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INVITED REVIEW

Endolysin-based biocontrol strategies against *Listeria monocytogenes* in food: A comprehensive review

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Abstract

Listeria monocytogenes poses significant challenges in the food industry due to its resistance in harsh environments and its ability to form biofilms. Endolysins represent a promising solution for controlling *L. monocytogenes* in the food industry, with potential applications during both production and storage. This review discusses various endolysins that effectively inhibit *L. monocytogenes*, emphasizing their optimal conditions and potential uses in food products. When used in combination with current methods such as nisin or high hydrostatic pressure, endolysins enhance food safety by providing synergistic antimicrobial effects. Additionally, cell binding domain enabled specific and rapid *L. monocytogenes* detection, expanding diagnostic applications through fluorescence tagging and biosensor integration. Advances in engineering endolysins have further improved their lytic efficiency and specificity, making them adaptable to diverse food safety challenges.

Keywords *L. monocytogenes* · Endolysin · Biocontrol · Rapid detection method · Food safety

Introduction

Listeria monocytogenes is a Gram-positive bacterium and a significant pathogen responsible for listeriosis (Osek et al., 2022). Along with *Salmonella* spp. and shiga toxin-producing *Escherichia coli*, it is one of the top three foodborne pathogens leading to hospitalizations in North America and Europe (Koopmans et al., 2023). The fatality rate of listeriosis ranges from 20 to 30%, with a global incidence of 0.1 to 11.3 cases per million people annually (Koopmans et al., 2023). The illness primarily affects pregnant women, neonates, the elderly, and immunocompromised individuals (Swaminathan and Gerner-Smidt, 2007). The economic burden of listeriosis is considerable, with healthcare and food safety costs estimated between $2.3 billion and $22 billion annually (Koopmans et al., 2023). Most listeriosis cases arise from the consumption of contaminated food products, including ready-to-eat products, soft cheeses, sliced vegetables, ice cream, and various meat products (Je et al., 2024; Koopmans et al., 2023). Because most of these foods are often consumed without heating, they present a heightened risk of *Listeria* contamination and subsequent infection (Je et al., 2024).

L. monocytogenes can survive in extreme environmental conditions such as low pH, low temperature, high salt concentrations, nutrient deprivation, and desiccation (Osek et al., 2022). Its ability to form biofilms further complicates its control during food processing (Osek et al., 2022). Consequently, *L. monocytogenes* is a critical concern throughout the food manufacturing and storage processes. Various physical, chemical, and biological strategies have been employed to mitigate the risk of pathogens and spoilage bacteria. Physical methods, including UV irradiation, high hydrostatic pressure (HHP), and thermal treatments, however, may adversely affect the texture and nutritional quality of food (Chavan et al., 2011; Guneser and Yuceer, 2012; Xia et al., 2022). The use of antibiotics is limited in food processing due to the spread of multidrug-resistant bacteria (Ventola, 2015). Additionally, disinfectants and food additives may pose health risk in humans, if not completely

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removed from food products (Trasande et al., 2018; Villanueva et al., 2015). These agents often lack specificity, potentially affecting beneficial bacteria along with the target pathogens (Khan et al., 2023). Recently, bacteriophages and their derived enzymes, such as endolysins have emerged as promising alternatives in the food safety interventions due to their targeted mode of action (Mahony et al., 2011; Schmelcher and Loessner, 2016). Khan et al. (2023) highlighted several advantages of endolysins (Khan et al., 2023). To briefly summarize, their high specificity ensures no effect on commensal microbiota and minimal risk of resistance development. Additionally, these enzymes have the ability to function under diverse environmental conditions and are capable of broader antibacterial activity compared to bacteriophages alone. Notably, GRN 000802 became the first endolysin targeting *Clostridium perfringens* to be recognized as Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) (Kazanavičiūtė et al., 2018). Given these attributes, this review aims to elucidate the properties of endolysins against *L. monocytogenes* and assess their potential applications in the food industry.

