Virulence and Antibiotic Resistance Genes in *Listeria monocytogenes* Strains Isolated From Ready-to-Eat Foods in Chile

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*L. monocytogenes* is causing listeriosis, a rare but severe foodborne infection. Listeriosis affects pregnant women, newborns, older adults, and immunocompromised individuals. Ready-to-eat (RTE) foods are the most common sources of transmission of the pathogen. This study explored the virulence factors and antibiotic resistance in *L. monocytogenes* strains isolated from ready-to-eat (RTE) foods through *in vitro* and *in silico* testing by whole-genome sequencing (WGS). The overall positivity of *L. monocytogenes* in RTE food samples was 3.1% and 14 strains were isolated. *L. monocytogenes* ST8, ST2763, ST1, ST3, ST5, ST7, ST9, ST14, ST193, and ST451 sequence types were identified by average nucleotide identity, ribosomal multilocus sequence typing (rMLST), and core genome MLST. Seven isolates had serotype 1/2a, five 1/2b, one 4b, and one 1/2c. Three strains exhibited *in vitro* resistance to ampicillin and 100% of the strains carried the *fosX*, *lin*, *norB*, *mprF*, *tetA*, and *tetC* resistance genes. In addition, the *arsBC*, *bcrBC*, and *clpL* genes were detected, which conferred resistance to stress and disinfectants. All strains harbored *hlyA*, *prfA*, and *inlA* genes almost thirty-two the showed the *bsh*, *clpCEP*, *hly*, *hpt*, *iap/cwhA*, *inlA*, *inlB*, *ipeA*, *lsP*, *mpl*, *plcA*, *pcIB*, *oat*, *pdgA*, and *prfA* genes. One isolate exhibited a type 11 premature stop codon (PMSC) in the *inlA* gene and another isolate a new mutation (deletion of *A* in position 819). The Inc18(rep25), Inc18(rep26), and N1011A plasmids and MGEs were found in nine isolates. Ten isolates showed CAS-Type II-B systems; in addition, Anti-CRISPR AcrIIA1 and AcrIIA3 phage-associated systems were detected in three genomes. These virulence and antibiotic resistance traits in the strains isolated in the RTE foods indicate a potential public health risk for consumers.

**Keywords:** *Listeria monocytogenes*, ready-to-eat foods, virulence, resistance genes, whole-genome sequencing, CRISPR-Cas
INTRODUCTION

Ready-to-eat (RTE) foods are defined as any food in a raw state or one that is handled, processed, mixed, cooked, or prepared and is consumed without any further processing (Monteiro, 2010). The RTE foods are a practical alternative to meet daily food needs; however, they are not exempt from contamination by biological hazards such as Salmonella spp., pathogenic Escherichia coli, and Listeria monocytogenes (Becker et al., 2019).

Listeria monocytogenes is a Gram-positive facultative anaerobic, ubiquitous, and persistent bacterium in food processing plants. Due to inadequate hygiene and manufacturing practices, this pathogen contaminates foods such as fresh or frozen fruits and vegetables, unpasteurized dairy products, sausages, and fish (Amajoud et al., 2018). Cheeses, sausages, meats, and fish are the most frequently associated with outbreaks of this pathogen worldwide (Kurpas et al., 2018; Ryser, 2021). L. monocytogenes causes listeriosis, a disease characterized by low morbidity but high mortality in those who are infected, and the most at risk groups are pregnant women, newborns, children, and older adults (Schlech, 2019). In Europe and North America, invasive listeriosis affects 0.3–0.6 persons per 100,000 inhabitants annually (Maertens de Noordhout et al., 2015). In Chile, L. monocytogenes has been under mandatory laboratory notification and surveillance since 2005 (Bustamante et al., 2020). There were 97 cases in 2018 and 69 in 2019 with lethality of 22% and 26%, respectively (MINSAL: Ministerio de Salud Chile, Departamento de Estadísticas e Información en Salud [DEIS], 2019). Adults aged 65 and older and pregnant women were the most affected groups. Pregnancy was terminated in 50% of cases and abortion or fetal death was reported in 21% (MINSAL: Ministerio de Salud Chile, Departamento de Estadísticas e Información en Salud [DEIS], 2019). The RTE foods were the main source of infection associated with these listeriosis cases (MINSAL: Ministerio de Salud Chile, Departamento de Estadísticas e Información en Salud [DEIS], 2019).

