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RECEIVED 06 February 2023

ACCEPTED 14 April 2023

PUBLISHED 10 May 2023

CITATION

Ramadan H, Al-Ashmawy M, Soliman AM,
Elbediwi M, Sabeq I, Yousef M, Algammal AM,
Hiott LM, Berrang ME, Frye JG and
Jackson CR (2023) Whole-genome sequencing
of *Listeria innocua* recovered from retail milk
and dairy products in Egypt.
Front. Microbiol. 14:1160244.
doi: 10.3389/fmicb.2023.1160244

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Whole-genome sequencing of *Listeria innocua* recovered from retail milk and dairy products in Egypt

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The similarity of the *Listeria innocua* genome with *Listeria monocytogenes* and their presence in the same niche may facilitate gene transfer between them. A better understanding of the mechanisms responsible for bacterial virulence requires an in-depth knowledge of the genetic characteristics of these bacteria. In this context, draft whole genome sequences were completed on five *L. innocua* isolated from milk and dairy products in Egypt. The assembled sequences were screened for antimicrobial resistance and virulence genes, plasmid replicons and multilocus sequence types (MLST); phylogenetic analysis of the sequenced isolates was also performed. The sequencing results revealed the presence of only one antimicrobial resistance gene, *fosX*, in the *L. innocua* isolates. However, the five isolates carried 13 virulence genes involved in adhesion, invasion, surface protein anchoring, peptidoglycan degradation, intracellular survival, and heat stress; all five lacked the *Listeria* Pathogenicity Island 1 (LIPI-1) genes. MLST assigned these five isolates into the same sequence type (ST), ST-1085; however, single nucleotide polymorphism (SNP)-based phylogenetic analysis revealed 422–1,091 SNP differences between our isolates and global lineages of *L. innocua*. The five isolates possessed an ATP-dependent protease (*clpL*) gene, which mediates heat resistance, on a *rep25* type plasmids. Blast analysis of *clpL*-carrying plasmid contigs showed approximately 99% sequence similarity to the corresponding parts of plasmids of *L. monocytogenes* strains 2015TE24968 and N1-011A previously isolated from Italy and the United States, respectively. Although this plasmid has been linked to *L. monocytogenes* that was responsible for a serious outbreak, this is the first report of *L. innocua* containing *clpL*-carrying plasmids. Various genetic mechanisms of virulence transfer among *Listeria* species and other genera could raise the possibility of the evolution of virulent strains of *L. innocua*. Such strains could challenge processing and preservation protocols and pose health risks from dairy products. Ongoing genomic research is necessary to identify these alarming genetic changes and develop preventive and control measures.

KEYWORDS

Listeria innocua, whole-genome sequencing, genetic context, *clpL* gene, phylogenetic analysis, milk, Egypt

Introduction

Food animals are considered a major reservoir for human infection with foodborne pathogens such as non-typhoidal *Salmonella*, *Campylobacter* species, *Escherichia coli*, and *Listeria* spp. (Heredia and García, 2018). Genus *Listeria* includes different species that are either pathogenic or non-pathogenic. *Listeria monocytogenes* and *L. ivanovii*, two pathogenic species belonging to *Listeria sensu strictu*, are the primary agents that cause listeriosis in humans and animals, respectively (Schardt et al., 2017). The pathogenicity of these two species is linked to an approximately 9 kb virulence gene cluster (Schmid et al., 2005). Other *Listeria* species, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. marthii*, and *L. grayi* are thought to be likely non-pathogenic *Listeria* species (Liu, 2013). Except for *L. grayi*, which belongs to the *Listeria sensu lato* group, all of the presumed non-pathogenic species were classified as *Listeria sensu strictu* and are considered saprophytes (Chiara et al., 2015).

The patterns of genome evolution in *Listeria sensu strictu* and *sensu lato* isolates were investigated in previous sequencing papers (Schmid et al., 2005; den Bakker et al., 2010; Chiara et al., 2015). Surprisingly, these papers documented the acquisition of virulence factors that were lacking in non-pathogenic species of *Listeria sensu strictu* and *sensu lato* at the time they were identified. Gene duplication, gene divergence, and lateral gene transfer—most often from sources outside of *Listeria*—are all common throughout the genus and are presumed to be the underlying mechanisms of bacterial evolution (Chiara et al., 2015).

Despite the fact that *L. innocua* is typically nonhemolytic, a previous study documented the existence of an atypical hemolytic *L. innocua* isolate, which contained every member of the *L. monocytogenes* *prfA*-regulated virulence gene cluster (*Listeria* pathogenicity island 1) but was avirulent in the mouse pathogenicity test (Johnson et al., 2004). Nevertheless, cases of animal listeriosis caused by *L. innocua* which shared a close genetic relationship with *L. monocytogenes* have been reported (Matto et al., 2022). Therefore, ongoing characterization of atypically pathogenic *Listeria* species, as well as changes in their taxonomy, are critical for development and testing of preservation and processing conditions that prevent their growth and spread in the food industry (Orsi and Wiedmann, 2016).

Whole-genome sequencing (WGS) is now recognized as the most effective approach for genetic characterization of pathogens compared to time-consuming and labor-intensive conventional molecular diagnostic techniques that include isolation, identification, and molecular confirmation of the microbe (Lüth et al., 2018; Uelze et al., 2020). WGS can be used to uncover traits such as those responsible for pathogenicity/virulence, antibiotic resistance, and dissemination of mobile genetic elements. WGS can further be applied to provide a more precise description of the taxonomic differences and phylogenetic relatedness between pathogens through multilocus sequence typing (MLST), clonal complex (CC) determination, core genome MLST (cgMLST) and single nucleotide polymorphism (SNP)-based phylogeny (Ramadan et al., 2021; Stessl et al., 2021; Parra-Flores et al., 2022).

Non-pathogenic *Listeria* spp. particularly *L. innocua*, have been reported in different studies from Egypt from different sources (El-Shenawy et al., 2011; Ismaiel et al., 2014; Dahshan et al., 2016), yet there is a lack of information about its genomic characterization. We aimed in the present study to characterize five *L. innocua* isolates from milk and dairy products. Our goal was to test the emergence of

virulence factors and genetic components that enable bacteria to survive, spread, and cause listeriosis.

