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Prevalence of *Listeria* Pathogenicity Islands in *Listeria innocua* Isolates from Meat, Meat Products, and Meat-Processing Environments in Poland

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Among the members of the genus *Listeria*, *Listeria innocua* is the species most commonly isolated from food products and food-processing environments. Although it is generally regarded as non-pathogenic, several reports have described cases of listeriosis caused by atypical *L. innocua* strains harboring virulence-associated genes. In this study, the presence of 28 virulence-associated genes was investigated by PCR in the genomes of 51 *L. innocua* isolates obtained from meat products and meat-processing environments in Poland. The hemolytic phenotype of the isolates was assessed as well. Among the *Listeria* pathogenicity island 1 (LIPI-1)-associated genes, *prfA* was detected in 10% of the isolates, while *plcA* and *hly* were each identified in 6% of the analyzed strains. The *actA1* gene was present in 4% of the isolates. Internalin genes were largely absent, as *inlA* and *inlB* were not detected, while *inlC* and *inlJ* were each identified in only 2% of the isolates. Among other genes, *sigB* was present in all isolates (100%), whereas *iap* and *flaA* were detected in 7 and 6 isolates, respectively. In contrast, genes associated with *Listeria* pathogenicity island 3 (LIPI-3) were considerably more prevalent. Genes located in the 5′ region of the island (*llsA, llsG, llsH,* and *llsX*) were detected in approximately 69–73% of the isolates and frequently co-occurred, whereas genes from the 3′ region were much less common (5–13%). A complete LIPI-3 island was identified in two isolates (4%), while partial LIPI-3 profiles were detected in 40 isolates (78%). Among *Listeria* pathogenicity island 4 (LIPI-4) genes, *licC* and *glvA* were detected in 100% and 94% of the isolates, respectively. In the hemolysis assay, no hemolytic activity was detected in any of the isolates. These findings underscore a heterogeneous distribution of pathogenicity island-associated genes in *L. innocua*.

Keywords: food safety, hemolysis *Listeria innocua,* pathogenicity, virulence

INTRODUCTION

Members of the genus *Listeria* are Gram-positive, bacteria that are ubiquitous in natural ecosystems as well as in agricultural and food-processing environments. Their ability to tolerate osmotic stress, grow at refrigeration temperatures, and form biofilms enables their persistence in food-processing facilities and along the production chain [Cebeci & Otlu, 2024]. Among the described species, *Listeria monocytogenes* is the main pathogen responsible for listeriosis in humans and animals [Kaszoni-Rückerl *et al*., 2020]. In contrast, *Listeria innocua* has long been considered non-pathogenic and is commonly used as a model organism due to its close genetic relatedness to *L. monocytogenes* [Matto *et al*., 2022]. It is frequently isolated from raw materials and food-processing environments, often co-occurring with *L. monocytogenes* and, in many cases, representing the predominant *Listeria* species detected [Arslan *et al*., 2020]. Despite its traditional classification as harmless, recent studies have suggested that certain atypical *L. innocua* strains may harbor virulence-associated determinants.

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These strains may arise through horizontal gene transfer or due to evolutionary divergence from a virulent ancestor shared with *L. monocytogenes* [Ramadan *et al*., 2023; Zanolli Moreno *et al*., 2012]. Phylogenetic analyses support this view, indicating that both species originated from a common ancestor, with the attenuated phenotype of *L. innocua* likely resulting from the loss of key virulence genes [Clayton *et al.*, 2014; Gana *et al.*, 2023]. The presence of pathogenicity islands, such as *Listeria* pathogenicity island 1 (LIPI-1), (*Listeria* pathogenicity island 3 (LIPI-3), and *Listeria* pathogenicity island 4 (LIPI-4), in atypical *L. innocua* isolates further supports their evolutionary and genetic relatedness to *L. monocytogenes* [Clayton *et al.*, 2014; Li M. *et al.*, 2021; Zanolli Moreno *et al.*, 2012]. Although these strains generally exhibit lower virulence than *L. monocytogenes* [Moura *et al.*, 2019], some hemolytic variants have demonstrated the ability to cross the intestinal barrier and disseminate within host tissues [Gwida *et al.*, 2020]. Clinical reports have linked atypical *L. innocua* to severe infections in immunocompromised individuals, including bacteremia [Perrin *et al.*, 2003], meningitis [Favaro *et al.,* 2014], ventriculoperitoneal shunt infection [Karli *et al.*, 2014], neonatal listeriosis [Arumugam *et al.*, 2021], meningoencephalitis [Liao *et al.*, 2022], and joint infection [Gupta *et al.*, 2024]. Animal infections, such as cerebral listeriosis in cattle, have also been described [Matto *et al.*, 2022; Rocha *et al.*, 2013].