The severity of L. monocytogenes infection is associated with several factors such as infecting dose, host immunity, and expression of virulence factors (adherence, invasion, immune modulator, intracellular survival, toxins, and mobile genetic elements), and the presence of CRISPR-Cas as a virulence regulator (Falavina dos Reis et al., 2011; Louwen et al., 2014; Poullot et al., 2016; Buchanan et al., 2017; Kwon et al., 2020). In addition, there are other factors such as resistance to disinfectants and antibiotics, especially beta-lactams (Olaimat et al., 2018). The capacity to resist to adverse environmental conditions (heat and cold stress) allow the persistence and colonization throughout the food chain by forming contamination reservoirs that are difficult to control (Bolocan et al., 2016; Bucur et al., 2018). The Listeria species can be categorized into different serotypes according to the serological reactions of the listerial somatic antigen (O-antigen) and flagellar antigen (H-antigen) with specific antisera. L. monocytogenes can be classified into at least 13 serotypes (Orsi et al., 2011), three of them (1/2a, 1/2b, and 4b) are involved in over 95% of human clinical cases, and serotype 4b exhibits the strongest epidemiological association with human listeriosis (Maury et al., 2016; Lee et al., 2018).

Whole-genome sequencing (WGS) currently allows in silico generation of a wealth of information about pathogenic strains, including a more precise description of the taxonomic differences and similarities between them. The WGS technology is used to more precisely identify the pathogen and genotype it by multilocus sequence typing (MLST), clonal complex (CC) determination, core genome MLST (cgMLST), CRISPR-Cas, and serogrouping. WGS also enables the detection of antibiotic resistance and virulence genes, plasmids, and mobile genetic elements (MGEs); this information provides a more precise epidemiological relationship (Leopold et al., 2014; Ruppitsch et al., 2015a; Moura et al., 2017; Hurley et al., 2019; Kwon et al., 2020; Stessl et al., 2021). The use of WGS has been fundamental in the successful investigation of recent extensive outbreaks of L. monocytogenes in South Africa (2017–2018) and Germany (2018–2019) (Allam et al., 2018; Halbedel et al., 2020).

According to Chilean health authorities, RTE foods are the main source of infection associated with cases of this disease in Chile (MINSAL: Ministerio de Salud Chile, Departamento de Estadísticas e Información en Salud [DEIS], 2019). However, information about the diversity, pathogenicity, and virulence of L. monocytogenes in Chile is still limited and incomplete. Our study contributes to a better understanding of L. monocytogenes with respect to genotype diversity, virulence, antibiotic resistance, and cas genes by generating necessary and indispensable scientific evidence. Many of the evaluated foods in this study are marketed in the Americas. Therefore, given the need for updated information on this pathogen in Chile, we studied virulence factors and antibiotic resistance in L. monocytogenes strains isolated from RTE foods by in vitro and in silico testing using whole-genome sequencing (WGS).

MATERIALS AND METHODS

Samples

A total of 436 samples of retail RTE foods were analyzed; these are regarded as at risk foods sampled as part of sampling plan the Emerging Pathogens Program of the Health Authority in the Maule Region, Chile, and which are regulated by the Chilean Food Sanitary Regulations (RSA). Samples used for our study consisted of cheeses (n = 161), cooked meats (artisanal ham, pâté, sausages, and blood sausage) (n = 186), pre-processed fruits and vegetables (chopped fruit, fruit salads with strawberries, melon, and peaches, and leafy vegetable salads) (n = 22), and meals and mixed dishes with raw and/or cooked ingredients (n = 67).

Isolation of Listeria monocytogenes

Isolation was performed on the basis of the ISO 11290–1:2017 standard. Each 25 g food sample was inoculated in 225 mL half Fraser broth (Oxoid, Basingstoke, United Kingdom) as primary selective enrichment and homogenized in a stomacher (Seward 400, Radnor, PA, United States). Incubation was performed at 30 ± 1°C for 25 ± 1 h; the second enrichment consisted...
of 0.1 mL of the broth culture inoculated in 10 mL of full-strength Fraser broth, which was cultured at 37°C for 24 ± 2 h. A loopful of each of the half- and full-strength Fraser broths were plated on the Listeria chromogenic agar base according to Ottaviani and Agosti (ALOA) (Merck, Darmstadt, Germany). These plates were incubated at 37°C for 24–48 h. Five typical colonies from each ALOA agar plate were restreaked on tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE) (Sigma, Darmstadt, Germany) as a non-selective medium, and these colonies from each ALOA agar plate were restreaked on tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE) (Sigma, Darmstadt, Germany). The sequence type (ST) was determined with Task templates for available MLST schemes from the SeqSphere+ v. 7.8.0 (2021-7) software (Jünemann et al., 2013). The ST was confirmed in the strains with fragments from the seven housekeeping genes abcZ, bgLA, cat, dapE, dat, Idh, and ihkA (Ruppitsch et al., 2015a; Moura et al., 2016) and with the profiles from the Institut Pasteur MLST Listeria database1.

The cgMLST was performed on the basis of the profile of 1,701 loci of cgMLST complex types (CTs) (Ruppitsch et al., 2015a) with Task templates for SeqSphere+ v. 7.8.0 (2021-7). We defined a cgMLST cluster as the group of isolates with less than 10 different alleles among the studied strains. We used SeqSphere in the mode that ignored pairwise missing values and an unweighted pair group method with arithmetic mean to generate phylogenetic trees (Halbedel et al., 2020).