Materials and methods

Listeria innocua isolates for whole-genome sequencing

Five *L. innocua* isolates (four from raw milk and one from yoghurt) were chosen from our previous study (Youssef et al., 2020) and subjected to WGS for further genomic characterization. Isolates were recovered from raw milk and dairy products (yoghurt and ice cream) purchased from local supermarkets and retail stores in Mansoura City, Egypt during the period between 2014 and 2018. A 25 mL sample from raw milk or 25 g from dairy products were homogenized with 225 mL of trypticase soy broth (TSB), and the sample-TSB mixture was then processed for culturing on *Listeria* selective media as previously described (Youssef et al., 2020). *Listeria* spp. isolates were submitted to the Bacterial Epidemiology and Antimicrobial Resistance (BEAR) Research Unit, United States Department of Agriculture (USDA), Athens, Georgia, United States, through a Material Transfer Research Agreement between Mansoura University and USDA (Agreement No. 58-6040-0-001-F). Isolates were revived by culture in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, United States) which was incubated for 24 h at 35°C. From incubated BHI broth, 10 µL were streaked onto Modified Oxford (MOX) agar with selective supplement (Oxoid, Basingstoke, Hampshire, United Kingdom) and plates were then incubated at 37°C for 24 h. Characteristic small black *Listeria* spp. colonies were picked from MOX agar and plated onto nutrient agar plates for further biochemical identification using Vitek (BioMérieux, Durham, NC, United States).

Genomic DNA extraction, DNA library preparation and whole-genome sequencing

Genomic DNA (gDNA) was extracted from *Listeria* isolates using the blood and tissue genomic DNA extraction kit (Qiagen, Germantown, MD, United States); purity of extracted gDNA was assessed using a NanoDrop™ spectrophotometer. The concentration of gDNA was determined using a Qubit® double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Life Technologies Inc., Carlsbad, CA, United States) on an Invitrogen Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer's instructions. Sequencing libraries were prepared using Nextera™ XT DNA Sample Preparation Kit and Nextera™ XT Index Kit (Illumina Inc., San Diego, CA, United States). Libraries were sequenced on an Illumina MiSeq platform using a MiSeq v2 reagent kit (Illumina Inc., San Diego, CA, United States) with 500 cycles to generate a paired-end read length of 2 × 250 bp. Quality check of raw reads was determined using FastQC tool,¹ and low

1 <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

quality reads were trimmed using trimmomatic with the following parameters: leading: 10, trailing: 10, sliding window: 4:20, and minlen: 40. Obtained reads were then *de novo* assembled using A5-miseq assembler (Coil et al., 2015) and assembly statistics that included genome length, number of contigs, coverage, GC% and N50 were determined.

Bioinformatics analysis

Assembled fasta files were uploaded into Galaxy server² and screened against different databases: ResFinder (Zankari et al., 2012), virulence factor database (VFDB) (Chen et al., 2016), and PlasmidFinder (Carattoli et al., 2014) using the ABRicate (version 1.0.1) tool³ for identification of resistance and virulence genes and plasmid replicons, respectively. Further *in silico* analysis was performed using Plasmid SPAdes and PLACNETw tools to separate plasmid contigs from WGS of the examined isolates (Vielva et al., 2017). The reconstructed plasmid sequences were then blasted against the National Center for Biotechnology Information (NCBI) database to determine the closely matched plasmids. A genetic comparison was performed between the reconstructed *L. innocua* plasmids from this study and plasmid sequences retrieved from NCBI using the BLAST Ring Image Generator (BRIG) tool.⁴ Linear comparison to determine the genetic environment of resistance and/or virulence genes located on *L. innocua* plasmids was also determined using the Easyfig⁵ tool (Sullivan et al., 2011). Other mobile genetic elements such as insertion sequences (IS) were searched for using the ISFinder⁶ tool.

To determine MLST types for the examined *Listeria* isolates, the assembled sequences were blasted against the *Listeria* sequence typing database available on BIGSdb.⁷ To put our isolates into context with global lineages of *L. innocua*, a single nucleotide polymorphism (SNP)-based phylogenetic analysis was performed using Snippy v4.4.4⁸ with the following variant calling parameters: minimum base quality 60, minimum read coverage 10 and minimum proportion for variant evidence 0.9. Our five isolates were compared to publicly available genomes ($n = 260$) of *L. innocua* recovered from different sources in the NCBI database (updated April 10th, 2021), and all the enrolled isolates were mapped to the reference *L. innocua* ATCC 33091 genome. Metadata for *L. innocua* sequences retrieved from NCBI database are listed in Supplementary Tables S1, S2. Output files from Snippy that determined SNPs variant calling were combined using Snippy core into a core SNPs alignment. Using the Randomized Accelerated Maximum Likelihood (RAXML) tool, maximum likelihood phylogenetic trees were generated from SNPs alignment, and the trees were then visualized with iTOL (Letunic and Bork, 2016).

Results

In this study, the 5 *L. innocua* isolates from milk and dairy products were subjected to WGS. Assembly statistics of the sequences of *L. innocua* are listed in Table 1. The genome size was 2.96 Mbp with GC% ranging from 37.2 to 37.3, which is consistent with average genome size and GC% of the complete genome of *L. innocua*. Draft genome sequences of the examined isolates were assembled into an average of 16 contigs (14 contigs for LI-36, 15 for L-I33 and LI-35, 16 for LI-34 and 18 for LI-32), with N50 of 544,812 bp for isolate LI-32, 544,816 bp for isolates LI-34 and LI-35 and 545,607 bp for isolates LI-33 and LI-36.

ResFinder analysis of the examined 5 *L. innocua* revealed absence of antimicrobial resistance genes; only *fosX* responsible for intrinsic resistance of *Listeria* spp. to fosfomycin was identified. Screening *L. innocua* contigs using the ABRicate tool in Galaxy against the virulence factor database (VFDB), with a minimum 80% identity and coverage showed the presence of multiple virulence genes belonging to adherence (*fbpA*, and *lap*), invasion (*iap/cwhA*, *gtcA*, and *lpeA*), surface protein anchoring (*lspA*), peptidoglycan modification (*oatA*, and *pdgA*), intracellular survival (*lplA1*, and *prsA2*) and heat shock proteins (*clpC*, *clpE*, and *clpP*). PlasmidFinder results exhibited the presence of a single plasmid replicon type (rep25) in the examined isolates (Table 1).

Using plasmid SPAdes and PLACNETw tools, plasmid contigs were distinguished from the draft genome sequences of the examined isolates. Blast analysis of the reconstructed plasmids from our *L. innocua* isolates showed high sequence similarity with plasmids of *L. monocytogenes*: clinical strain 2015TE24968 (accession no. CP015985.1) isolated from an outbreak of invasive listeriosis in Italy in 2015 and environmental strain N1-011A (accession no. CP006611.1) isolated from the United States. Noticeably, the reconstructed *L. innocua* plasmids (pLI-32, pLI-33, pLI-34, pLI-35, pLI-36) carried the *clpL* gene which mediates heat resistance (Figure 1). Blast analysis of *clpL* carrying contigs from our isolates showed approximately 99% sequence similarity to the corresponding parts of plasmids of *L. monocytogenes* strain 2015TE24968 and strain N1-011A. Genetic context of *clpL* in our *L. innocua* and the retrieved NCBI sequences of *L. monocytogenes* revealed the presence of insertion sequence ISLmo8 and ISLmo9 downstream and upstream to the *clpL* gene, respectively (Figure 2).