Fundamentals of endolysins

Endolysins generally consist of two functional domains: the enzymatically active domain (EAD) at the C-terminal and the cell wall-binding domain (CBD) at the N-terminal, connected by a short linker (Loessner, 2005). The EAD is responsible for degrading the bacterial cell wall by cleaving specific chemical bonds in the peptidoglycan layer, while CBD provides specificity by recognizing and binding to distinct components of the bacterial cell wall. These modular domains work synergistically to achieve efficient bacterial cell lysis, making endolysins highly specific and effective antimicrobial agents. EADs can be classified into peptidase, amidase, and glycosyl hydrolase, depending on their cleavage sites within the peptidoglycan (Khan et al., 2023; Romero et al., 2018). Peptidases hydrolyze peptide bonds in the stem peptides of peptidoglycan. Amidases catalyze the hydrolysis of the amide bond between N-acetylmuramic acid and the peptide chain. Glycosyl hydrolases cleave the glycosidic bonds between sugar molecules in the peptidoglycan backbone. Of the twenty-nine endolysins targeting *L. monocytogenes*, twenty are classified as peptidases, seven as amidases, and two as glycosyl hydrolases across various bacteriophages (Romero et al., 2018). CBD recognizes teichoic acids, specific polysaccharides, or other unique surface molecules present in the peptidoglycan layer. In *L. monocytogenes*, the CBD is tailored to bind to the cell wall teichoic acids, ensuring the high selectivity of endolysins toward this pathogen (Eugster et al., 2011).

The structural differences between Gram-positive and Gram-negative bacteria significantly impact the action of endolysins. Gram-positive bacteria possess a thick peptidoglycan layer that is directly exposed on the outside of the cell, making more accessible to endolysin. As a result, endolysin can lyse Gram-positive bacteria immediately upon external application. In contrast, Gram-negative bacteria are protected by an outer membrane that shields their peptidoglycan layer, making it more challenging for endolysins to penetrate. Consequently, for Gram-negative bacteria, additional agents such as holins, chelators and organic acids are often required to facilitate lysis (Schmelcher and Loessner, 2016). However, organic acids like citric and malic acids could inactivate endolysins under acidic conditions (Holle and Miller, 2021; Van Tassell et al., 2017). For these reasons, the primary application of endolysins has been in controlling and inhibiting Gram-positive bacterial pathogens.

Endolysins targeting *L. monocytogenes* and their host range

The inactivation of *L. monocytogenes* using purified endolysins and EADs has been investigated for the host range and optimal inactivation conditions (Table 1). Endolysins such as PlyP100, PlyP35, PlyP40, and Ply511 were derived from virulent bacteriophages P100, P35, P40, and A511, respectively (Dorscht et al., 2009; Holle and Miller, 2021). The commercialized Listex P100, developed from bacteriophage P100, has been approved by the U.S. FDA and the European Food Safety Authority (EFSA) as a natural food preservative specifically targeting *L. monocytogenes.* PlyP100, PlyP40, and Ply511 exhibited broad activity against various *L. monocytogenes* serotypes and certain *Listeria* species (Grallert et al., 2014; Loessner et al., 1995; Van Tassell et al., 2017). Additionally, PlyP100 and PlyP40 could lyse *Bacillus subtilis* (Loessner et al., 1995; Van Tassell et al., 2017). PlyP35 demonstrated the ability to target a wide range of *L. monocytogenes*, particularly serogroups 1/2 and 3, as well as some strains from serogroups 4 to 6. (Schmelcher et al., 2011). Endolysins derived from temperate bacteriophages, such as Ply118 and Ply500 from A118 and A500, also exhibited broad lytic activity against *L. monocytogenes* serogroups 4 and 1/2, with additional activity against *B. polymyxa*, *B. subtilis*, and *B. megaterium* (Dorscht et al., 2009; Loessner et al., 1995). PlyPSA, from temperate bacteriophage PSA, lysed *L. monocytogenes* serogroup 4, although its lytic activity was lower than PlyP100 and PlyP40 (Holle and Miller, 2021; Korndörfer et al., 2006). PlyP825, an endolysin of P825, had shown a lytic activity against all *Listeria* serotypes (Grallert et al., 2014). The 293-amidase, an EAD of temperate phage vB_LmoS_293,

