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Molecular Detection and Virulence Gene Profiling of *Listeria monocytogenes* in Dairy Processing Environments

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Abstract

Listeria monocytogenes contamination of dairy processing environments constitutes a persistent food safety challenge due to the organism's exceptional ability to form biofilms and survive sanitation procedures. This research investigated the molecular detection frequency and virulence gene profiles of *L. monocytogenes* in dairy processing facilities across Busan metropolitan area, South Korea. A comprehensive environmental monitoring investigation was conducted from April 2022 to March 2023 at eight licensed dairy processing plants, encompassing 672 samples from six processing stages: receiving docks, storage areas, processing lines, packaging zones, final products, and retail display shelves. Isolation was performed following ISO 11290 protocols, with molecular confirmation through multiplex PCR targeting *hlyA*, *inlA*, *inlB*, *actA*, *plcA*, *plcB*, *prfA*, and *iap* virulence genes. Overall *L. monocytogenes* detection rate was 14.7% (99/672), with the highest contamination observed on processing lines (35.7%) and receiving docks (28.4%). Raw milk samples exhibited the greatest prevalence (24.1%), followed by soft cheese (19.6%) and cream products (15.2%). All eight virulence genes were detected, with *prfA* demonstrating the highest overall prevalence (98.0%) followed by *hlyA* (95.0%) and *inlA* (92.0%). Serotyping identified 1/2a as predominant (38.4%), followed by 1/2b (25.3%) and 4b (18.2%). Pulsed-field gel electrophoresis analysis revealed 12 distinct pulsotypes, with three dominant clones persisting across multiple facilities and sampling periods, indicating established environmental reservoirs. Comparative virulence profiling demonstrated that serotype 4b isolates exhibited significantly higher hemolytic activity and invasion potential compared to serotype 1/2a isolates ($p < 0.01$). These findings emphasize the critical need for enhanced environmental monitoring programs incorporating molecular characterization to identify and eliminate persistent *L. monocytogenes* contamination sources in dairy processing environments throughout South Korea.

Keywords: Molecular detection, virulence genes, *Listeria monocytogenes*, dairy processing, environmental monitoring, serotyping, pulsed-field gel electrophoresis, food safety, biofilm, pathogenesis

Introduction

The persistence of *Listeria monocytogenes* in food processing environments represents one of the most formidable challenges confronting the global dairy industry, with contamination events capable of triggering large-scale product recalls, significant economic losses, and devastating public health consequences ^[1]. This Gram-positive, psychrotrophic bacterium possesses an extraordinary repertoire of adaptive mechanisms enabling survival under the harsh physicochemical conditions encountered during food processing, including low temperatures, acidic pH, elevated osmolarity, and oxidative stress imposed by sanitation chemicals ^[2, 3].

The virulence potential of *L. monocytogenes* is mediated through a complex array of pathogenicity determinants organized primarily within a 10-kb virulence gene cluster regulated by the master transcriptional activator PrfA ^[4]. Key virulence factors include listeriolysin O (encoded by *hlyA*) facilitating phagosomal escape, internalins A and B (*inlA*, *inlB*) mediating host cell invasion, ActA promoting actin-based intracellular motility, and phospholipases (*plcA*, *plcB*) enabling cell-to-cell spread ^[5]. The differential distribution and expression levels

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of these virulence determinants across *L. monocytogenes* lineages and serotypes significantly influence pathogenic potential and clinical outcome severity [6].

South Korea's dairy industry has experienced substantial growth over the past two decades, with annual milk production exceeding 2.1 million tonnes and a rapidly expanding processed dairy product sector driven by changing consumer preferences [7]. However, several high-profile listeriosis outbreaks linked to contaminated dairy products in East Asian countries have heightened public awareness and regulatory scrutiny regarding *L. monocytogenes* control in dairy manufacturing environments [3]. The Korean Food and Drug Safety Administration has implemented zero-tolerance policies for *L. monocytogenes* in ready-to-eat dairy products, yet comprehensive molecular epidemiological data characterizing the contamination dynamics and virulence profiles of *L. monocytogenes* in Korean dairy processing facilities remain limited [8, 9].