The allelic profile for the seven housekeeping genes retrieved from MLST 2.0 was determined as follows: *abcZ*, 188; *bglA*, 157; *cat*, 182; *dapE*, 223; *dat*, 136; *ldh*, 353; *lhkA*, 148 that assigned the 5 *L. innocua* isolates, regardless of isolate source, into the same sequence type ST-1085. We performed a SNP-based phylogenetic analysis to compare our isolates with the global lineages of *L. innocua*. Findings of SNP-based phylogeny revealed that no specific trends have been observed for clustering isolates based on their source. This was observed from the clustering of examined isolates in a clade with *L. innocua* isolates from different sources, food, and environment (Figure 3A). To zoom in on the differences among isolates within this clade, isolates were separately subjected to a SNP-based phylogeny (Figure 3B). No more than 10 SNP differences were identified among this study isolates, indicating that the five isolates from milk and yoghurt are clonal. The number of SNPs between our isolates and closely related *L. innocua* isolates (SAMN12370801, SAMN12374550, SAMN14487858, SAMN10075820, SAMN17265727, SAMN17153928, and

2 <https://usegalaxy.org/>

3 <https://github.com/tseemann/abricate>

4 <http://sourceforge.net/projects/brig>

5 <http://mjsull.github.io/Easyfig/>

6 <https://www-is.biotoul.fr/blast.php>

7 <http://bigsdw.web.pasteur.fr/listeria/>

8 <https://github.com/tseemann/snippy>

TABLE 1 Assembly statistics, multilocus sequence typing (MLST), resistance and virulence genes and plasmid replicons of *clpL*-producing *Listeria innocua* recovered from milk and dairy products from Egypt.

Isolates genomic features	LI-32	LI-33	LI-34	LI-35	LI-36
Source/Sample ID	Raw milk/M1	Raw milk/M2	Yoghurt/Y2	Raw milk/M7	Raw milk/M7
Season/year of isolation	Summer/2014	Summer/2014	Summer/2018	Fall/2014	Fall/2014
Genome length (Mbp)	2.96	2.96	2.96	2.96	2.96
No. of contigs	18	15	16	15	14
N50 (bp)	544,812	545,607	544,816	544,816	545,607
Median coverage	115	124	87	90	109
GC content	37.3%	37.2%	37.3%	37.3%	37.2%
MLST	ST-1085	ST-1085	ST-1085	ST-1085	ST-1085
Resistance genes	<i>fosX</i>	<i>fosX</i>	<i>fosX</i>	<i>fosX</i>	<i>fosX</i>
Virulence genes	<i>fbpA, lap, iap/cwhA, gtcA, lpeA, lspA, oatA, pdgA, lplA1, prsA2, clpC, clpE, clpP</i>	<i>fbpA, lap, iap/cwhA, gtcA, lpeA, lspA, oatA, pdgA, lplA1, prsA2, clpC, clpE, clpP</i>	<i>fbpA, lap, iap/cwhA, gtcA, lpeA, lspA, oatA, pdgA, lplA1, prsA2, clpC, clpE, clpP</i>	<i>fbpA, lap, iap/cwhA, gtcA, lpeA, lspA, oatA, pdgA, lplA1, prsA2, clpC, clpE, clpP</i>	<i>fbpA, lap, iap/cwhA, gtcA, lpeA, lspA, oatA, pdgA, lplA1, prsA2, clpC, clpE, clpP</i>
Plasmid replicon type	<i>rep25</i>	<i>rep25</i>	<i>rep25</i>	<i>rep25</i>	<i>rep25</i>
Accession No.	JAQHRC000000000	JAQHRB000000000	JAQHRA000000000	JAQHQQ000000000	JAQHQQ000000000

fbpA, fibronectin-binding protein; *lap*, listeria adhesion protein; *iap/cwhA*, cell wall hydrolase; *gtcA*, cell wall teichoic acid glycosylation protein; *lpeA*, lipoprotein promoting entry protein; *lspA*, lipoproteins-specific signal peptidase II; *oatA*, O-acetyltransferase gene; *pdgA*, peptidoglycan N-deacetylase; *lplA1*, lipote protein ligase A1; *prsA2*, post-translocation chaperone; *clpC*, ATP-dependent proteases.

SAMN12374702) ranged from 422 to 1,091 (Supplementary Table S3); where all isolates were assigned to the same ST type (ST-1085) and were sourced from food except isolate SAMN14487858 that was recovered from the environment.