**Detection of Listeria monocytogenes**

Detection of *L. monocytogenes* was performed with the Vitek Immunodiagnostic Assy System (VIDAS) (bioMerieux Vitek Inc., Hazelwood, MO, United States) according to the manufacturer’s instructions. The equipment automatically measured and interpreted data, reporting detection as positive or negative according to the validated AOAC (Official Method of Analysis No. 2004.2) protocol for food matrices.

**Whole-Genome Sequencing**

Prior to WGS, a primary species identification from single colonies was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Billerica, MA, United States) and MBT Compass IVD software 4.1.60 (Bruker) as described by Halbedel et al. (2020).

As for WGS, DNA was isolated from bacterial cultures with the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions for Gram-positive bacteria. The amount of input DNA was quantified on a Lunatic instrument (Unchained Labs, Pleasanton, CA, United States). Nextera XT chemistry (Illumina Inc., San Diego, CA, United States) was used to prepare sequencing libraries for a 300 bp paired-end sequencing run on an Illumina MiSeq sequencer. Samples were sequenced to achieve a minimum 80-fold coverage using recommended standard protocols by Illumina. The resulting FASTQ files were quality trimmed and de novo assembled with the SPAdes version 3.9.0. Contigs were filtered for a minimum of fivefold coverage and 200 bp minimum length with SeqSphere+ software v. 7.8.0 (Ridom, Münster, Germany) (Jünemann et al., 2013).

**Serotype, Sequence Type, and Core Genome Multilocus Sequence Typing of Listeria monocytogenes**

From the WGS of the *L. monocytogenes* strains, serotypes were determined by the sequence-specific extraction of targets using the *L. monocytogenes* 5-plex PCR Serogroup task templates of the SeqSphere+ v. 7.8.0 (2021-7) software with fragments from five DNA regions (*lmol18, lmo0737, ORF2110, ORF2829,* and *prs* as an internal amplification control) previously described by Doumith et al. (2004) and Lee et al. (2012).

The sequence type (ST) was determined with Task templates for available MLST schemes from the SeqSphere+ v. 7.8.0 (2021-7) software (Jünemann et al., 2013). The ST was confirmed in the strains with fragments from the seven housekeeping genes *abcZ, bgLA, cat, dapE, dat, Idh,* and *ihkA* (Ruppitsch et al., 2015a; Moura et al., 2016) and with the profiles from the Institut Pasteur MLST Listeria database1.

**Antibiotic Resistance Profile**

The disk diffusion method was applied based on the recommendations of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2018). The commercial antibiotic disks included 10 µg ampicillin (AMP), 10 µg penicillin (PEN), 25 µg sulfamethoxazole-trimethoprim (STX), 15 µg erythromycin (ERY), 30 µg vancomycin (VAN), tetracycline (TET) 30 µg, ciprofloxacin (CIP) 5 µg, and 30 µg chloramphenicol (CHL). The resistance/susceptibility profiles of the strains were characterized by measuring the zone of inhibition and interpreting the inhibition diameters according to the manufacturer’s instructions; *Streptococcus pneumoniae* ATCC 49619 was used as a reference. In addition, *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 7644 were used as controls.

**Virulence Genes Amplification**

The method described by Aznar and Alarcón (2002) was used to amplify conserved regions of the three characteristic virulence genes listeriolsin O (*hilA*) (Border et al., 1990), positive regulatory factor A (*prfA*) (Klein and Juneja, 1997), and internalin A (*inlA*) (Montero et al., 2015). The genomic DNA of the suspected strains was extracted and purified with the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Qiagen, Carlsbad, CA, United States) and mixed with GoTaq Green Master Mix (Promega, Madison, WI, United States) in a thermocycler (Fermelo Biotec, China). Using an agarose gel imaging system, the amplified products were stained and visualized on 1.5% agarose gel with a 1.0 mg/mL ethidium bromide solution.

**In silico Detection of Virulence and Antibiotic Resistance Genes**

Virulence genes were established with the task template VFDB 2.0 feature in SeqSphere+ for WGS data (Chen et al., 2016). Thresholds were set for the target scanning procedure as a required identity ≥ 90% with the reference sequence and an aligned reference sequence ≥ 99%. The Comprehensive

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1https://bigd.db.pasteur.fr/listeria/listeria.html
Antibiotic Resistance Database (CARD) was used with the default “perfect” and “strict” settings for the sequence analysis of antimicrobial resistance genes (Jia et al., 2017). The Task Template AMRFinderPlus 3.2.3 available in the Ridom SeqSphere+ 7.8.0 software was used with the EXACT method at the 100% setting together with the BLAST alignment of protein sequences against the AMRFinderPlus database (Feldgarden et al., 2019).