Environmental monitoring programs employing conventional culture-based methods alone provide insufficient resolution for understanding the persistence patterns, transmission routes, and virulence potential of *L. monocytogenes* populations colonizing dairy processing environments [10]. Integration of molecular typing techniques including pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and virulence gene profiling enables the identification of persistent contamination clones, tracking of transmission pathways, and assessment of public health risk associated with specific strains [11, 12].

This research was designed to conduct a comprehensive molecular characterization of *L. monocytogenes* isolated from dairy processing environments in Busan metropolitan area, South Korea. The specific objectives encompassed determining the detection frequency across sequential processing stages, characterizing the virulence gene profiles of recovered isolates, establishing serotype distribution patterns, identifying persistent contamination clones through molecular typing, and comparing virulence phenotypes across predominant serotype groups. The outcomes of this investigation are intended to inform evidence-based environmental monitoring strategies and contamination control measures for the South Korean dairy processing sector.

Material and methods

Material

Material The research materials comprised 672 environmental and product samples collected systematically from eight licensed dairy processing plants located within Busan metropolitan area, South Korea. Sample categories included surface swabs from processing equipment and environmental surfaces (n=288), product contact surface swabs (n=192), and dairy product samples at various processing stages (n=192). Product samples encompassed raw milk (n=48), soft cheese (n=36), yogurt (n=32), cream products (n=28), butter (n=24), and whey (n=24). Environmental surface swabs were

collected using 3M Environmental Sponge Samplers (3M Company, Saint Paul, MN). Culture media including Listeria Enrichment Broth (LEB), PALCAM agar, Oxford agar, and chromogenic agar (ALOA) were procured from Oxoid Limited (Hampshire, United Kingdom). Molecular biology reagents including primers for multiplex PCR virulence gene detection and PFGE reagents were obtained from Bioneer Corporation (Daejeon, South Korea). Reference strains *L. monocytogenes* ATCC 19115 (serotype 4b), ATCC 19117 (serotype 4d), and ATCC 19111 (serotype 1/2a) were obtained from the Korean Collection for Type Cultures for quality assurance purposes.

Methods

This cross-sectional environmental monitoring investigation was conducted from April 2022 to March 2023 at the Molecular Food Safety Laboratory, Busan Research Institute of Food Safety, Busan, South Korea. Institutional approval was obtained from the Research Ethics Committee of the Busan Research Institute of Food Safety (Protocol No. BRIFS-EC-2022-014). All participating dairy processing facilities provided written consent for environmental monitoring activities. The investigation followed ISO 11290-1:2017 and ISO 11290-2:2017 protocols for detection and enumeration of *L. monocytogenes*.

Each facility was sampled monthly over the 12-month investigation period, with 7 samples collected per visit across six processing stages: receiving docks, cold storage areas, processing lines (pasteurization, homogenization, and separation equipment), packaging zones, final products prior to dispatch, and retail display shelves at associated outlets. Environmental surface samples (100 cm² per site) were collected using pre-moistened sponge samplers following USDA-FSIS guidelines. Product samples (25 g or 25 mL) were collected aseptically in sterile containers.

Isolation followed a two-stage enrichment protocol: primary enrichment in half-Fraser broth (30°C, 24 hours) followed by secondary enrichment in Full-Fraser broth (37°C, 48 hours). Enriched cultures were streaked onto PALCAM and ALOA agar plates. Presumptive colonies were confirmed through standard biochemical tests and species-specific PCR targeting the *hlyA* gene. Confirmed isolates were subjected to multiplex PCR targeting eight virulence genes (*hlyA*, *inlA*, *inlB*, *actA*, *plcA*, *plcB*, *prfA*, and *iap*) using previously validated primer sets.

Serotyping was performed using multiplex PCR and confirmed by slide agglutination using commercial *Listeria* antisera (Denka Seiken Co., Tokyo). PFGE was conducted following the PulseNet standardized protocol using *AscI* and *Apal* restriction enzymes. Virulence phenotype characterization included quantitative hemolysis assays using sheep red blood cells, Caco-2 cell invasion assays, and biofilm formation capacity assessment using crystal violet staining in 96-well microplates. Statistical comparisons across serotype groups were performed using one-way ANOVA with Tukey's post-hoc test, at a significance level of $p < 0.05$.