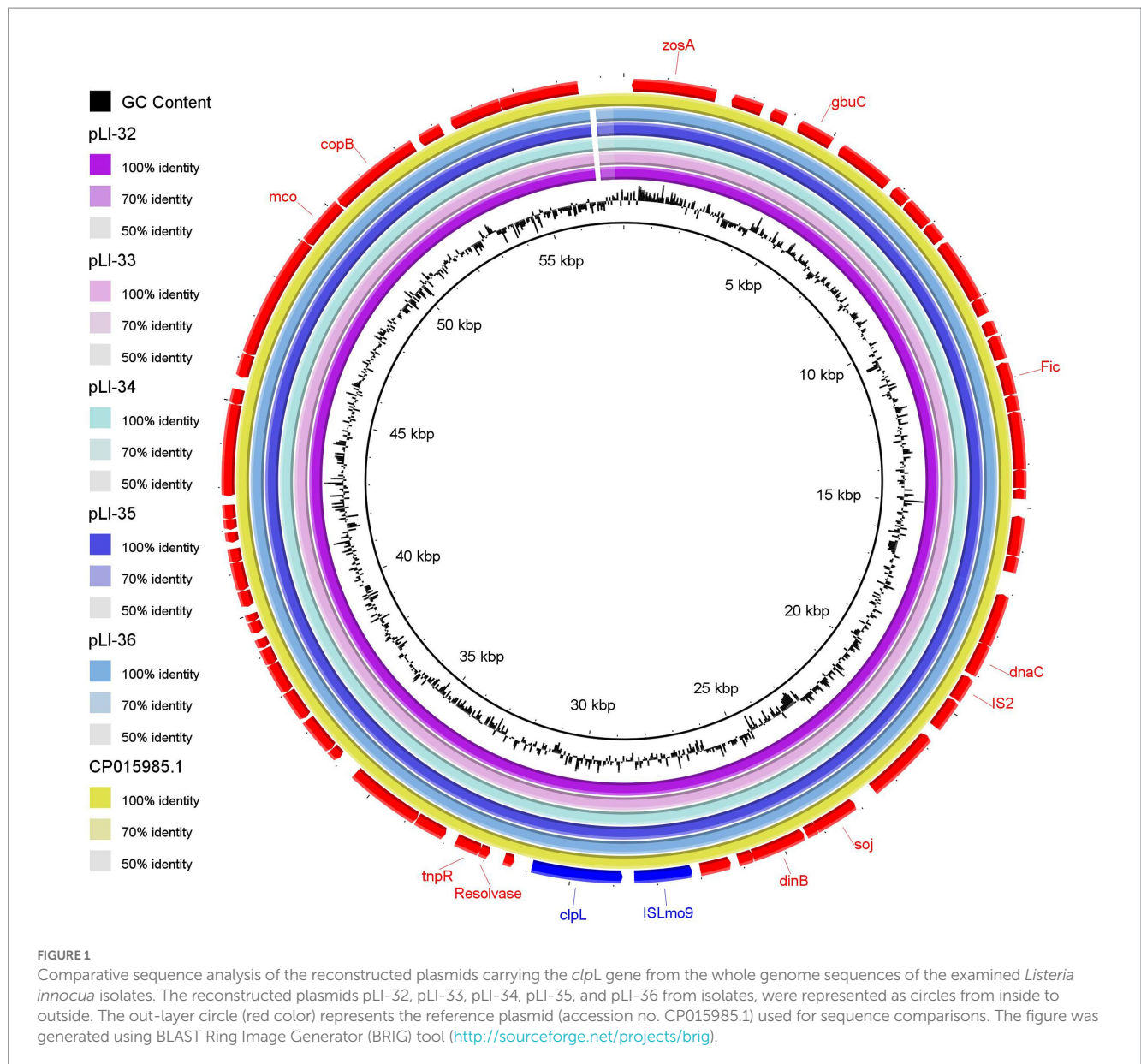
Discussion

Listeria infection is a significant foodborne disease that can be transmitted to humans primarily *via* the food chain. Many food sources are implicated in *Listeria* foodborne infections, including milk and milk products, an important source of protein available to many groups of consumers (Bintsis, 2017; Kim et al., 2018). The pathogenic potential of *L. monocytogenes* is explained by its possession of certain virulence genes that increases bacterial fitness and survivability (Abdelhamed et al., 2022; Osek et al., 2022); however, the pathogenic potentiality of some non-pathogenic *Listeria* spp. that are isolated at a high rate requires further research to explain. Here, we performed the comparative genome sequence analysis of *L. innocua* recovered from dairy and dairy products in Egypt.

As indicated from WGS, we observed absence of antimicrobial resistance genes except FosX. This gene that confers resistance to fosfomycin has been identified in previous studies from *Listeria* spp. (Scotti et al., 2018; Wilson et al., 2018; Parra-Flores et al., 2022). In both pathogenic and non-pathogenic *Listeria* spp., a FosX enzyme that is expressed by the *fosX* gene confers intrinsic fosfomycin resistance. However, when infected with pathogenic *L. monocytogenes*, the host signals activate *prfA* virulence regulons, *hpt*, and *prfA* and epistatically promote greater fosfomycin influx into the bacterial cell, suppressing *fosX*-mediated resistance (Scotti et al., 2018). Current whole genome sequenced *L. innocua* isolates showed absence of both *prfA* virulence regulons, *hpt* and *prfA*, indicating that fosfomycin treatment would not be effective under infection circumstances. ResFinder results also revealed absence of other antimicrobial resistance genes in isolates

from this study. The few antimicrobial resistance genes found in these *L. innocua* isolates were generally consistent with past findings (Hof, 1991; Charpentier and Courvalin, 1999), and the most recent study conducted in the United States between 2010 and 2021 (Jorgensen et al., 2021; Hanes and Huang, 2022). As reported in these studies, most *L. monocytogenes* as well as strains of other *Listeria* spp., were found to be susceptible to a wide range of antimicrobials and there was no increase in antimicrobial resistance genes except for cephalosporin, fosfomycin, lincosamide and tetracycline resistance genes. Despite the few occurrences of antimicrobial resistance genes among the examined *L. innocua* isolates, which is considered a good sign for infection treatment, the spread of antimicrobial resistance among different bacterial species remains a major problem in Egyptian dairy farms (Tartor et al., 2021; Badawy et al., 2022). Further studies on larger sets would be helpful to determine the state of antimicrobial resistance in *Listeria* from milk and dairy products, since high antimicrobial resistances among different bacterial species from dairy farms in Egypt prevail.

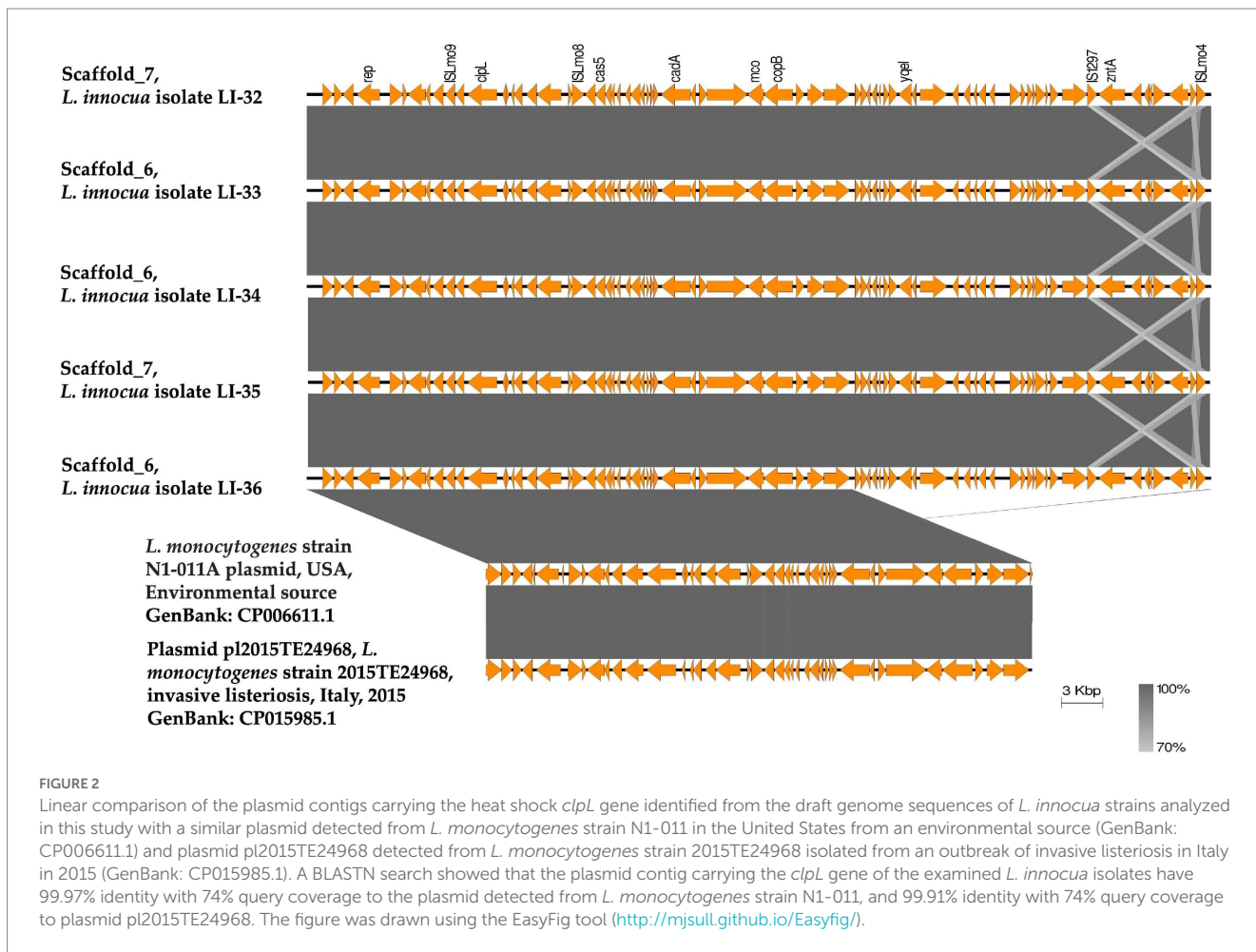
L. monocytogenes pathogenesis requires the coordinated expression of six genes, namely *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*, which are primarily assembled in the 9 kb *Listeria* Pathogenicity Island 1 (LIPI-1) (Vázquez-Boland et al., 2001). The *prfA* gene encodes a transcriptional activator, *prfA*, which directly or indirectly induces the transcription of over 140 genes, including the other five genes found in LIPI-1 (Paramithiotis et al., 2014). Both *plcA* and *plcB* encode phospholipases C (phosphatidylinositol and phosphatidylcholine), which, in conjunction with listeriolysin O (LLO), protect *Listeria* from cytoplasmic phagosomes (Schlüter et al., 1998). The *mpl* encodes a zinc-metalloprotease required for pro-*plcB* maturation (Raveneau et al., 1992), and *actA* is a multifunctional virulence factor (Travier et al., 2013). Our findings revealed the existence of 13 virulence genes among the examined isolates, belonging to adherence (*fbpA*, and *lap*), invasion (*iap/cwhA*, *gtcA*, and *lpeA*), surface protein anchoring (*lspA*), peptidoglycan