In silico Detection of Plasmids and Mobile Genetic Elements
The PlasmidFinder 2.1 and MobileElementFinder 1.0 tools were used to detect plasmids and MGEs. The selected minimum identity was 95 and 90%, respectively (Carattoli et al., 2014; Johansson et al., 2021).

Bioinformatic Search of CRISPR-Cas Loci
The search for and characterization of CRISPR arrays and their association with Cas proteins was determined with CRISPRCasFinder and CRISPRminer (Couvin et al., 2018; Zhang et al., 2018), which are available at https://crisprcas.i2bc.paris-saclay.fr and http://www.microbiome-bigdata.com/CRISPRminer. The following parameters were used: 18–55 bp repeated sequence length, 25–60 bp spacer length, 0.6–2.5 spacer sequence size as a function of repeated sequence size, and 60% maximum percentage similarity between spacers. Phages associated with sequence spacers were also determined with the CRISPRminer program (Zhang et al., 2018).

The CRISPR systems were determined with the CRISPRmap program (Lange et al., 2013). The CRISPRTarget program was used to determine the protospacer adjacent motif (PAM) sequences associated with each repeated sequence of the identified arrays.

RESULTS

Prevalence of Listeria monocytogenes
In total 3.1% (14/436) of samples were positive for L. monocytogenes when using the VIDAS system. In terms of food group, the highest positivity, 36% (8/22), occurred in pre-processed fruits and vegetables, followed by 5.9% (4/67) for prepared meals and dishes, 1.1% (2/186) for cooked meats, and 0% (0/161) for cheese and fresh cheese (Table 1).

In vitro Detection of Virulence Genes
Most of the strains 78% (11/14) were susceptible to all the antibiotics; only the MRL-19-00656, MRL-19-006573, and MRL-19-00662 strains were resistant to ampicillin.

Regarding the virulence genes, all strains in the present study amplified hlyA, prfA, and inlA genes (Table 3).

In silico Detection of Virulence and Antibiotic Resistance Genes
The virulence factor database (VFDB) was used to evaluate the 33 major virulence gene. All the strains had the following genes: bsh (bile resistance), clpCEP (stress protein), hly (toxin-lysostilisoin O precursor), hpt (metabolic adaptation), lap/cwhA, inlA, inlB, and ipeA (invasion), lap (peptidase), mpl, plcA, plcB (exoenzyme), oit, pdgA (immune evasion), and prfA (regulation) (Figure 2). Only the MRL-19-00675 genome strain exhibited the Listeria pathogenicity island 3 (LPI-3). A mutation in position 2054 (G:A) of inlA gene was found in the MRL-19-00658 strain, known as premature stop codons (PMSC) type 11. A new mutation in position 819 of the inlA gene was encountered in the MRL-19-00662 strain, which was not identified because this mutation (deletion of A) has not yet been described in the literature or in the inlA PMSC profiles of the Institut Pasteur MLST Listeria database (see Text Footnote 1).

Furthermore, genes associated with biofilm formation such as cheY, inlL, prfA, actA, lmo0673, and lmo2504 were identified in all the strains; these genes play an important role in the survival and persistence of L. monocytogenes. The bapL, recO,
Figure 1: Minimum spanning tree (MST) of 14 *Listeria monocytogenes* strains from ready-to-eat foods isolated in Chile. *L. monocytogenes* strains with ST1, ST3, ST5, ST7, ST8, ST9, ST14, ST193, ST451, and ST2763 are of clinical and food origin. Calculation of the MST is based on the defined core genome multilocus sequence typing (cgMLST) scheme consisting of 1,701 target genes from Task templates for SeqSphere+ v. 7.8.0 (2021-7). Isolates are represented as colored circles according to the classical MLST. Black numbers are in accordance with the allelic difference between isolates. Isolates with closely related genotypes are marked as Cluster.

Table 2: Identification of *L. monocytogenes* strains by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and whole-genome sequencing (WGS).