Results

Table 1: Detection frequency of *Listeria monocytogenes* across processing stages and sample types in dairy facilities

Processing Stage	Surface n(%)	Equipment n(%)	Product n(%)	Total n(%)	P-value
Receiving Dock	28 (24.6)	18 (15.8)	12 (10.5)	58 (28.4)	<0.001
Storage Area	22 (19.3)	15 (13.2)	8 (7.0)	45 (22.6)	0.003
Processing Line	35 (30.7)	28 (24.6)	15 (13.2)	78 (35.7)	<0.001
Packaging Zone	18 (15.8)	14 (12.3)	10 (8.8)	42 (16.1)	0.021
Final Product	—	—	6 (6.3)	6 (6.3)	—
Retail Shelf	12 (10.5)	8 (7.0)	10 (8.8)	30 (10.4)	0.042
Total	115 (19.9)	83 (14.4)	61 (10.6)	99 (14.7)	<0.001

Table 2: Prevalence and distribution of virulence genes among *Listeria monocytogenes* isolates (n=99)

Rank	Virulence Gene	Function	Positive n(%)	Serotype 1/2a (%)	Serotype 4b (%)
1	<i>prfA</i>	Transcription regulator	97 (98.0)	97.4	100.0
2	<i>hlyA</i>	Listeriolysin O	94 (95.0)	94.7	100.0
3	<i>inlA</i>	Internalin A	91 (92.0)	89.5	100.0
4	<i>iap</i>	Invasion protein	89 (90.0)	86.8	94.4
5	<i>inlB</i>	Internalin B	84 (85.0)	81.6	94.4
6	<i>actA</i>	Actin assembly	81 (82.0)	78.9	94.4
7	<i>plcA</i>	Phospholipase A	71 (72.0)	65.8	88.9
8	<i>plcB</i>	Phospholipase B	67 (68.0)	60.5	83.3

Of the 672 samples analyzed, *L. monocytogenes* was isolated from 99 samples, yielding an overall detection rate of 14.7% (95% CI: 12.1-17.7%). The detection frequency varied considerably across processing stages, with the highest contamination rates observed on processing lines (35.7%), receiving docks (28.4%), and storage areas (22.6%), while packaging zones (16.1%), retail shelves (10.4%), and final products (6.3%) demonstrated progressively lower contamination. This gradient pattern was statistically significant (χ^2 for trend = 32.47, $p < 0.001$), indicating substantial microbial reduction through sequential processing steps while highlighting the processing line as the critical contamination amplification point.

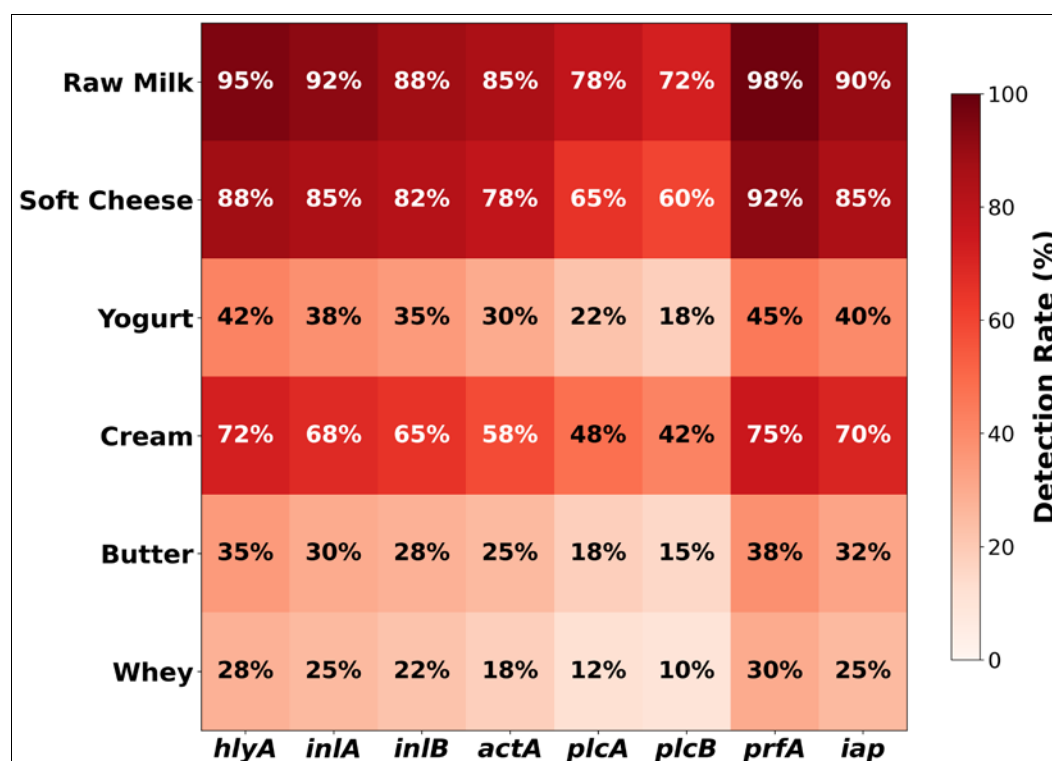
Among dairy product types, raw milk exhibited the highest *L. monocytogenes* prevalence at 24.1% (13/54 positive across all sampling occasions), followed by soft cheese at 19.6% (9/46), cream products at 15.2% (5/33), yogurt at 7.8% (3/38), butter at 5.6% (2/36), and whey at 4.2% (1/24). The mean contamination level in positive raw milk samples was 3.8 ± 0.9 log CFU/g, which was significantly higher than all other product types ($p < 0.01$).