modification (*oatA*, and *pdgA*), intracellular survival (*lplA1*, and *prsA2*) and heat shock proteins (*clpC*, *clpE*, and *clpP*). These genes that encode for minor or accessory virulence factors, were detected in most *L. innocua* strains. Nevertheless, *L. innocua* isolates carrying the above virulence profile were found associated with clinical cases in different hosts such as ruminants (Matto et al., 2022), and birds (Moura et al., 2019). Fortunately, none of the important LIPI-1 genes were found in the currently studied *L. innocua*. Virulence genes such as *hly*, which is responsible for the β-hemolysis associated with *L. monocytogenes* and some atypical hemolytic *L. innocua* (Johnson et al., 2004), were not identified in these *L. innocua* isolates.

Listeria spp. strains might acquire genes via mobile genetic elements, enhancing their capacity to endure and colonize a variety of food processing environments. These resistance and adaptation genes can increase tolerance of *Listeria* to a variety of stressors, such as sanitizers, antimicrobials, and environmental toxins, as well as extremes in salinity, acidity, and temperature. Transfer of such genes may also explain the widespread nature of *Listeria* in both natural

and agricultural settings (Korsak and Szuplewska, 2016; Hingston et al., 2017; Pöntinen et al., 2017). Several genes that are responsible for the survivability and pathogenicity of *Listeria* spp., are plasmid mediated which can be transferred within and between *Listeria* spp. (Kuenne et al., 2010; Schmitz-Esser et al., 2021). Based on their replication protein *repA*, *Listeria* plasmids mostly belong to groups 1 and 2 which are commonly known in plasmid database (PLSDB) as *rep25* and *rep26*, respectively. The examined *L. innocua* isolates were found possessing plasmids of *rep25* replicon type; this replicon type was previously identified in *L. monocytogenes* strain 2015TE24968, causing a severe listeriosis outbreak in Central Italy (Orsini et al., 2018) and environmental strain N1-011A (GenBank accession number CP006611.1) isolated in the United States. The ATP-dependent protease (*clpL*), which increases heat resistance, was initially discovered in *L. monocytogenes* harboring plasmid-borne *clpL*. It was revealed that *clpL* gene introduction into a naturally heat-sensitive strain of *L. monocytogenes* greatly boosted the recipient strain's heat resistance, but plasmid-borne *clpL* deletion



resulted in dramatically lower heat resistance in the wild-type strain (Pöntinen et al., 2017). To our knowledge, this is the first time that plasmid-borne *clpL* has been reported in *L. innocua*. The presence of such plasmid-borne *clpL* in *L. innocua* isolated from dairy products may allow them to survive at high processing temperatures, necessitating a stricter food product control protocol. It is also critical to understand the role of these plasmid-mediated genes in the pathogenicity and persistence of *Listeria* in challenging and *in vitro* environments. Fortunately, due to the similarities in ecology, genome, and physiological traits between both *L. monocytogenes* and *L. innocua*, researchers are able to control *L. monocytogenes* by using *L. innocua* surrogates and identifying hidden metabolomic tools for surviving stressors (He et al., 2021; Wu et al., 2021). Considering this, further research into the metabolic changes caused by the plasmid-borne *clpL* gene in *L. innocua* will be required.

WGS has become a powerful tool used for routine epidemiological surveillance of infectious diseases, outbreak investigations, and tracing transmission routes (Oakeson et al., 2018; Brown et al., 2021). MLST and SNP-based phylogeny are frequently used for bacterial subtyping, that enables precise source attribution and identification of the origin of pathogen associated-outbreaks (Stessl et al., 2021). In the present study, the 5 *L. innocua* isolates sourced from milk and yoghurt were

assigned to the same sequence type, ST-1085. SNP-based phylogeny confirmed the MLST findings of the sequenced isolates, and few SNP differences (less than 10 SNPs) were identified among isolates indicating the circulation of the same *L. innocua* clone in raw milk and dairy product. Comparing our isolates to the global lineages of *L. innocua*, our isolates showed close similarity with *L. innocua* isolates (422-1,091 SNPs) that were recovered from food and the environment. This highlights the importance of WGS-based phylogeny for monitoring the potential sources of foodborne pathogens.