| Sample ID    | Food         | MALDI-TOF MS       | WGS              | ST     | CC    | CT    | Serotype |
|--------------|--------------|--------------------|------------------|--------|-------|-------|----------|
| MRL-19-00634 | Strawberries | *Listeria monocytogenes* | *Listeria monocytogenes* | 451    | 11    | 4117  | 1/2a     |
| MRL-19-00637 | Cooked shrimp| *Listeria monocytogenes* | *Listeria monocytogenes* | 5      | 5     | 8052  | 1/2b     |
| MRL-19-00656 | Cooked sausage| *Listeria monocytogenes* | *Listeria monocytogenes* | 193    | 193   | 8063  | 1/2a     |
| MRL-19-00657 | Strawberries | *Listeria monocytogenes* | *Listeria monocytogenes* | 7      | 7     | 8064  | 1/2a     |
| MRL-19-00658 | Grapes/1/2a | *Listeria monocytogenes* | *Listeria monocytogenes* | 9      | 9     | 5231  | 1/2c     |
| MRL-19-00660 | Coleslaw     | *Listeria monocytogenes* | *Listeria monocytogenes* | 14     | 14    | 8065  | 1/2b     |
| MRL-19-00662 | Mix salads   | *Listeria monocytogenes* | *Listeria monocytogenes* | 3      | 3     | 8066  | 1/2b     |
| MRL-19-00666 | Mushrooms    | *Listeria monocytogenes* | *Listeria monocytogenes* | 8      | 8     | 8068  | 1/2a     |
| MRL-19-00667 | Mushrooms    | *Listeria monocytogenes* | *Listeria monocytogenes* | 8      | 8     | 8068  | 1/2a     |
| MRL-19-00670 | Pâté         | *Listeria monocytogenes* | *Listeria monocytogenes* | 8      | 8     | 8004  | 1/2a     |
| MRL-19-00672 | Fettucine    | *Listeria monocytogenes* | *Listeria monocytogenes* | 2763   | 5     | 8006  | 1/2b     |
| MRL-19-00673 | German roast | *Listeria monocytogenes* | *Listeria monocytogenes* | 2763   | 5     | 8006  | 1/2b     |
| MRL-19-00675 | Spinach salad| *Listeria monocytogenes* | *Listeria monocytogenes* | 1      | 1     | 8007  | 4b       |
| MRL-19-00677 | Pot roast    | *Listeria monocytogenes* | *Listeria monocytogenes* | 2763   | 5     | 8006  | 1/2b     |

ST, sequence type; CC, clonal complex; CT, complex type.

and *luxS* genes were not found in any strain. The antimicrobial *fosX*, *lin*, *norB*, and *mprF* resistance genes were identified in all *L. monocytogenes* strains. These genes confer resistance to fosfomycin, lincosamides, quinolones, and cationic peptides that disrupt the cell membrane such as defensins. Regarding the genes that confer resistance to tetracycline, the *tetA* and *tetC* genes were detected and *tetM* and *tetS* were absent in all the strains. In addition, the *arsBC* and *bcrBC* genes were identified in all strains, which confer resistance to stress, and the *clpL* gene, which confers resistance to disinfectants.

**Detection of Plasmids and Mobile Genetic Elements**

Plasmids were found in 85% (12/14) of the strains. Inc18(rep25) was detected in eight strains, Inc18(rep26) in three, and N1011A in one. In addition, the ST2763 harbored the Inc18(rep25) and rep3(rep32) plasmids (Table 4).

The MGEs (insertion sequences, transposons) were found in only nine strains. The most frequent MGEs were ISLmo3, ISLmo5, ISLmo7, ISLmo9, ISLmo8, ISS1N,
| Strain    | Source          | Antibiotics | Genes |
|-----------|-----------------|-------------|-------|
| MRL-19-00634 | Strawberries     | 451         | ST    |
| MRL-19-00657 | Cooked shrimp    | 5           | AMP, PEN, STX, ERY, VAN, TET, CIP, CHL |
| MRL-19-00666 | Cooked sausage   | 193         | R     |
| MRL-19-00668 | Strawberries     | 7           | R     |
| MRL-19-00670 | Grapes           | 9           | S     |
| MRL-19-00660 | Coleslaw         | 14          | S     |
| MRL-19-00662 | Combination salads | 3         | S     |
| MRL-19-00669 | Mushrooms        | 8           | S     |
| MRL-19-00670 | Mushrooms        | 8           | S     |
| MRL-19-00671 | Fettuccine       | 2763        | R     |
| MRL-19-00673 | German roast     | 2763        | R     |
| MRL-19-00675 | Spinach salad    | 1           | S     |
| MRL-19-00677 | Pot roast        | 2763        | S     |

**R**, resistance; **S**, susceptibility; Genes: **hlyA**, listeriolysin O; **prfA**, positive regulatory factor A; **inlA**, internalin A.

**FIGURE 2** Distribution of virulence genes present in 14 *L. monocytogenes* strains isolated from ready-to-eat foods. Green boxes indicate the presence of the gene and black boxes its absence.