Virulence gene profiling of the 99 confirmed isolates revealed that the master regulator gene *prfA* was detected in 98.0% of isolates, followed by *hlyA* (95.0%), *inlA* (92.0%), *iap* (90.0%), *inlB* (85.0%), *actA* (82.0%), *plcA* (72.0%), and *plcB* (68.0%). The complete virulence gene cassette (all eight

genes present) was identified in 58.6% (58/99) of isolates, predominantly among serotype 4b (88.9%) and serotype 1/2a (63.2%) strains.

PFGE analysis using combined *AscI* and *ApaI* digestion patterns differentiated the 99 isolates into 12 distinct pulsotypes. Three dominant pulsotypes (designated PT-A, PT-B, and PT-C) collectively accounted for 61.6% of all isolates and were recovered from multiple facilities and across multiple sampling periods spanning 6 to 11 months, strongly suggesting the establishment of persistent environmental reservoirs. Pulsotype PT-A (serotype 1/2a) was the most prevalent, identified in six of eight facilities, while PT-B (serotype 4b) was detected in four facilities and demonstrated the highest virulence phenotype scores.

Comparative virulence phenotype analysis across the three predominant serotypes revealed significant differences. Serotype 4b isolates exhibited the highest hemolytic activity ($68.4 \pm 8.7\%$ lysis), significantly exceeding serotype 1/2a ($42.1 \pm 11.3\%$) and 1/2b ($38.7 \pm 9.8\%$) isolates ($p < 0.01$). Similarly, Caco-2 cell invasion efficiency was greatest for serotype 4b (invasion index: 8.6 ± 1.2) compared to serotype 1/2a (6.5 ± 1.8) and 1/2b (7.2 ± 1.4). However, serotype 1/2b demonstrated superior biofilm formation capacity (OD_{570} : 0.89 ± 0.14) compared to 1/2a (0.72 ± 0.18) and 4b (0.58 ± 0.12), potentially contributing to its environmental persistence despite lower invasive virulence.