Virulence in pathogenic *Listeria* species is mediated by a coordinated set of adhesion, invasion, and intracellular survival factors enabling colonization of the gastrointestinal tract and translocation across the intestinal barrier [Wiktorczyk-Kapischke *et al.*, 2023]. The core virulence determinants are primarily located within LIPI-1, which comprises genes encoding two phospholipases (*plcA* and *plcB*), the pore-forming toxin (*hly*), a metalloprotease (*mpl*), and the actin-polymerization factor (*actA*) responsible for intracellular motility. Expression of LIPI-1 genes is regulated by the transcriptional activator PrfA (encoded by *prfA* gene) [Lee *et al*., 2023]. Among the genes located within LIPI-1, the most critical is *hly*, which encodes listeriolysin O (LLO), a toxin responsible for β-hemolytic activity. *L. innocua*, which typically lacks the *hly* gene, is therefore generally considered a non-hemolytic species. However, atypical *L. innocua* strains exhibiting hemolytic activity have also been described [Johnson *et al*., 2004]. Conversely, rare, atypical *L. monocytogenes* strains, in which spontaneous loss of virulence genes resulted in a nonhemolytic phenotype, have been reported by Olaimat *et al*. [2018]. These observations highlight the necessity for continuous monitoring of hemolytic properties and associated virulence determinants. Another important group of virulence-associated proteins are internalins, *i.e.*, surface proteins mediating adhesion and invasion of host cells. The best-characterized members include *inlA* and *inlB*, while additional internalins, such as *inlC*, *inlJ*, *inlH*, *inlK*, *inlL*, *inlF*, and *inlP*, have also been implicated in host interaction and dissemination [Liu *et al.,* 2007]. Other factors contributing to pathogenic potential include the invasion-associated protein (*iap*), flagellin (*flaA*), and the alternative sigma factor B (*sigB*), which regulates the general stress response and enhances bacterial survival under adverse environmental conditions [Osek & Wieczorek, 2022]. In addition to LIPI-1, hypervirulent *Listeria* strains may harbor LIPI-3 and LIPI-4. LIPI-3 consists of eight genes (*llsA*, *llsG*, *llsH*, *llsX*, *llsB*, *llsD*, *llsP*, and *llsY*) encoding listeriolysin S (LLS), *i.e.*, a second cytolytic peptide that exhibits properties of both a bacteriocin and a hemolytic cytotoxic factor. LLS contributes to the modulation of the intestinal microbiota and promotes gastrointestinal colonization [Clayton *et al.*, 2014; Cotter *et al.*, 2008; Lee *et al*., 2023]. Notably, the *llsX* gene is commonly regarded as a molecular marker for the presence of the LIPI-3 [Wiktorczyk-Kapischke *et al*., 2023]. LIPI-4, composed of six genes, encodes a cellobiose-family phosphotransferase system (PTS) and has been associated with enhanced neural and placental tropism, suggesting a role in central nervous system and maternal-neonatal infections [Lee *et al.*, 2023; Li M. *et al.*, 2021]. Although virulence factors have been extensively investigated in *L. monocytogenes*, knowledge regarding their distribution in other *Listeria* species remains limited.

Listeriosis remains a significant cause of mortality worldwide. Identifying and characterizing virulence factors is essential for both basic scientific research and ongoing medical challenges. Therefore, this study aimed to investigate the occurrence of selected virulence-associated genes and the hemolytic phenotype of *L. innocua* isolates recovered from meat products and meat-processing environments in Poland.

MATERIALS AND METHODS

Bacterial isolates and genetic material

A total of 51 *L. innocua* isolates were included in this investigation. Of these, 32 were recovered from raw poultry, pork, and beef, as well as from processed meat products, including bacon, ham, and sausages. The remaining 19 isolates originated from meat-processing environments and were recovered from swab samples collected from production lines, processing equipment, walls, and floors. The isolates were obtained through collaboration with an accredited microbiological diagnostic laboratory in Poland. Due to the confidential nature of the source information, detailed data regarding the exact origin of the isolates and the individual meat-processing facilities cannot be disclosed. All isolates were obtained in Poland during 2020–2021. Genomic DNA was extracted with the Genomic Mini kit (A&A Biotechnology, Gdańsk, Poland) following the supplier's protocol and served as the template for PCR analyses. Isolates stored at –80°C in brain heart infusion (BHI; Oxoid, Warsaw, Poland) supplemented with glycerol were used for the evaluation of hemolytic activity. Prior to the experiments, the taxonomic identity of all isolates had been verified as *L. innocua via* polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and multiplex polymerase chain reaction (multiplex PCR). Exact methodologies were reported previously [Li F. *et al.*, 2021; Paillard *et al*., 2003].