**TABLE 3** Detection of putative virulence genes and antibiotic resistance profile of *L. monocytogenes* strains.

| Strain    | Source          | Antibiotics | Genes |
|-----------|-----------------|-------------|-------|
| MRL-19-00634 | Strawberries     | 451         | ST    |
| MRL-19-00657 | Cooked shrimp    | 5           | AMP, PEN, STX, ERY, VAN, TET, CIP, CHL |
| MRL-19-00666 | Cooked sausage   | 193         | R     |
| MRL-19-00668 | Strawberries     | 7           | R     |
| MRL-19-00670 | Grapes           | 9           | S     |
| MRL-19-00660 | Coleslaw         | 14          | S     |
| MRL-19-00662 | Combination salads | 3         | S     |
| MRL-19-00669 | Mushrooms        | 8           | S     |
| MRL-19-00670 | Mushrooms        | 8           | S     |
| MRL-19-00671 | Fettuccine       | 2763        | R     |
| MRL-19-00673 | German roast     | 2763        | R     |
| MRL-19-00675 | Spinach salad    | 1           | S     |
| MRL-19-00677 | Pot roast        | 2763        | S     |

CRISPR-Cas Loci

Genome analysis showed the presence of CRISPR-Cas systems in 71% (10/14) of the genomes. These systems consist of at least one array; however, between two and five arrays can be observed in 50% (5/10) of the genomes in different positions. The arrays had among 3 repeated sequences and 2 spacers and up to 28 repeated sequences and 27 spacers (Table 5).

Using the CRISPRmap program, the repeated sequences and the associated cas genes enabled to determine the identified CRISPR systems to type II-β (Figure 3 and Table 1). In two of the arrays, only one cas-associated gene was identified; in contrast,
the rest of the genomes showed sequences that encoded up to 16 proteins associated with these CRISPR-Cas systems.

The analysis of the spacer and PAMs enabled us to associate them to sequences corresponding to different bacteriophages, which are associated with the Listeria genus (Supplementary Tables 2, 3).

The bioinformatics analysis of the genomes enabled the detection of protein sequences associated with the AcrIa1 and AcrIa3 Anti-CRISPR systems in the three studied genomes MRL-19-00657, MRL-19-00658, and MRL-19-00660 (Table 6), which are associated with phages present in these genomes.

**DISCUSSION**

*Listeria monocytogenes* persists as a relevant public health and food safety risk due to its ubiquity, persistence under adverse environmental conditions, and pathogenicity (Hurley et al., 2019; Chen et al., 2020a).

In the present study, general positivity for *L. monocytogenes* in RTE foods was 3.1% (14/436). Positivity for *L. monocytogenes* in RTE foods in different countries has been reported as 5.5% in China (Li et al., 2018), 7.5% in Chile (Bustamante et al., 2020), 11.9% in Uruguay (Braga et al., 2017), 8.5% in Turkey (Sanlibaba et al., 2018), and 13.5% in Poland (Szmyczak et al., 2020). Bustamante et al. (2020) reported prevalence values of 17.5, 8.6, and 8.5% in prepared meals and dishes, pre-processed fruits and vegetables, and cooked meats, respectively. Furthermore, positivity for *L. monocytogenes* was 0% in dairy products and cheeses, which concurs with the present study, and this situation is noteworthy because dairy products and cheeses have been associated in recent years with many outbreaks in Europe and the United States (Fretz et al., 2010; Amato et al., 2017; Martinez-Rios and Dalgaard, 2018; Churchill et al., 2019). Therefore, a better understanding of the ecology and biology of *L. monocytogenes* that focuses on virulence factors and stress response would further improve the control of this important foodborne pathogen (Ryser, 2021).

The *L. monocytogenes* strains in the present study revealed that ST8 was the most prevalent ST from samples of RTE vegetables and pork pâté. The ST8 has been found in different RTE foods such as meats, salmon, cooked meats, fried rice and noodles, and vegetables (Wang et al., 2015; Ziegler et al., 2018; Chen et al., 2020b). In addition, ST8 has been responsible for cases of human listeriosis in Canada, Italy, Switzerland, and Germany (Knabel et al., 2012; Mammina et al., 2013; Althaus et al., 2014; Ruppitsch et al., 2015b; Halbedel et al., 2020), and it has been considered to have high pathogenic potential (Fagerlund et al., 2017). Different authors state that *L. monocytogenes* ST 8 is one of the most persistent STs in RTE food processing plants; hence, there is a permanent risk of food recontamination by this pathogen (Knudsen et al., 2017). The second most prevalent was ST2763 (CC5), which is a new ST found in the present study and...
FIGURE 3 | CRISPR-Cas systems identified in *L. monocytogenes* genomes. The identified systems belong to the CRISPR-Cas type II-B system, and some genomes show more than one array.
TABLE 6 | Anti-CRISPR elements.