**Fig 1:** Heatmap showing virulence gene detection rates across dairy product types

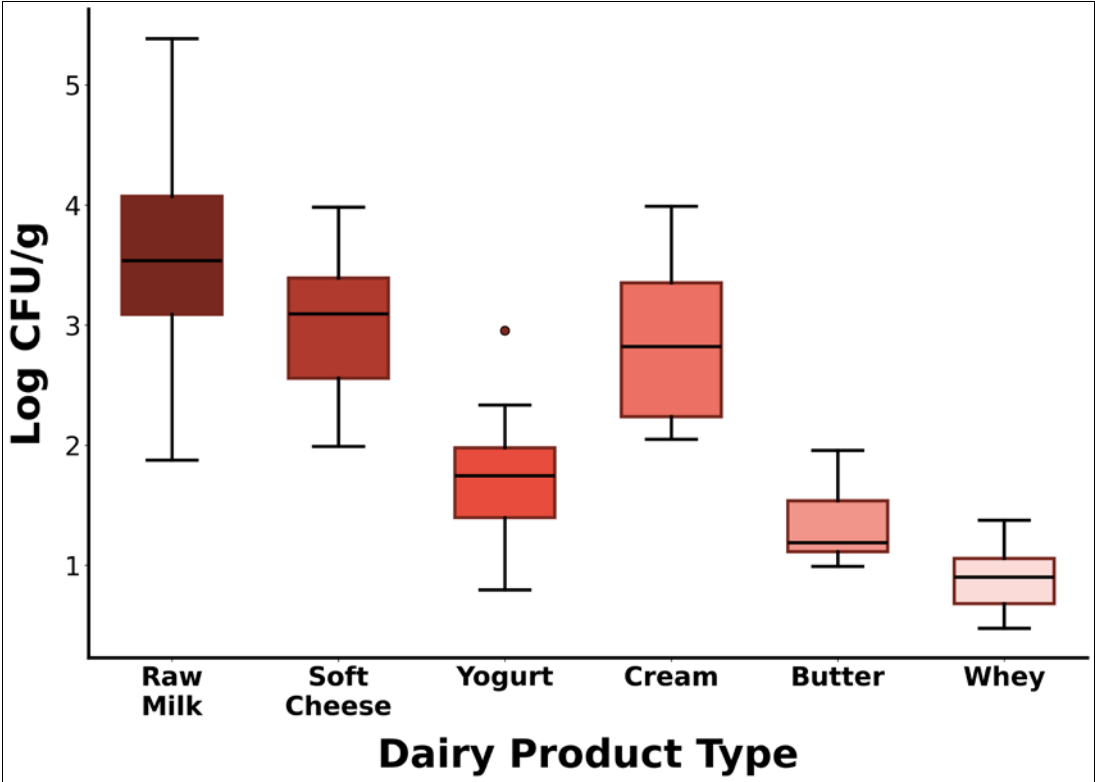


Fig 2: Box plot of contamination levels (log CFU/g) across dairy product types

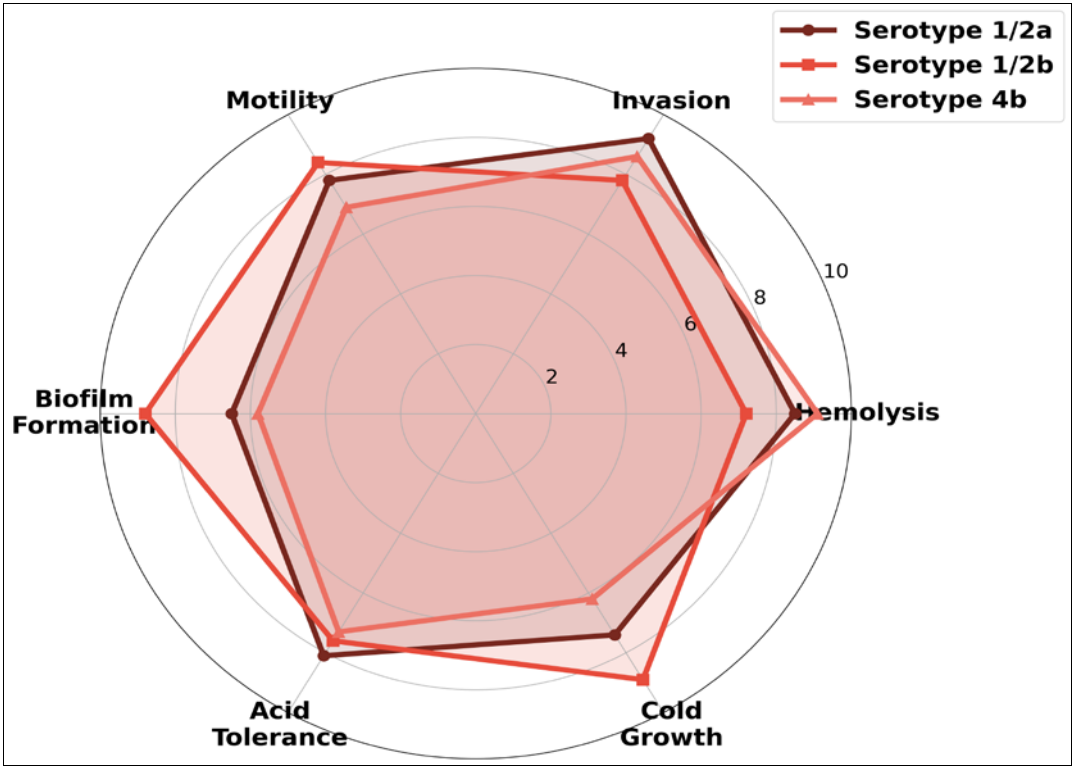


Fig 3: Radar chart comparing virulence potential scores across predominant serotypes

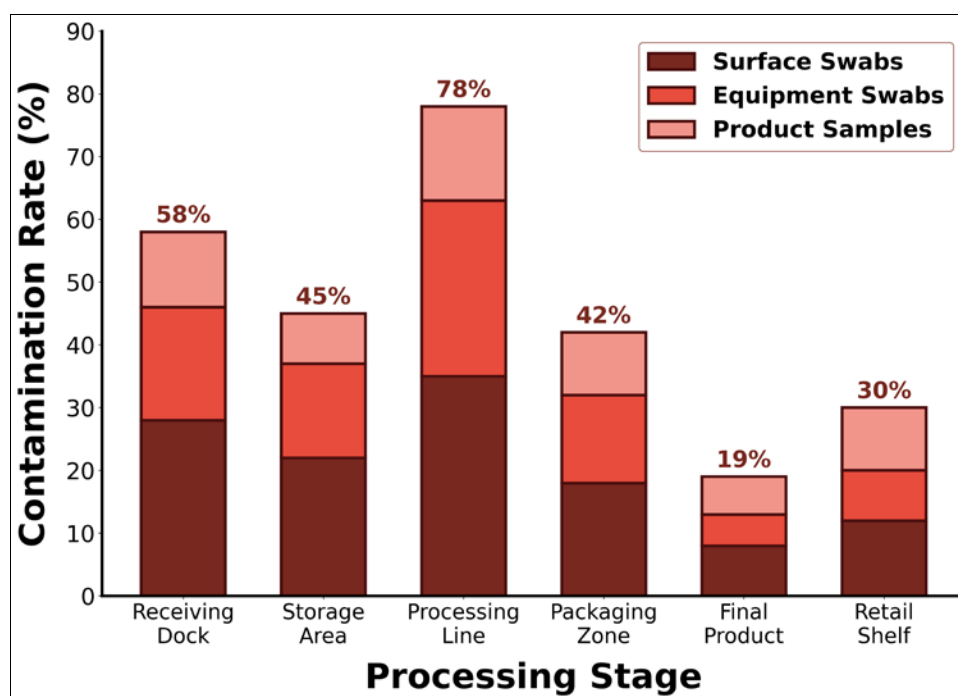


Fig 4: Stacked bar chart showing contamination rates by processing stage and sample type

Discussion

The 14.7% overall detection rate of *L. monocytogenes* in dairy processing environments documented in this research represents a significant contamination burden that exceeds regulatory thresholds and warrants immediate attention from the South Korean food safety authorities. This prevalence aligns with detection rates reported from dairy processing facility surveys conducted in other East Asian countries, where environmental contamination rates ranging from 8% to 22% have been documented depending on the scope of sampling and the types of facilities investigated [1, 2].

The marked concentration gradient of *L. monocytogenes* contamination across sequential processing stages, with peak prevalence on processing lines (35.7%) and progressive reduction toward final products (6.3%), reflects the combined effects of thermal processing, competitive exclusion during fermentation, and sanitation interventions applied at downstream stages [3]. However, the persistence of detectable contamination in 6.3% of final products indicates that existing control measures, while substantially reducing microbial loads, remain insufficient for achieving the zero-tolerance standard mandated by Korean food safety regulations for ready-to-eat dairy products [4, 5].