Table 1 Endolysins against *L. monocytogenes*

Endolysin	Origin	EAD	Target serovars	Optimal conditions			References
				NaCl	pH	Temperature	
PlyP100	P100^{M,V*}	Peptidase	All serovars *Listeria* spp. *B. subtilis*	1.5–2.0%	7–8	37–50 °C	Dorscht et al. (2009), Holle and Miller (2021), Van Tassell et al. (2017)
PlyP35	P35^{S,V}	Peptidase	Serovars 1/2, 3 *Listeria* spp.	300 mM	8–9	< 40 °C	Dorscht et al. (2009), Schmelcher et al. (2012)
PlyP40	P40^{S,V}	Muramidase	All serovars *Listeria* spp. *B. subtilis*	0%	5	25–37 °C	Dorscht et al. (2009), Holle and Miller (2021), Romero et al. (2018)
Ply511	A511^{M,V}	Amidase	All serovars *Listeria* spp.	200 mM	7.5	< 40 °C	Dorscht et al. (2009), Grallert et al. (2014)
Ply500	A500^{S,T}	Peptidase	Serovar 4, 5, 6 *Listeria* spp. *Bacillus* spp.	300 mM	9–10	< 40 °C	Dorscht et al. (2009), Schmelcher et al. (2012)
Ply118	A118^{S,T}	Peptidase	Serovars 1/2, 3, 7 *Bacillus* spp.	300 mM	8–9	< 50 °C	Dorscht et al. (2009), Schmelcher et al. (2012)
PlyPSA	PSA^{S,T}	Amidase	Serovar 4	1.0–2.0%	8	37 °C	Holle and Miller (2021), Korndörfer et al. (2006)
Ply825	P825^{U,U}	Peptidase	All serovars *Listeria* spp.	250 mM	6.5–8.5	–	Grallert et al. (2014)
293-Amidase	Phage vB_LmoS_293^{S,T}	Amidase	Serovar 4	–	–	–	Pennone et al. (2019)
Lys25	FWLLm3^{M,U}	Amidase	Serovar 1/2, 4 *Listeria* spp.	–	–	–	Zhang et al. (2012)

*M: Myoviridae S: Siphoviridae V: virulent T: temperate U: unknown

selectively killed *L. monocytogenes* serotypes 4b and 4e (Pennone et al., 2019). This enzyme exhibited a significant biofilm prevention capability, suggesting its potential in controlling *L. monocytogenes* biofilms in food processing environments (Pennone et al., 2019). Lys25, derived from the *L. monocytogenes* FWLLm3 bacteriophage isolated from ruminant feces in Christchurch, New Zealand, showed activity against all 18 tested *L. monocytogenes* isolates as well as *L. innocua* and *L. welshimeri* (Zhang et al., 2012).

Although, endolysins are generally considered specific due to their CBD, studies have confirmed that some can target multiple genera or families, indicating a broader range of antimicrobial potential. PlySs2, a bacteriophage lysin against *Streptococcus suis*, has been shown to reduce *L. monocytogenes* HER1184 and 1083 by more than 5 log units (Gilmer et al., 2013). Endolysins such as P100, Ply118, P40, and Ply500 also inhibited *Bacillus* species, suggesting their potential applications extend beyond *Listeria* species (Loessner et al., 1995; Van Tassell et al., 2017). Modifying the host range of endolysins through the removal of CBDs or domain shuffling has been explored. D27L, derived from the bacteriophage ΦCD27 targeting *C. difficile*, was able to lyse *L. ivanovii*, *B. cereus*, *B. subtilis*, and *B. amuloliquefaciens* (Mayer et al., 2011). A truncated version of the endolysin, $CD27L_{1–179}$, retaining only the catalytic domain, exhibited a broader host range against *L. monocytogenes* serotype 4b and *L. innocua* (Mayer et al., 2011; Romero et al., 2018). Through domain shuffling between PlyPSA and Ply118, EADPSA-CBD118 gained the ability against *L. monocytogenes* serotype 1/2c, with higher reduction against serotype 4b. Conversely, EAD118-CBDPSA exhibited higher activity against serotype 4b than PlyPSA (Schmelcher et al., 2011). These findings emphasize the potential of domain shuffling to fine-tune endolysin specificity and activity against different bacterial strains.

Optimal conditions for endolysins: ionic concentration, pH, and temperature

Endolysins exhibit increased stability and lytic activity under specific environmental conditions, particularly optimal ionic concentration, pH, and temperature (Table 1). Enhanced lytic activity was observed in moderate NaCl concentrations ranging from 200 to 300 mM (Grallert et al., 2014; Holle and Miller, 2021; Schmelcher et al., 2012). However, PlyP40 displayed its maximum enzymatic activity in the absence of NaCl, with its activity reduced to one-tenth at 2.5% NaCl (Holle and Miller, 2021). The optimal pH for endolysins varied significantly depending on the specific enzyme.