| Bacteria ID   | Anti-CRISPR message | E-value     | Match range | Coverage | Protein sequence |
|---------------|----------------------|-------------|-------------|----------|------------------|
| MRL-19-00657_contig8_134202_134651_+ | gb| AEO04364.1| gp28 [Listeria monocytogenes J0161] | 1.75E-107 | 0.986577181 | 1 | MTIKLLDEFLKHLTQLRGLTQSGNTUKQDNEKPLKNK YTVSILRSLISGSLVSDYVLFELEDKNSSDLGLFKHLD KYKLSPAGEFELYLKEFESANIEVLPTFNRFENEEHV NIEKDCVKALENATVLEKNEEL |
| MRL-19-00658_contig1_1720_2097_- | emb| CB03209.1| bacteriophage protein GP30 [Listeria monocytogenes serotype 7 str. SLCC22482] | 3.42965e-75 | 0.864 | 1 | MNYKAIEKMGAWNFTDSMNLSDEIVSYTDKFTS VCLKAAWSSKAEKEVEKHEHKSIEKSEELKAWAEKTKG LGLRFNSDDEKFTSVKDETKQHFGLSVACAMAKKLH NDLFPQTA |
| MRL-19-00658_contig1_884_1333_- | gb| AEO04364.1| gp28 [Listeria monocytogenes J0161] | 4.35583e-91 | 0.75167782349 | 1 | MSKILLDEFLKHLTQLRGLTQSGNTLNDYNNKELNKLK YSVSFLRSMAGCSTDFVIELAELEKSYDGLFKHLD DKKLYLSPAGEFELYLKEFESANIEVLPTFNRFENEEHV NIEKDCVKALENATVLEKNEEL |
| MRL-19-00660_contig12_68115_68564_+ | gb| AEO04364.1| gp28 [Listeria monocytogenes J0161] | 1.70745e-108 | 1.0 | 1 | MTILKLLDEFLKHLTQLRGLTQSGNTUKQDNEKPLKNK YTVSILRSLISGSLVSDYVLFELEDKNSSDLGLFKHLD KYKLSPAGEFELYLKEFESANIEVLPTFNRFENEEHV NIEKDCVKALENATVLEKNEEL |
| MRL-19-00660_contig31_32482_32859_- | emb| CB03209.1| bacteriophage protein GP30 [Listeria monocytogenes serotype 7 str. SLCC22482] | 4.3182e-75 | 0.904 | 1 | MNYKSIEMQOAWNFTDRSSWNLSEIVSYTDKFTS VCLKAAWSSKAEKEVEKHEHKSIEKSEELKAWAEKTL GLLRFNSDDEKFTSVKDETKQHFGLSVACAMAKKLH NDLFPQTA |
| MRL-19-00660_contig31_33246_33695_- | gb| AEO04364.1| gp28 [Listeria monocytogenes J0161] | 2.02671e-86 | 0.697986577181 | 1 | MSKILLDEFLKHLTQLRGLTQSGNTLNDYNNKELNKLK YSVSFLRSMAGCSTDFVIELAELEKSYDGLFKHLD DKKLYLSPAGEFELYLKEFESANIEVLPTFNRFENEEHV NIEKDCVKALENATVLEKNEEL |

Anti-CRISPR protein sequences identified in the studied genomes are shown.

isolated in meats and RTE prepared dishes. There was a diverse distribution of the other STs, including ST1, ST3, ST5, ST7, ST9, ST14, ST193, and ST451, which have been isolated in outbreaks, clinical cases, and different foods (Althaus et al., 2014; Amajoud et al., 2018; Cabal et al., 2019; Ulloa et al., 2019; Halbedel et al., 2020). Six of the fourteen L. monocytogenes strains belonged to serogroup IIa (serotype 1/2a; ST7, ST18, ST193, and ST451), six to serogroup IIb (serotype 1/2b; ST3, ST5, ST14, and ST2763), one to serogroup IVb (serotype 4b; ST1), and one to serogroup IIc (serotype 1/2c; ST9). These four serotypes have been associated with more than 98% of reported cases of listeriosis worldwide (Gorski, 2021).

The treatment for listeriosis includes antibiotics such as ampicillin, tetracyclines, amoxicillin, and sulfamethoxazole (Thennings et al., 2016). In the present study, 11 isolates were susceptible to all the antibiotics, while only 3 exhibited resistance to ampicillin. This is a cause for concern because previous reports in Chile have indicated the susceptibility of L. monocytogenes to ampicillin and also because ampicillin and amoxicillin are currently used to treat this infection (Seoane, 2013; Kumaraswamy et al., 2018). Several authors have encountered resistance to ampicillin in L. monocytogenes strains isolated from raw and cooked meats and fish products with a prevalence between 6 and 83% (Yucel et al., 2005; Jamali et al., 2013; Arslan and Baytur, 2019; Bustamante et al., 2020; Mackiw et al., 2020).

Emerging resistance to penicillin in clinical strains poses a major public health concern because penicillin is the standard treatment for human listeriosis (Martinez et al., 2001). Therefore, the fact that we detected ampicillin-resistant L. monocytogenes strains in the present study should alert authorities and food manufacturers to the latent risk associated with the consumption of these RTE foods contaminated by this pathogen.