Detection of virulence-associated genes

Conventional PCR was performed to detect selected virulence-associated genes in *L. innocua* isolates. The analysis of the *actA*

gene was conducted using two primer pairs, designated *actA1* and *actA2*. Additionally, PCR assays targeting LIPI-4 genes were subjected to optimization; reproducible amplification was achieved for *licC* and *glvA*, whereas optimization attempts for the remaining genes did not yield reproducible results across all isolates. Therefore, these genes were excluded from further analysis. Each PCR reaction contained 0.2 U of RUN DNA polymerase (A&A Biotechnology) with the corresponding reaction buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP) (A&A Biotechnology), and the appropriate primer pair (**Table 1**), synthesized by Genomed S.A. (Warsaw, Poland). Approximately 10 ng of template DNA were added to each reaction, and the final reaction volume was adjusted to 10 µL. PCR amplification was carried out using a Biometra TOne thermal cycler (Biometra, Göttingen, Germany). Three *L. monocytogenes* isolates obtained from food samples were used as positive controls. Following amplification, PCR fragments were examined by agarose gel electrophoresis using Midori Green Advance (1×; NIPPON Genetics EUROPE, Düren, Germany). Gel images were acquired with the Gel Doc imaging system (Bio-Rad, Hercules, CA, USA). For each target gene, a representative fragment of the expected length was selected for purification with the Gel-Out Concentrator kit (A&A Biotechnology)

Table 1. Detailed information about PCR conditions.

Virulence genes	Primers name	Target	Primers' sequence	Annealing temperature (°C)	Amplicon length (bp)	Reference
Listeria pathogenicity island 1 (LIPI-1)	*prfA*	Listeriolysin-positive regulatory protein	F: 5′-GATACAGAAACATCGGTTGGC-3′ R: 5′-GTGTAATCTTGATGCCATCAGG-3′	49	274	D'Agostino *et al.* [2004]
	plcA	Phosphatidylinositol-specific phospholipase C	F: 5′-CTGCTTGAGCGTTCATGTCTCATCCCCC-3′ R: 5′-CATGGGTTTCACTCTCCTTCTAC-3′	60	1,484	Notermans *et al.* [1991]
	plcB	Phosphatidylicholin-specific phospholipase C	F: 5′-GCAAGTGTTCTAGTCTTTCCGG-3′ R: 5′-ACCTGCCAAAGTTTGCTGTGA-3′	55	795	Nishibori *et al.* [1995]
	mpl	Metalloprotease	F: 5′-GGCTCATTTCACTATGACGG-3′ R: 5′-GCTTCCCAAGCTTCAGCAACT-3′	55	1,473	
	hly	Listeriolysin O	F: 5′-GCAGTTGCAAGCGCTTGGAGTGAA-3′ R: 5′-GCAACGTATCCTCCAGAGTGATCG-3′	60	210	Park *et al.* [2006]
	actA1	Actin polymerization protein	F: 5′-CGCCGCGGAAATTAAAAAAAGA-3′ R: 5′-ACGAAGGAACCGGGCTGCTAG-3′	62	839 (or 950)	Jallewar *et al.* [2007]
	actA2	Actin polymerization protein	F: 5′-GACGAAAATCCCGAAGTGAA-3′ R: 5′-CTAGCGAAGGTGCTGTTTCC-3′	63	268 (or 385)	Jaradat *et al.* [2002]
Other virulence--associated genes	*sigB*	Sigma factor	F: 5′-TCATCGGTGTCACGGAAGAA-3′ R: 5′-TGACGTTGGATTCTAGACAC-3′	51	310	Bae *et al.* [2012]
	iap	Invasion associated protein	F: 5′-ACAAGCTGCACCTGTTGCAG-3′ R: 5′-TGACAGCGTGTGTAGTAGCA-3′	56	131	Furrer *et al.* [1991]
	flaA	Flagellin	F: 5′-TTACTAGATCAAACTGCTCC-3′ R: 5′-AAGAAAAGCCCCTCGTCC-3′	54	538	Borucki & Call [2003]
Internalins	*inlA*	Internalin A	F: 5′-ACGAGTAACGGGACAAATGC-3′ R: 5′-CCCGACAGTGGTGCTAGATT-3′	55	800	Liu *et al.* [2007]
	inlJ	Internalin J	F: 5′-TGTAACCCCGCTTACACAGTT-3′ R: 5′-AGCGGCTTGGCAGTCTAATA-3′	55	238	
	inlB	Internalin B	F: 5′-CATGGGAGAGTAACCCAACC-3′ R: 5′-GCGGTAACCCCTTTGTCATA-3′	57	500	Zhang & Knabel [2005]
	inlC	Internalin C	F: 5′-CCCACAATCAAATAAGTGACCTT-3′ R: 5′-CTGGGTCTTTGACAGTATTTGTT-3′	57	400	
Listeria pathogenicity island 3 (LIPI-3)	*llsX*	Listeriolysin L	F: 5′-TTATTGCATCAATTGTTC TAGGG -3′ R: 5′-CCCCTATAAACATCATGCTAGTG-3′	55.5	200	Vilchis-Rangel *et al.* [2019]
	llsA		F: 5′-CGATTTCACAATGTGATAGGATG-3′ R: 5′-GCACATGCACCTCATAAC-3′	52	280	Clayton *et al.* [2014]
	llsG		F: 5′-GAGACTGGGCTTACTTGC-3′ R: 5′-TACCTCCTGTTCACTGCTTG-3′	50	415	Zhang *et al.* [2019]
	llsH		F: 5′-ATGATGTTCGCTATGGTT-3′ R: 5′-ACATTCCTACTGGCATCA-3′	45	421	
	llsB		F: 5′-TTACAATCAACCACCAGG-3′ R: 5′-AGTGAACCGAATGACAGA-3′	45	334	

