplemented with different concentrations of $CdCl_2$ (400, 200, 100, 70, 50, 25, and 12.5 µg/mL). Each *L. monocytogenes* isolate was

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adjusted to ~10^8 CFU/mL and inoculated onto the $CdCl_2$ plates. The plates were then incubated at 37 ºC / 24 h for triplicate. Resistance to cadmium was interpreted as ≥ 70 µg/mL [49,50].

4.3.2. Phenotypic Antibiotic Sensitivity and Resistance Analysis

The resistance and susceptibility of *L. monocytogenes* strains to antibiotics were determined using the agar diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) [74]. Bacterial suspensions adjusted to 0.5 McFarland were inoculated onto MHA, incorporated the antibiotics, and incubated at 35 ºC / 24 h. Thirteen were selected among eleven classes of antimicrobials: phenicols [chloramphenicol (CL, 30 µg)]; cephalosporines (1st generation) [cephalothin (CF, 30 µg)]; licosamide [clindamycin (CLM, 30 µg)]; sulphonamide [trimethoprim-sulfamethoxazole (SXT, 2.5/23.75 µg)]; cyclic peptides [tetracycline (TE, 30 µg)]; macrolide [erythromycin (E, 15 µg)]; aminoglycoside [gentamicin (GE, 10 µg)]; fluoroquinolone [ciprofloxacin (CPF, 5 µg)]; cephalosporines (3rd generation) [cefotaxime (CFX, 30 µg)]; glycopeptide [vancomycin (VA, 30 µg)]; β-lactam [penicillin (P, 10 U), ampicillin (AM, 10 µg), and dicloxacillin (DC, 1 µg)] (BBL™ Sensi-Disc™). The inhibition zones were interpreted as Resistance (R), Intermediate resistance (I) and Susceptible (S) according to CLSI [28]. *L. monocytogenes* ATCC 19111 was used as the positive control. The multiple antibiotic resistance (MAR) index of *L. monocytogenes* isolates was determined using the methods by Krumperman [41] and Blasco *et al.* [75].

4.3.3. Biofilm Formation Assay

The strains' ability to form biofilms was evaluated in polystyrene microtiter plates (Corning® 96-Well Assay Microplate, Lowell, MA, USA) using crystal violet (CV) staining, following the protocol described by Avila-Novoa *et al.* [76]. For each strain, 230 µL of TSB and 20 µL bacterial suspension (~10^8 CFU/mL) were added to polystyrene microtiter plates and incubated at 30 °C for 240 h. The planktonic bacteria were removed using 200 µL of phosphate-buffered saline (PBS; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl, pH 7.4). The biofilm was fixed with 200 µL of methanol for 10 min, dried at 55 ºC for 15 min, and stained with 200 µL of 0.1% crystal violet for 45 min. Excess stain was rinsed off with PBS and resolubilized with 200 µL of 95% ethanol. Absorbance was measured at 595 nm (OD_{595}), using the Multiskan FC. The assay was performed in triplicate, including positive and negative controls. The cutoff OD (ODc) was determined using the protocol described by Stepanović *et al.* [77].

5. Conclusions

The present study provides data regarding the genetic diversity of *L. monocytogenes* in fruits and vegetables in Mexico and the possible impact on its population associated with the genetic determinants involved in the severity of the pathology and resistance mechanisms of antibiotics that are used within the therapeutic scheme of the patient or veterinary medicine, in addition to the environmental impact that fertilizer residues have on antimicrobial resistance. This is to raise awareness of a continuous improvement in treatments and sanitary prerequisites such as agricultural practices, food farming practices, and standard operating procedures for sanitation. A recommendation is to validate and rotate disinfectants to reduce the risk of *L. monocytogenes* niches being established in the environment and tolerance to disinfectants that promote the survival of *L. monocytogenes* biofilms.

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