PlyP40 performed best under acidic conditions, with peak activity at a pH 5 (Romero et al., 2018). In contrast, PlyP100 and Ply511 exhibited maximum lytic activity in neutral to slightly alkaline pH range of 7–8 (Grallert et al., 2014; Van Tassell et al., 2017). Others such as PlyPSA, PlyP35, Ply500, and Ply118 were more effective in alkaline environments, showing increased activity at pH levels of 8 or above (Holle and Miller, 2021; Schmelcher et al., 2012). Notably, PlyP100 was highly sensitive to pH levels, with minimal activity outside the pH range of 6–10 (Van Tassell et al., 2017). Similarly, PlyPSA demonstrated reduced activity at pH 6, highlighting the sensitivity of its enzymatic function to pH (Holle and Miller, 2021). Temperature also plays a critical role in endolysin function. Most endolysins exhibited optimal activity below 40 °C. Ply511 completely lost its activity at 60 °C, indicating relatively lower thermal stability than other endolysins (Schmelcher et al., 2012). On the other hand, PlyP35 remained stable after incubation at 90 °C for 30 min (Schmelcher et al., 2012). Ply118 and Ply511 also retained 40% and 60% activity, respectively, after being exposed to 90 °C for 5 min (Schmelcher et al., 2012). In food processing and storage, factors such as pH, temperature, and ionic concentrations must be carefully controlled, as they can significantly affect endolysin activity. By combining endolysins with different optimal conditions, it is possible to achieve stable and effective antimicrobial activity across a broader range of environments. This strategy not only enhances food safety and shelf life but also enables adaptation to various processing conditions for optimal treatment.

Endolysin applications in food products

Endolysins have been extensively studied for their efficacy in controlling *L. monocytogenes* in various food products (Table 2). Ply118, Ply500, and Ply511 have demonstrated significant activity on iceberg lettuce, reducing *L. monocytogenes* by up to 2.4 log CFU/g after 6 days at 6 °C and 12 °C (Schmelcher et al., 2012). LysZ5 activity was tested in soy milk at 4 °C at a concentration of 40 U/mL, leading to undetectable levels within just 3 h, while a lower concentration of 15 U/mL achieved a 2 log CFU/g reduction compared to the control (Zhang et al., 2012). Fresh cheese, due to its high moisture content, near-neutral pH, and minimal processing, is particularly susceptible to contamination. Studies examining endolysin application in fresh cheese models at 4 °C, demonstrated their potential in food safety applications. The application of 250 µg/g of PlyP100 and PlyP40 reduced in *L. monocytogenes* by 2.5 and 0.7 log CFU/g, respectively, compared to untreated controls after 4 weeks (Holle and Miller, 2021). However, despite this initial reduction, *L. monocytogenes* continued to grow steadily throughout storage, indicating that endolysins could not fully inhibit bacterial growth over extended periods. Additionally, 750 µg/g of PlyP100 reduced *Listeria* counts by 0.5 log CFU/g from an initial inoculation of 5 log CFU/g, with activity persisting for more than 3 weeks (Van Tassell et al., 2017). At a initial inoculation of 1 log CFU/g, no growth recovery was observed with 10 U/g of PlyP100, demonstrating sustained activity when applied both initially and during storage (Ibarra-Sánchez et al., 2018). Further studies