Table 1 cont. Detailed information about PCR conditions.

Virulence genes	Primers name	Target	Primers' sequence	Annealing temperature (°C)	Amplicon length (bp)	Reference
	llsD		F: 5'-TATGGTGGTATGGAGGGT-3' R: 5'-ATCACCCTGCTTATTTCA-3'	45	562	
	llsP		F: 5'-TTTCCAGGTATGCTTCTT-3' R: 5'-CAATTACGGTGGTTCTCA-3'	45	554	
	llsY		F: 5'-ATTAGAATAGGAACGCAGAC-3' R: 5'-TCATAGCACCCAGTTTCG-3'	50	581	
Listeria pathogenicity island 4 (LIPI-4)	*licA*	Phosphotransferase system (PTS) sugar transporter enzyme IIA (EIIA) subunit	F: 5'-GCCTCTTCCTCGTTTCTA-3' R: 5'-GACTTAACTAAATCGCAGTA-3'	45	227	Zhang *et al.* [2019]
	licB	Phosphotransferase system (PTS) sugar transporter enzyme IIB (EIIB) subunit	F: 5'-ATTGCGGCATCTGAGAAA-3' R: 5'-CAGCGATTAGAATTGGTACTGC-3'	55	232	
	licC	Phosphotransferase system (PTS) sugar transporter enzyme IIC (EIIC) subunit	F: 5'-GGGATTCCGAAACTACCT-3' R: 5'-CGAGTGCTCCTGTAACCC-3'	47	736	
	Lm900558-70012	Phosphotransferase system (PTS)-associated protein	F: 5'-TGGTAACAATGCCTGCTT-3' R: 5'-GCTGAAAGCCCACTGTAT-3'	50	369	
	Lm900558-70013	Transcriptional antiterminator	F: 5'-TATTCAGTGGTTACGAGGCT-3' R: 5'-CTCCGCCGAAATCTGGTA-3'	50	403	
	glvA	Maltose-6'-phosphate--glucosidase	F: 5'-TTACTATTGCTGGCGGAGGA-3' R: 5'-TGCTCACGACCATCCATT-3'	47	847	

and subsequently subjected to Sanger sequencing by Genomed S.A. (Warsaw, Poland). The resulting sequences were compared against reference entries in the NCBI BLAST database (version 2.15.0) to verify the identity of the amplified targets.

Hemolysis assay

Hemolytic activity was assessed using Columbia Agar supplemented with 5% sheep blood (Oxoid, Warsaw, Poland). Bacterial isolates stored as glycerol stocks were first streaked onto BHI agar and incubated at 35°C for 24 h. Subsequently, cultures were re-streaked onto blood agar plates and incubated at 35°C for 24 h and 48 h, with hemolysis evaluated after each incubation period. *Listeria ivanovii* ATCC 19119 was used as a positive control.