In conclusion, the sequencing results revealed that current isolates of *L. innocua* lacked the *Listeria* Pathogenicity Island 1 (LIPI-1) gene, the *hly* gene, and any antimicrobial resistance genes, except for the *fosX* gene. However, the absence of *fosX* gene suppressing regulons, *hpt*, and *prfA* suggests that fosfomycin treatment would be ineffective under infection conditions. Furthermore, the 5 *L. innocua* isolates possessed 13 virulence genes involved in adhesion, invasion, surface protein anchoring, peptidoglycan degradation, intracellular survival, and heat stress. Though these genes encode for minor or accessory virulence factors, the close genetic relationship with *L. monocytogenes* and the potential for horizontal virulence gene transfer raises the possibility of evolution of virulent *L. innocua* strains. The

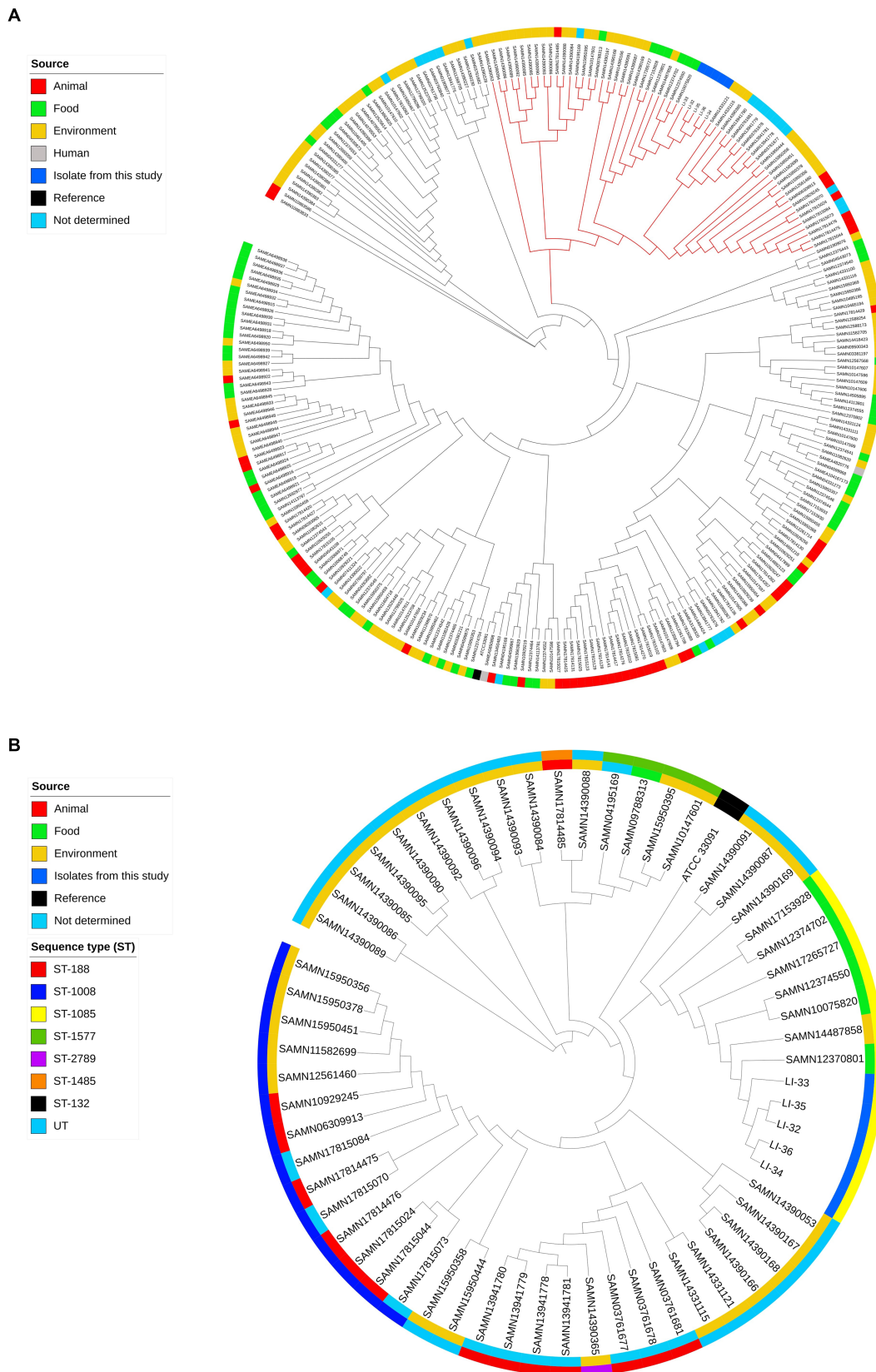


FIGURE 3
(A) Phylogenomic analysis of *Listeria innocua* isolates and publicly available genomes ($n=260$) of *L. innocua* recovered from different sources in the NCBI database (updated April 10th, 2021); all the enrolled isolated were mapped to the reference *L. innocua* ATCC 33091 genome. The clade with branches in red denotes clustering of closely related NCBI *L. innocua* ($n=56$) to our isolates. **(B)** Isolates in this clade were further subjected to SNP-based phylogeny for understanding closer genetic relationships among these isolates. From inside-out, the first circle indicates the source of isolates. The second circle indicates the sequence types (STs).

ATP-dependent protease (*clpL*), mediating elevated heat resistance of strains which carry it, was reported for the first time in the currently studied *L. innocua*, specifically that *clpL* was carried by plasmids. The first documentation of *clpL*-carrying plasmids in *L. innocua*, along with the common virulence and antimicrobial resistance genes, and the ongoing various genetic mechanisms of virulence transfer among *Listeria* and other genus, could endanger current industrial processing and preservation protocols and present health risks from dairy products. Therefore, ongoing genomic studies are necessary to identify these alarming genetic changes and research is needed to develop and test preventive and control measures.

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 at: <https://www.ncbi.nlm.nih.gov/>, Bioproject ID. PRJNA921817.

Author contributions

HR, MA-A, and CJ conceived and designed the study. HR performed the microbiology work and whole-genome sequencing of isolates, analyzed the genome data, and wrote the original draft of the manuscript. AS and ME participated to the analysis of WGS data. IS contributed to the data analysis and manuscript drafting. MY and LH participated to microbiology work. AA contributed to the data analysis. HR, MA-A, MB, JF, and CJ secured funding and provided project administration. HR, MA-A, AS, ME, IS, AA, MB, JF, and CJ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