Table 2 Endolysin treatment in food products

Endolysin	Combination with other methods	Tested foods	Conditions			Reduction compared to the control (CFU/mL)	References
			Concentration	Temperature	Incubation time		
LysZ5	–	Milk	15 U/mL	4 °C	3 h	> 1.5 log	Zhang et al. (2012)
			40 U/mL			> 5.0 log	
PlyP40	–	Fresh cheese	250 µg/g	4 °C	4 wk	< 1.0 log	Holle and Miller (2021)
PlyP100	–	Fresh cheese	250 µg/g	4 °C	4 wk	> 2.5 log	Holle and Miller (2021)
	–		750 µg/g	4 °C	4 wk	> 3.0 log	Van Tassell et al. (2017)
	–		10 U/g	4 °C	1 wk	> 1.0 log	Ibarra-Sánchez et al. (2018)
	250 ug/g nisin				4 wk	> 6.0 log	
	250 ug/g nisin H27/31 K		2.5 U/g	4 °C	4 wk	> 5.5 log	Ibarra-Sánchez et al. (2021)
PlyP825	400 MPa HHP*	Milk	34 µg/mL	25 °C	10 min	> 2.5 log	Misiou et al. (2018)
		Mozzarella cheese				> 0.5 log	
		Smoked salmon				< 1.0 log	

*HHP: high hydrostatic pressure

on milk with varying fat content (1.5% and 3.5%) revealed that Ply825, Ply511, and Ply40 were effective, achieving over 4.5 log CFU/ml reduction of *L. monocytogenes* EGDe in milk with 1.5% fat at 30 °C for 3 h, and maintained superior lytic activity with 3.5% fat as well (Grallert et al., 2014).

Synergistic effects of endolysins with other intervention methods

Nisin A, a preservative approved by the World Health Organization in 1969 and by the FDA in 1988, is widely employed in the food industry for its ability to inhibit the growth of Gram-positive bacteria, including *L. monocytogenes* (Chan et al., 2023). During the cheese aging process, pH levels tend to increase towards neutrality, causing the reduction of nisin's stability and solubility, which in turn diminishes its antimicrobial activity (Ibarra-Sánchez et al., 2021). Endolysin PlyP100 is stable and effective at neutral pH, making it a complementary agent when combination with nisin A. In Queso Fresco, a combination of 2.5 U/g PlyP100 and 250 µg nisin A reduced *L. monocytogenes* below the detection limits after 28 days (Ibarra-Sánchez et al., 2021, 2018). Other endolysins, such as PlyP40, Ply511, and PlyP825, have shown synergistic effects when treated with HHP. A combination of 0.16 µg/mL of these endolysins with 200 MPa HHP reduced *L. monocytogenes* counts by approximately 5.0–5.5 log CFU/mL (van Nassau et al., 2017). In contrast, treatment with either HHP or 0.16 µg/mL of endolysins alone exhibited little to no anti-listerial effects, indicating HHP's crucial role in enhancing endolysin efficacy, particularly at lower concentrations (van Nassau et al., 2017). Additionally, PlyP40 was more effective during the exponential phase of bacterial growth than in the stationary phase (van Nassau et al., 2017). A HHP treatment combining 3.4 µg/mL PlyP825 with 400 MPa HHP effectively inactivated *L. monocytogenes* in milk and mozzarella but had no effect on smoked salmon (Misiou et al., 2018).

Endolysin application via nanoparticles

Immobilizing endolysins on nanoparticles has shown promise in enhancing their antimicrobial activity by improving stability, extending their activity duration, and increasing the available surface area for bacterial interaction (Massani et al., 2024; Solanki et al., 2013; Stone et al., 2022). Ply500 was conjugated to silica nanoparticles (SNP), which are cost-effective and classified as GRAS by the FDA (Solanki et al., 2013). Ply500-SNPs maintained stability at 25 °C for 15 days, whereas the native Ply500 completely lost the activity against *L. innocua* ATCC33090 (Solanki et al., 2013). Furthermore, Ply500 was incorporated into a film matrix using 2-hydroxyethyl methacrylate and polyethylene glycol dimethacrylate. This approach preserved the integrity of Ply500 without leaching and exhibited excellent inhibitory effects against *L. innocua*, confirming its potential for antibacterial applications (Solanki et al., 2013). In a separate study, Stone et al. (2022) developed endolysin and amidase nanoparticles derived from phage vB_LmoS_293. These enzymes were fused to polyhydroxyalkanoate bionanoparticles (PHA_BNPs), creating PHA_lysin293_BNPs and PHA_amidase293_BNPs (Stone et al., 2022). PHA_lysin293_BNPs and PHA_amidase293_BNPs demonstrated effective inhibition of *L. monocytogenes* serotype 4e (Stone et al., 2022).