RESULTS AND DISCUSSION

Listeriosis is a significant foodborne disease transmitted primarily through contaminated food, including meat and meat products [Zanolli Moreno *et al*., 2014]. While the pathogenicity of *L. monocytogenes* is well characterized in terms of its virulence gene [Freitag *et al.*, 2009], the virulence potential of non-pathogenic *Listeria* species remains poorly understood. Therefore, this study analyzed the presence of 28 virulence-associated genes in 51 *L. innocua* isolates by PCR. Detailed results regarding the presence of individual virulence-associated genes in each isolate, together with the corresponding source of isolation, are presented in **Figure 1**.

Presence of LIPI-1 genes and internalin-associated genes

Genes associated with the LIPI-1 were detected sporadically among the analyzed isolates (**Figure 1**). The *prfA* gene was identified in 5 isolates (10%) from raw meat samples and food-processing environments, while *plcA* and *hly* were each detected in 3 isolates (6%). The *actA1* gene was present in 2 isolates (4%), whereas *plcB*, *actA2*, and *mpl* were not detected in any strain. In contrast, the stress-response regulator *sigB*, which is not part of LIPI-1, was present in all isolates (100%). Additionally, the *iap* gene was identified in 7 isolates (14%), and *flaA* in 6 isolates (12%). Notably, none of the analyzed isolates carried a complete LIPI-1.

The prevalence of LIPI-1 genes observed in the present study was generally lower than that reported in several previous investigations. For example, *plcA* was detected in 97.4% of *L. innocua* isolates recovered from beef, fish, raw milk, and traditional cheese samples in Turkey, followed by *iap* (35.8%) and *hlyA* (15.3%) [Cebeci *et al.*, 2024]. In the present study, *plcA* and *hly* were identified in only 6% of the isolates, while *iap* was detected in 15%, indicating a substantially lower prevalence of these virulence-associated determinants. The occurrence of the *iap* gene appears to vary considerably among studies. A markedly higher prevalence was reported, with *iap* identified in 36 isolates from the poultry production chain [Gwida *et al.*, 2020]. Similarly, the gene was detected in isolates originating from cattle and sheep [Matto *et al.*, 2022], raw milk and yogurt samples [Ramadan *et al.*, 2023] and grain samples [Yong *et al.*, 2024]. The prevalence of *plcA*