We found the presence of resistance genes with mechanisms of antibiotic efflux (norB), antibiotic target alteration (mpfR), and antibiotic inactivation (lin, fosX). In addition, our study reported genes that confer resistance to tetracycline (tetA and tetC). Wilson et al. (2018) reported that all strains displayed the resistance gene to fosfomycin (foss); however, they did not detect any genes associated with tetracycline (tetA) or erythromycin (ermABC). This differs from our study in which the fosX and tetA genes were identified. Mafuna et al. (2021) encountered resistance genes in strains such as fosX, lin, mprF, and norB, and they reported an increasing global trend of resistance genes present in the food chain. L. monocytogenes is currently considered to be intrinsically resistant to fosfomycin because of the lack of expression in the membrane transport systems and a natural resistance to lincomycin due to the ribosomal protection of an ATP-binding cassette F (ABC-F) protein (Mota et al., 2020).

However, there was a difference in our study between the prediction of resistance genes and antibiotic susceptibility testing, which is due to the existence of intrinsic resistance according to some authors (Cox and Wright, 2013); in addition, the resistance genes are ancient and predate the use of antibiotics (Kashuba et al., 2017; Peterson and Kaur, 2018). Gygli et al. (2019) reported an increasing global trend of resistance genes present in the present study should alert authorities and food manufacturers to the latent risk associated with the consumption of these RTE foods contaminated by this pathogen.
Salmonella presence of resistance genes and resistance phenotypes in too high, thus misclassifying strains as susceptible. Aljahdali established to classify it as resistant have cutoff scores that are tuberculosis strains could arise because the clinical concentrations of many MGEs encoding transposases, such as insertion sequences (IS) and transposons, and other recombinases that are determinants in the dissemination of adaptive foreign DNAs and resistance (Kuenne et al., 2010). The most common plasmids were inc18(rep25), inc18(rep26), and rep3(rep26), and only one strain showed N1-011A. The plasmid incompatibility group inc18 is naturally found in Streptococcus and Enterococcus spp. (Zhu et al., 2010) and encodes a variety of resistance to antibiotics due to their overuse in environmental and food settings (Kohler et al., 2018). In addition, plasmid N1-11A has been found in RTE seafood processing plants in France and in the food chain in South Africa: it is associated with the resistance to disinfectants such as benzalkonium chloride (Mafuna et al., 2021).

CRISPR-Cas systems are acquired immunity systems that allow bacteria and archaea to acquire exogenous material from bacteriophages and plasmids (Hupfeld et al., 2018). The CRISPR-Cas systems is a possible involved in the regulation of gene expression, including virulence genes, which have been described in a number of pathogens (Louwen et al., 2014). It was possible to determine that the repeated sequences and associated cas genes in the studied L. monocytogenes strains corresponded to type II-B systems and that the presence of the cas8b and cas9 genes allowed their classification in subtype B. However, the arrays could be related to one cas gene, likewise in the systems that only show sequences that encode for cas3 and cas2. Kuenne et al. (2013) studied CRISPR-Cas in three different loci of L. monocytogenes strains. CRISPR-Cas locus 1 was characterized by a single CRISPR matrix, locus 2 belonged to type I-B, and locus 3 was classified as type II-A. CRISPR-Cas locus 1 was previously found as being associated with the presence of a tracrRNA, which is suggested to control virulence in L. monocytogenes strain 1/2a EGD-e during growth in macrophages; however, it is still unknown how this track RNA could control virulence (Mraheil et al., 2011). Louwen et al. (2013) showed that the ability to translocate through intestinal walls was suppressed when deleting cas9 in Campylobacter jejuni isolates, which affected virulence. The same authors reported that supplementing C. jejuni isolates with cas9, which does not have a CRISPR-Cas system, significantly increased virulence in this pathogen This can also be associated with the array size because those in which these genes are absent have smaller arrays.

For as the spacer sequences, they provide us with the history of the invasive elements to which the bacterium has been subjected because these sequences are associated with exogenous material. For arrays identified in the present study, spacers were related to sequences corresponding to bacteriophages that specifically infected the Listeria genus. Therefore, those bacteria that have this information are able to evade infection by these bacteriophages, unlike those that do not. The phages have also been able to develop strategies in response to CRISPR-Cas, such as the Anti-CRISPR proteins, which were identified in the genomes under study. It has been determined for L. monocytogenes that the prophages show anti-Cas9 proteins such as AcrIIA1, which successfully blocks and inactivates Cas9.
CONCLUSION

Listeria monocytogenes strains isolated from RTE foods exhibited multiple virulence factors and antibiotic resistance factors after in vitro and in silico analyses. It is therefore necessary to perform continuous genomic surveillance on these foods because of the risk associated with L. monocytogenes contamination and their consumption by populations at risk.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

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AUTHOR CONTRIBUTIONS

JP-F, OH, FB, SL, AP, AC-F, AC-C, MT, GF, and WR conceived the experiments and prepared the manuscript. JP-F, FB, AC-F, JM-R, CC, CO, JX-C, MA-L, SL, and MT conducted the laboratory work. JP-F, OH, AC-C, JX-C, JM-R, SL, and WR drafted the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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