CBD application for *L. monocytogenes* detection

Fluorescent protein-tagged CBDs offered a specific and rapid method for detecting *L. monocytogenes*. Green fluorescent protein (GFP)-tagged CBDs from bacteriophages P35, A511, P40, A118, A006, A500, and PSA demonstrated high sensitivity and selectivity at picomolar to nanomolar concentrations (Schmelcher et al., 2010). These CBDs were also combined with cyan fluorescent protein, RedStar (RS) protein, blue and yellow fluorescent protein (Schmelcher et al., 2010). When used in pairs or combinations, the fluorescent protein-tagged CBD enabled highly effective differentiation of *Listeria* serotypes. Further developments by Schmelcher et al. (2011) created chimeric enzymes by fusing GFP with CBD500, CBD118, CBDP35, and the linker region of PlyPSA (Schmelcher et al., 2011). While some combinations showed reduced fluorescence intensity, most displayed enhanced detection with a wider range of serovars. Notably, CBD500-500 exhibited approximately a 50-fold increase in binding affinity for the cell wall compared to the original CBD500 and showed greater activity at ionic strengths above 1 M (Schmelcher et al., 2011).

In addition, an electrochemical detection approach has also been developed using CBD500, derived from Ply500, immobilized on a gold screen-printed electrode (Tolba et al., 2012). This system employs electrochemical impedance spectroscopy to detect changes in impedance, corresponding to the presence of *Listeria* cells. The detection limit was determined to be 4.04 log CFU/mL in pure culture and 5.04 log CFU/mL in 2% milk, although some non-specific responses were observed with *E. coli* (Tolba et al., 2012).

CBD application for recovery of *L. monocytogenes* cells in food products

CBD500 and CBD118 were immobilized on M-270 Epoxy Dynabeads, resulting in the attachment and recovery of over 90% of *L. monocytogenes* cells within 40 min (Kretzer et al., 2007). This approach demonstrated greater sensitivity and faster detection than standard ISO enrichment techniques when applied to artificially and naturally contaminated foods, including sliced iceberg lettuce, Camembert soft cheese, red smear cheese, smoked salmon, minced meat, turkey breast, and milk (Kretzer et al., 2007). Furthermore, the method was utilized to concentrate *L. monocytogenes* cells prior to PCR and plating in raw milk, yielding a 27.67%-point higher recovery rate through plate counting than real-time PCR (Walcher et al., 2010). The same research group further optimized the method by incorporating the reporter bacteriophage A511::*luxAB*, enhancing detection sensitivities in various food matrices such as chocolate milk, mozzarella, iceberg lettuce, shrimp, minced meat, smoked salmon, smoked turkey breast, and soft cheese (Kretzer et al., 2018). Additionally, the concentration process using CBD-P40-coated beads achieved over 90% cell recovery in standardized assays (Schmelcher et al., 2010). When applied to milk and Camembert cheese, these beads enabled rapid and efficient concentration of *Listeria* cells, allowing for effective detection using RS-CBDP35 and GFP-CBD500 (Schmelcher et al., 2010).

Conclusion

L. monocytogenes is a lethal pathogen capable of surviving in various harsh environments. As discussed, endolysins derived from bacteriophages have demonstrated the ability to inhibit growth and induce cell death of this pathogenic bacterium during food storage processes. Their rapid detection can also prevent potential outbreaks and aid in tracing contamination sources. While commercial bacteriophages such as P100 are employed in controlling *L. monocytogenes*, endolysins have yet to be extensively applied in food hygiene practices. Numerous studies have validated the exceptional efficacy of endolysins, along with their promising applications facilitated by gene recombination technologies. This signals a positive outlook for their integration into food safety. However, despite the proven effectiveness of endolysins in laboratory settings, additional research is essential to translate these findings into industrial applications, ensuring their effective integration into large-scale food processing environments. Moreover, considering the diverse physicochemical properties of food, which can impact the efficiency of endolysins, it is critical to investigate their characteristics under various conditions. Developing universally applicable methods for the use of endolysins in food hygiene will be vital for enhancing food safety and public health outcomes. Future research should focus on standardizing endolysin-based approaches, addressing regulatory considerations, and developing cost-effective production methods to support large-scale implementation. Integrating endolysins into existing food safety frameworks could significantly enhance microbial control strategies, reducing the risk of foodborne outbreaks. By overcoming these challenges, endolysins have the potential to become a key tool in improving food safety and public health.

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