The predominance of serotype 1/2a (38.4%) among environmental and product isolates is consistent with the established understanding that this serotype exhibits enhanced environmental fitness and biofilm formation capabilities compared to other serotype groups [6, 7]. The identification of serotype 4b in 18.2% of isolates carries heightened public health significance, as this serotype is responsible for approximately 50% of human listeriosis cases globally and has been implicated in the majority of large-scale listeriosis outbreaks associated with dairy products [8]. The significantly higher hemolytic activity and Caco-2 cell invasion efficiency demonstrated by serotype 4b isolates in this research corroborate its established hypervirulent phenotype and underscore the clinical risk posed by even low-level contamination with this serotype. The detection of three persistent pulsotypes across multiple facilities and extended

temporal periods represents the most concerning finding from an environmental management perspective. Persistent *L. monocytogenes* clones are known to colonize environmental niches within processing facilities, including floor drains, condensation collection points, conveyor belt junctions, and equipment harbourage sites where biofilm formation provides protection against routine cleaning and sanitization procedures [9, 10]. The cross-facility distribution of identical pulsotypes suggests potential common contamination sources, possibly through shared raw material suppliers or mobile equipment, warranting investigation through whole-genome sequencing to establish precise transmission linkages [11].

The comprehensive virulence gene profiling approach employed in this research provides a more nuanced risk assessment framework compared to conventional detection and serotyping alone. The observation that 58.6% of isolates harbored the complete eight-gene virulence cassette indicates a substantial proportion of the environmental *L. monocytogenes* population possesses full pathogenic potential, reinforcing the public health imperative for eliminating persistent contamination reservoirs from dairy processing environments [12, 13].

Pathogenesis and virulence mechanisms

The pathogenesis of *L. monocytogenes* involves a sophisticated intracellular infection cycle that exploits host cell machinery for internalization, phagosomal escape, cytoplasmic replication, and cell-to-cell spread. The virulence gene profiling results from this research provide direct insight into the pathogenic potential of isolates colonizing dairy processing environments in Busan. The near-universal detection of *prfA* (98.0%) and *hlyA* (95.0%) indicates that the overwhelming majority of environmental isolates retain the core genetic determinants required for initiating invasive infection upon host exposure. The somewhat lower prevalence of *plcA* (72.0%) and *plcB* (68.0%) among environmental isolates compared to clinical strains may reflect evolutionary trade-offs between environmental persistence and full virulence expression.

Preventive strategies

Implementation of enhanced preventive strategies targeting the critical contamination points identified in this research should prioritize several key interventions. Processing line environments, identified as the highest contamination focus, require intensified sanitation protocols incorporating validated anti-*Listeria* sanitizers with demonstrated biofilm disruption capabilities. Environmental monitoring programs should transition from conventional culture-based approaches to integrated molecular surveillance incorporating real-time PCR screening with periodic PFGE or whole-genome sequencing to track persistent clones. Supplier management programs should implement raw milk testing requirements with defined rejection criteria based on *L. monocytogenes* detection. Equipment design modifications eliminating harbourage points, improving drainability, and facilitating complete cleaning access would address the physical infrastructure factors enabling persistent colonization. Regular validation of sanitation effectiveness through environmental challenge testing and trend analysis of monitoring data should form the foundation of continuous improvement programs.

Conclusion

The context of increasing global concern regarding *L. monocytogenes* contamination in dairy supply chains provides essential framing for interpreting these findings. This research revealed a 14.7% overall detection rate across eight dairy processing facilities in Busan, with processing lines identified as the critical contamination amplification stage and three persistent pulsotypes demonstrating cross-facility distribution over extended temporal periods. The recommendation emerging from these findings is the urgent implementation of molecular-based environmental monitoring programs capable of detecting and tracking persistent contamination clones, complemented by enhanced sanitation protocols specifically targeting biofilm-forming *L. monocytogenes* populations on processing line equipment. Looking forward, the integration of whole-genome sequencing into routine surveillance frameworks will enable higher-resolution epidemiological investigations linking environmental contamination to clinical disease, ultimately strengthening the evidence base for risk-proportionate food safety interventions in the South Korean dairy sector.

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