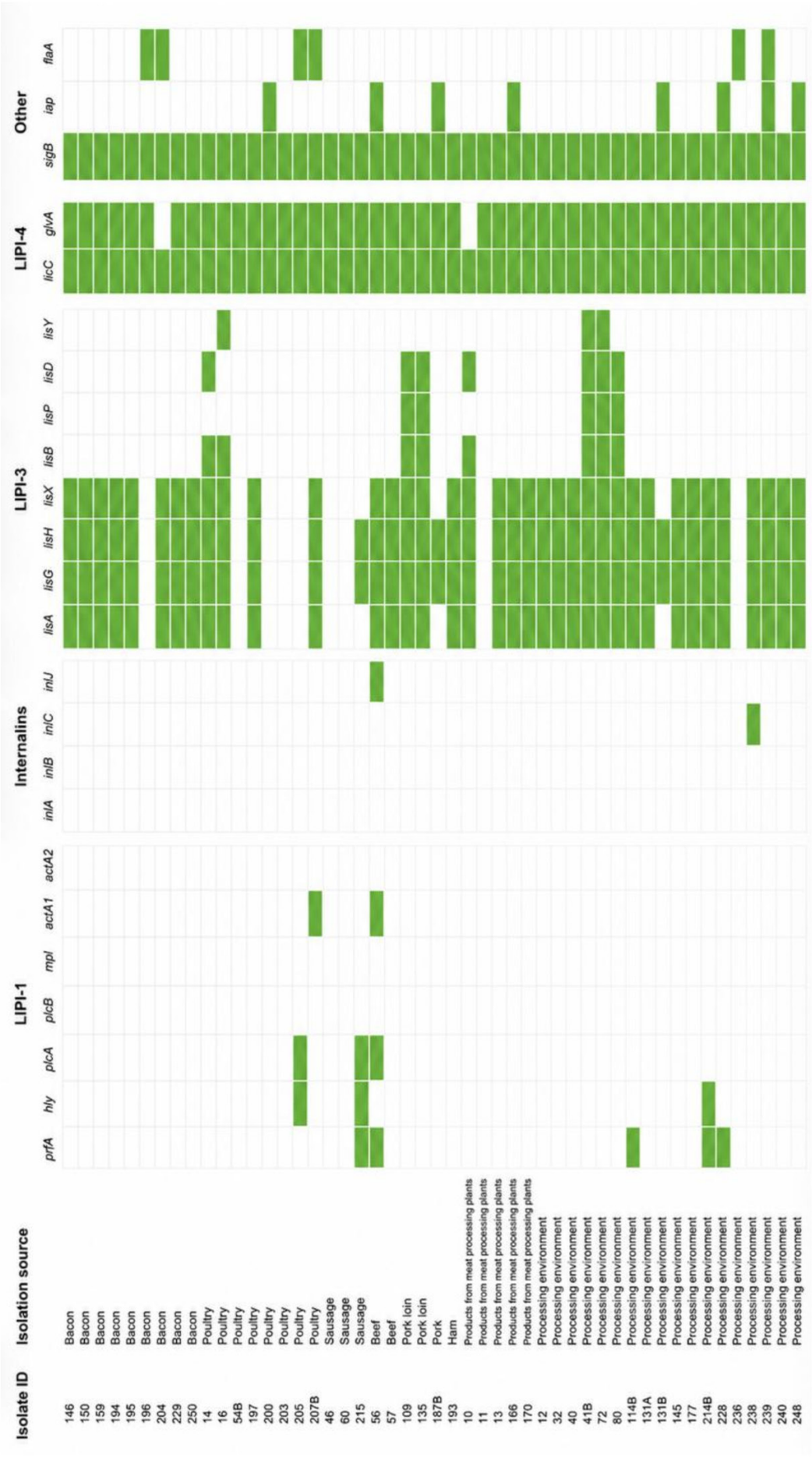

Figure 1. Presence of selected virulence-associated genes among *Listeria innocua* isolates from meat, meat products, and meat-processing environments analyzed in this study. Colored boxes indicate the presence (green) or absence (white) of each gene.

and *hly* has been reported to vary considerably among *L. innocua* isolates, with one study detecting both genes in all five isolates recovered from swine slaughterhouses and meat markets, whereas another identified them in only 9 of the 42 isolates collected from fecal samples of healthy birds in Finland [Moura *et al.*, 2019; Zanolli Moreno *et al.*, 2014]. In Polish research, *hly* was identified in 16.7% of isolates from fish and shrimp, while *plcA* and *iap* were absent [Zakrzewski *et al.*, 2020]. In contrast, no presence of *hly*, *plcA*, or *iap* was detected in *L. innocua* isolates from food and food-contact surfaces [Mafuna *et al.*, 2022; Rossi *et al.*, 2020]. The discrepancies between studies may reflect differences in isolate origin, genomic diversity, and the dynamic nature of virulence-associated gene acquisition or loss within *L. innocua* populations.

The present study demonstrated a very limited distribution of internalin-associated genes among the analyzed *L. innocua* isolates (**Figure 1**). Neither *inlA* nor *inlB* was detected in any of the examined strains. In contrast, *inlC* was identified in a single isolate recovered from a food-processing environment, while *inlJ* was detected in one isolate originating from a beef sample (2% each).

Similarly, low prevalence of internalin genes has been reported in other studies. In the study, five atypical *L. innocua* isolates were described, all of which harbored *inlA* and *inlJ*, suggesting that certain atypical strains may carry a broader repertoire of virulence-associated genes [Zanolli Moreno *et al.*, 2012]. The presence of *inlA* in only a single isolate obtained from food and food-manufacturing plants has also been reported [Rossi *et al.*, 2020]. Conversely, no *inlA, inlB, inlC, inlJ,* or *inlK* genes were detected among isolates from the poultry production chain [Gwida *et al.*, 2020]. Other studies have also reported the absence of major virulence determinants in *L. innocua*. Isolates from fresh and frozen soft fruits lacked LIPI-1 and internalin genes [Wartha *et al.*, 2023], while strains from slaughterhouses were found to be devoid of LIPI-1, internalins, and LIPI-3 elements [Qi *et al.*, 2024].

Although LIPI-1 and internalin-associated genes were generally detected sporadically and most often occurred individually, two isolates stood out due to their notably expanded virulence gene profiles. Isolate ID56, recovered from beef, harbored six virulence-associated genes (*prfA*, *sigB*, *iap*, *plcA*, *actA*, and *inlJ*), representing one of the most complex profiles identified in this study. Similarly, isolate ID 205, originating from poultry, carried four virulence-associated genes (*sigB*, *plcA*, *hly*, and *flaA*). The presence of multiple virulence determinants within single isolates is noteworthy and may indicate an increased adaptive or pathogenic potential, warranting further investigation to assess their functionality and possible contribution to virulence.