References

- Abdelhamed, H., Ramachandran, R., Narayanan, L., Islam, S., Ozan, O., Freitag, N., et al. (2022). Role of FruR transcriptional regulator in virulence of *Listeria monocytogenes* and identification of its regulon. *PLoS One* 17:e0274005. doi: 10.1371/journal.pone.0274005
- Badawy, B., Elafify, M., Farag, A. M. M., Moustafa, S. M., Sayed-Ahmed, M. Z., Moawad, A. A., et al. (2022). Ecological distribution of virulent multidrug-resistant *Staphylococcus aureus* in livestock, environment, and dairy products. *Antibiotics* 11:1651. doi: 10.3390/antibiotics11111651
- Bintsis, T. (2017). Foodborne pathogens. *AIMS Microbiol.* 3, 529–563. doi: 10.3934/microbiol.2017.3.529
- Brown, B., Allard, M., Bazaco, M. C., Blankenship, J., and Minor, T. (2021). An economic evaluation of the whole genome sequencing source tracking program in the U.S. *PLoS One* 16:e0258262. doi: 10.1371/journal.pone.0258262
- Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/aac.02412-14
- Charpentier, E., and Courvalin, P. (1999). Antibiotic resistance in *Listeria* spp. *Antimicrob. Agents Chemother.* 43, 2103–2108. doi: 10.1128/aac.43.9.2103
- Chen, L., Zheng, D., Liu, B., Yang, J., and Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res.* 44, D694–D697. doi: 10.1093/nar/gkv1239
- Chiara, M., Caruso, M., D'Erchia, A. M., Manzari, C., Fraccalvieri, R., Goffredo, E., et al. (2015). Comparative genomics of *Listeria sensu lato*: genus-wide differences in evolutionary dynamics and the progressive gain of complex, potentially pathogenicity-related traits through lateral gene transfer. *Genome Biol. Evol.* 7, 2154–2172. doi: 10.1093/gbe/evv131
- Coil, D., Jospin, G., and Darling, A. E. (2015). A5-MISEQ: an updated pipeline to assemble microbial genomes from illumina MiSeq data. *Bioinformatics* 31, 587–589. doi: 10.1093/bioinformatics/btu661
- Dahshan, H., Merwad, A. M. A., and Mohamed, T. S. (2016). *Listeria* species in broiler poultry farms: potential public health hazards. *J. Microbiol. Biotechnol.* 26, 1551–1556. doi: 10.4014/jmb.1603.03075
- den Bakker, H. C., Bundrant, B. N., Fortes, E. D., Orsi, R. H., and Wiedmann, M. (2010). A population genetics-based and phylogenetic approach to understanding the evolution of virulence in the genus *Listeria*. *Appl. Environ. Microbiol.* 76, 6085–6100. doi: 10.1128/AEM.00447-10
- El-Shenawy, M., El-Shenawy, M., Mañes, J., and Soriano, J. M. (2011). *Listeria* spp. in street-vended ready-to-eat foods. *Interdiscip. Perspect. Infect. Dis.* 2011:968031. doi: 10.1155/2011/968031
- Hanes, R. M., and Huang, Z. (2022). Investigation of antimicrobial resistance genes in *Listeria* spp. monocytes from 2010 through to 2021. *Int. J. Environ. Res. Public Health* 19:5506. doi: 10.3390/ijerph19095506
- He, Y., Zhao, X., Chen, L., Zhao, L., and Yang, H. (2021). Effect of electrolysed water generated by sodium chloride combined with sodium bicarbonate solution against *Listeria innocua* in broth and on shrimp. *Food Control* 127:108134. doi: 10.1016/j.foodcont.2021.108134
- Heredia, N., and García, S. (2018). Animals as sources of food-borne pathogens: a review. *Anim. Nutr.* 4, 250–255. doi: 10.1016/j.aninu.2018.04.006
- Hingston, P., Chen, J., Dhillon, B. K., Laing, C., Bertelli, C., Gannon, V., et al. (2017). Genotypes associated with *Listeria monocytogenes* isolates displaying impaired or enhanced tolerances to cold, salt, acid, or desiccation stress. *Front. Microbiol.* 8:369. doi: 10.3389/fmicb.2017.00369

Funding

This work has been funded by the U.S. Department of Agriculture (USDA) project 6040-32000-079-00-D.

Acknowledgments

The authors acknowledge the technical help of Eric Adams, USDA, Athens, GA.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1160244/full#supplementary-material>