Presence of LIPI-3 genes

In contrast to the limited distribution of LIPI-1 and internalin-associated genes, LIPI-3 genes were considerably more prevalent among the analyzed *L. innocua* isolates. Genes located in the 5′ region of the LIPI-3 were detected at high frequencies. The *llsA* and *llsX* genes were each identified in 36 isolates (69%) and consistently co-occurred within the same strains. Similarly, *llsG* and *llsH* were detected in 37 isolates (73%) and were always present together. Notably, all isolates harboring *llsA* and *llsX* also carried *llsG* and *llsH*, indicating a strong tendency for co-occurrence of genes located in the 5′ region of the island. In contrast, genes located in the 3′ region of LIPI-3 were detected much less frequently. The *llsB* and *llsD* genes were each identified in 7 isolates (13%) and consistently co-occurred. The *llsP* gene was detected in 5 isolates (10%), whereas *llsY* was found in only 3 isolates (6%). A complete LIPI-3, defined as the presence of all eight analyzed LIPI-3 genes, was identified in 2 isolates (4%), both originating from distinct food-processing environment samples. Partial LIPI-3 profiles, characterized by the presence of at least one LIPI-3 gene, were detected in 40 isolates (78%), whereas 11 isolates (21%) did not harbor any of the analyzed LIPI-3 genes.

The predominance of LIPI-3 genes observed in the present study is consistent with previous findings demonstrating that these genes are more prevalent in *L. innocua* than other pathogenicity islands. Clayton *et al.* [2014] analyzed a total of 64 *L. innocua* isolates recovered from food products, of which 45 strains (70.3%) were positive for *llsA*. Eleven isolates carried a complete LIPI-3, whereas 25 exhibited a truncated LIPI-3 profile lacking *llsB*, *llsY*, *llsD*, and *llsP*, corresponding to the presence of *llsA*, *llsG*, *llsH*, and *llsX*. Nine isolates harbored only *llsA*, and 19 strains lacked all LIPI-3 genes. Notably, the LIPI-3-positive isolates described in that study were non-hemolytic, consistent with the findings of the present study [Clayton *et al.*, 2014]. In another investigation focusing on non-pathogenic *Listeria* spp. isolated from food and food-processing facilities, three *L. innocua* isolates harboring a complete LIPI-3 were identified among 36 analyzed strains [Mafuna *et al.*, 2022]. A larger survey conducted in South Africa examined 110 *L. innocua* isolates originating from cattle farms, beef abattoirs, and retail outlets. The *llsA* (66 isolates), *llsG* (66 isolates), *llsH* (65 isolates), and *llsX* (65 isolates) genes were frequently detected, whereas genes located in the 3′ region- *llsB* (7 isolates), *llsD* (8 isolates), *llsP* (6 isolates), and *llsY* (8 isolates), were considerably less prevalent [Gana *et al.*, 2023]. These findings corroborate the results of the present study, confirming the broader distribution of LIPI-3 compared with other pathogenicity islands in *L. innocua*. Hence, a consistent pattern emerges across studies, including the present one, demonstrating higher prevalence and frequent co-occurrence of genes located in the 5′ region of the island, accompanied by reduced detection of genes positioned toward the 3′ end. The structure of LIPI-3, therefore, appears to vary among *L. innocua* isolates, ranging from complete islands to partial remnants or total absence, reflecting ongoing genomic variability within the species. Notably, LIPI-3 has been reported to be considerably more widespread in *L. innocua* than in *L. monocytogenes*, where it is generally confined to selected hypervirulent lineages [Lee *et al.*, 2023; Zhang *et al.*, 2019].

Presence of selected LIPI-4 genes

Among the LIPI-4 genes, *licC* and *glvA* were successfully analyzed. The *licC* gene was detected in all examined isolates

(51/51; 100%), while *glvA* was identified in 48 isolates (94%). The remaining LIPI-4 genes were not included in the present analysis.