- Hof, H. (1991). Therapeutic activities of antibiotics in listeriosis. *Infection* 19, S229–S233. doi: 10.1007/bf01644039
- Ismail, A. A. R., Ali, A. E. S., and Enan, G. (2014). Incidence of Listeria in Egyptian meat and dairy samples. *Food Sci. Biotechnol.* 23, 179–185. doi: 10.1007/s10068-014-0024-5
- Johnson, J., Jinneman, K., Stelma, G., Smith, B. G., Lye, D., Messer, J., et al. (2004). Natural atypical *Listeria innocua* strains with *Listeria monocytogenes* pathogenicity island 1 genes. *Appl. Environ. Microbiol.* 70, 4256–4266. doi: 10.1128/aem.70.7.4256-4266.2004
- Jorgensen, J., Bland, R., Waite-Cusic, J., and Kovacevic, J. (2021). Diversity and antimicrobial resistance of Listeria species and *L. monocytogenes* clones from produce handling and processing facilities in the Pacific northwest. *Food Control* 123:107665. doi: 10.1016/j.foodcont.2020.107665
- Kim, S. W., Haendiges, J., Keller, E. N., Myers, R., Kim, A., Lombard, J. E., et al. (2018). Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). *PLoS One* 13:e0197053. doi: 10.1371/journal.pone.0197053
- Korsak, D., and Szuplewska, M. (2016). Characterization of nonpathogenic Listeria species isolated from food and food processing environment. *Int. J. Food Microbiol.* 238, 274–280. doi: 10.1016/j.ijfoodmicro.2016.08.032
- Kuene, C., Voget, S., Pischmarov, J., Oehm, S., Goesmann, A., Daniel, R., et al. (2010). Comparative analysis of plasmids in the genus Listeria. *PLoS One* 5:e12511. doi: 10.1371/journal.pone.0012511
- Leticic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–W245. doi: 10.1093/nar/gkw290
- Liu, D. (2013). Molecular approaches to the identification of pathogenic and nonpathogenic Listeriae. *Microbiol. Insights* 6, 59–69. doi: 10.4137/mbi.S10880
- Lüth, S., Kleta, S., and Al Dahouk, S. (2018). Whole genome sequencing as a typing tool for foodborne pathogens like *Listeria monocytogenes* – the way towards global harmonisation and data exchange. *Trends Food Sci. Technol.* 73, 67–75. doi: 10.1016/j.tifs.2018.01.008
- Matto, C., D'Alessandro, B., Mota, M. I., Braga, V., Buschiazio, A., Giannechini, E., et al. (2022). *Listeria innocua* isolated from diseased ruminants harbour minor virulence genes of *L. monocytogenes*. *Veter. Med. Sci.* 8, 735–740. doi: 10.1002/vms3.710
- Moura, A., Disson, O., Lavina, M., Thouvenot, P., Huang, L., Leclercq, A., et al. (2019). Atypical hemolytic *Listeria innocua* isolates are virulent, albeit less than *Listeria monocytogenes*. *Infect. Immun.* 87:e00758. doi: 10.1128/iai.00758-18
- Oakeson, K. F., Wagner, J. M., Rohrwasser, A., and Atkinson-Dunn, R. (2018). Whole-genome sequencing and bioinformatic analysis of isolates from foodborne illness outbreaks of campylobacter jejuni and *Salmonella enterica*. *J. Clin. Microbiol.* 56, e00161–e00118. doi: 10.1128/JCM.00161-18
- Orsi, R. H., and Wiedmann, M. (2016). Characteristics and distribution of Listeria spp., including Listeria species newly described since 2009. *Appl. Microbiol. Biotechnol.* 100, 5273–5287. doi: 10.1007/s00253-016-7552-2
- Orsini, M., Cornacchia, A., Patavino, C., Torresi, M., Centorame, P., Acciari, V. A., et al. (2018). Whole-genome sequences of two *Listeria monocytogenes* Serovar 1/2a strains responsible for a severe Listeriosis outbreak in Central Italy. *Genome Announc.* 6:e00236. doi: 10.1128/genomeA.00236-18
- Osek, J., Lachtara, B., and Wiczorek, K. (2022). *Listeria monocytogenes* – how this pathogen survives in food-production environments? *Front. Microbiol.* 13:866462. doi: 10.3389/fmicb.2022.866462
- Paramithiotis, S., Hadjilouka, A., and Drosinos, E. H. (2014). “Listeria Pathogenicity Island 1. Structure and function” in *Listeria Monocytogenes: food sources, prevalence and management strategies*. ed. E. C. Hambrick, 265–282.
- Parra-Flores, J., Holý, O., Bustamante, F., Lepuschitz, S., Pietzka, A., Contreras-Fernández, A., et al. (2022). Virulence and antibiotic resistance genes in *Listeria monocytogenes* strains isolated from ready-to-eat foods in Chile. *Front. Microbiol.* 12:796040. doi: 10.3389/fmicb.2021.796040
- Pöntinen, A., Aalto-Araneda, M., Lindström, M., and Korkeala, H. (2017). Heat resistance mediated by pLM58 plasmid-borne *clpL* in *Listeria monocytogenes*. *mSphere* 2:e00364. doi: 10.1128/mSphere.00364-17
- Ramadan, H., Soliman, A. M., Hiott, L. M., Elbediwi, M., Woodley, T. A., Chattaway, M. A., et al. (2021). Emergence of multidrug-resistant *Escherichia coli* producing CTX-M, MCR-1, and FosA in retail food from Egypt. *Front. Cell. Infect. Microbiol.* 11:681588. doi: 10.3389/fcimb.2021.681588
- Raveneau, J., Geoffroy, C., Beretti, J. L., Gaillard, J. L., Alouf, J. E., and Berche, P. (1992). Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene. *Infect. Immun.* 60, 916–921. doi: 10.1128/iai.60.3.916-921.1992
- Schardt, J., Jones, G., Müller-Herbst, S., Schauer, K., D'Orazio, S. E. F., and Fuchs, T. M. (2017). Comparison between Listeria sensu stricto and Listeria sensu lato strains identifies novel determinants involved in infection. *Sci. Rep.* 7:17821. doi: 10.1038/s41598-017-17570-0
- Schlüter, D., Domann, E., Buck, C., Hain, T., Hof, H., Chakraborty, T., et al. (1998). Phosphatidylcholine-specific phospholipase C from *Listeria monocytogenes* is an important virulence factor in murine cerebral listeriosis. *Infect. Immun.* 66, 5930–5938. doi: 10.1128/iai.66.12.5930-5938.1998
- Schmid, M. W., Ng, E. Y., Lampidis, R., Emmerth, M., Walcher, M., Kreft, J., et al. (2005). Evolutionary history of the genus Listeria and its virulence genes. *Syst. Appl. Microbiol.* 28, 1–18. doi: 10.1016/j.syapm.2004.09.005
- Schmitz-Esser, S., Anast, J. M., and Cortes, B. W. (2021). A large-scale sequencing-based survey of plasmids in *Listeria monocytogenes* reveals global dissemination of plasmids. *Front. Microbiol.* 12:653155. doi: 10.3389/fmicb.2021.653155
- Scortti, M., Han, L., Alvarez, S., Leclercq, A., Moura, A., Lecuit, M., et al. (2018). Epistatic control of intrinsic resistance by virulence genes in Listeria. *PLoS Genet.* 14:e1007525. doi: 10.1371/journal.pgen.1007525
- Stessl, B., Wagner, M., and Ruppitsch, W. (2021). Multilocus sequence typing (MLST) and whole genome sequencing (WGS) of Listeria monocytogenes and *Listeria innocua*. *Methods Mol. Biol.* 2220, 89–103. doi: 10.1007/978-1-0716-0982-8_7
- Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039
- Tartor, Y. H., Gharieb, R. M. A., Abd El-Aziz, N. K., El Damaty, H. M., Enany, S., Khalifa, E., et al. (2021). Virulence determinants and plasmid-mediated Colistin resistance MCR genes in gram-negative bacteria isolated from bovine milk. *Front. Cell. Infect. Microbiol.* 11:761417. doi: 10.3389/fcimb.2021.761417
- Travier, L., Guadagnini, S., Gouin, E., Dufour, A., Chenal-Francois, V., Cossart, P., et al. (2013). ActA promotes *Listeria monocytogenes* aggregation, intestinal colonization and carriage. *PLoS Pathog.* 9:e1003131. doi: 10.1371/journal.ppat.1003131
- Uelze, L., Grützke, J., Borowiak, M., Hammerl, J. A., Juraschek, K., Deneke, C., et al. (2020). Typing methods based on whole genome sequencing data. *One Health Outlook* 2:3. doi: 10.1186/s42522-020-0010-1
- Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., et al. (2001). Listeria pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14, 584–640. doi: 10.1128/cmr.14.3.584-640.2001
- Vielva, L., de Toro, M., Lanza, V. F., and de la Cruz, F. (2017). PLACNETw: a web-based tool for plasmid reconstruction from bacterial genomes. *Bioinformatics* 33, 3796–3798. doi: 10.1093/bioinformatics/btx462
- Wilson, A., Gray, J., Chandry, P. S., and Fox, E. M. (2018). Phenotypic and genotypic analysis of antimicrobial resistance among *Listeria monocytogenes* isolated from Australian food production chains. *Genes* 9:80. doi: 10.3390/genes9020080
- Wu, J., Zhao, L., Lai, S., and Yang, H. (2021). NMR-based metabolomic investigation of antimicrobial mechanism of electrolysed water combined with moderate heat treatment against *Listeria monocytogenes* on salmon. *Food Control* 125:107974. doi: 10.1016/j.foodcont.2021.107974
- Youssef, M., Ramadan, H., and Al-Ashmawy, M. (2020). Prevalence of Listeria species in raw milk, ice cream and yogurt and effect of selected natural herbal extract on its survival. *Mansoura Veter. Med. J.* 21, 99–106. doi: 10.21608/mvmj.2020.21.317
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261