LIPI-4 is a relatively recently described pathogenicity island and remains the least extensively investigated among the known *Listeria* pathogenicity islands. In *L. monocytogenes*, its occurrence is largely restricted to the hypervirulent clonal complex CC4 [Lee *et al.*, 2023]. Data regarding its distribution in *L. innocua* remains limited compared with LIPI-1 and LIPI-3. Structurally, LIPI-4 encodes a PTS system: *licC*, encoding the enzyme IIC component (EIIC) of the phosphotransferase system (PTS) sugar transporter, responsible for substrate recognition and facilitates sugar transport into the cell, and *glvA*, encoding maltose-6'-phosphate glucosidase [Zhang *et al.*, 2019]. Both genes were highly prevalent among the examined isolates, with *licC* detected in all strains and *glvA* in the vast majority. Although genes associated with the LIPI-4 region were frequently detected, the limited number of target genes successfully amplified did not allow confirmation of the presence of a complete and functional LIPI-4 in the studied collection. Previous studies have reported the occurrence of intact LIPI-4 in *L. innocua*, including three food-derived isolates harboring the complete cluster [Li M. *et al.*, 2021], as well as a high prevalence of the full island among 36 analyzed strains [Mafuna *et al.*, 2022]. The frequent detection of *licC* and *glvA* suggests enhanced metabolic adaptability of the analyzed isolates, particularly in the utilization of specific carbohydrates. Such capabilities may support persistence in food environments, improve competitiveness against other microorganisms, and potentially facilitate host colonization. At the same time, the widespread presence of these genes cautiously indicates that LIPI-4 related elements may be common in *L. innocua*. However, further investigations are necessary to determine the presence of the remaining LIPI-4 genes and to clarify the functional significance of the detected determinants.

Hemolysis assay

No hemolytic activity was observed among the analyzed *L. innocua* isolates, despite the presence of the *hly* gene in three isolates (205, 214B, and 215), indicating a lack of functional expression of this virulence determinant under the tested conditions. The discordance between the presence of *hly* and the absence of a clear hemolytic phenotype represents a particularly intriguing finding. Several explanations may account for this observation, including mutations within the *hly* coding sequence impairing listeriolysin O functionality, nonsense mutations leading to truncated protein products, or alterations within the promoter region affecting gene transcription. Additionally, impaired activity or mutations in the transcriptional regulator *prfA* could disrupt proper activation of LIPI-1 genes. To clarify the molecular basis of this discrepancy, further investigations should include sequencing of the *hly* gene and its regulatory regions, as well as analyses of gene expression. Although *L. innocua* is generally regarded as a non-hemolytic species, hemolytic isolates have occasionally been reported. An atypical strain carrying a complete set of LIPI-1 genes exhibited hemolytic activity but failed to induce disease in murine models, suggesting that hemolysis alone does not necessarily confer full pathogenic potential [Johnson *et al.*, 2004]. In contrast, other studies demonstrated that hemolytic *L. innocua* isolates harboring LIPI-1 and *inlA* may exhibit virulence, including the ability to cross the intestinal epithelium and disseminate systemically, albeit to a lesser extent than *L. monocytogenes* EGDe [Moura *et al.*, 2019]. Weakly hemolytic *hly*-positive isolates and additional hemolytic LIPI-1 positive strains from pork, seafood, and poultry have also been described [Milillo *et al.*, 2012; Volokhov *et al.*, 2007; Zanolli Moreno *et al.*, 2014]. Although exposure to atypical hemolytic *L. innocua* strains appears to be rare, both the present findings and previous reports indicate that phenotypic assessment of hemolysis may not always provide reliable results. The emergence of atypical isolates, including strains exhibiting low-hemolytic activity, further complicates species identification when conventional methods are applied. The present findings highlight that the presence of the *hly* gene alone is not sufficient to infer hemolytic phenotype in *L. innocua*, emphasizing the importance of combining genotypic and phenotypic approaches in virulence assessment.

CONCLUSIONS

This study provides the first comprehensive characterization of 28 virulence-associated genes in *L. innocua* isolates recovered from raw and processed meat products as well as meat-processing environments in Poland. The findings indicate a sporadic occurrence of genes clustered within the LIPI-1 and internalin-associated genes. In contrast, LIPI-3 genes, present either as a complete island or in truncated forms, were more widespread, demonstrating a predominance compared with other pathogenicity islands. The universal presence of *sigB*, together with the frequent detection of *licC* and *glvA*, may suggest enhanced metabolic adaptability of the analyzed isolates and could support their persistence in food and food-processing environments. Although *L. innocua* is generally regarded as non-pathogenic, its occurrence in food matrices warrants attention, particularly due to its frequent coexistence with *L. monocytogenes* and its potential role as a reservoir of virulence-associated genes. Importantly, the detection of virulence-associated genes at the genomic level does not necessarily imply their functional expression or pathogenic potential. As the present study was limited to DNA-based identification of these determinants, further investigations focusing on transcriptional activity and functional analyses are required to more accurately assess their potential contribution to the pathogenicity of *L. innocua*. Furthermore, the implementation of whole-genome sequencing (WGS) in future studies could further strengthen this approach by providing comprehensive

information on the genetic background, virulence-associated genes, and evolutionary relationships among *L. innocua* isolates. These findings emphasize the importance of including *L. innocua* in food safety monitoring and risk assessment frameworks.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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