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Unlocking the Potential of Bacteriophage Endolysins: A Promising Alternative for Combating Antibiotic Resistance in Poultry

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ABSTRACT

Bacteriophages are viruses that infect bacteria, exploiting their cellular machinery for replication. They employ a lytic system to disrupt the bacterial cell wall. Towards the end of the lytic cycle, phage proteins known as holins form pores in the cytoplasmic membrane, allowing endolysins to enter the peptidoglycan and hydrolyze it, resulting in bacterial lysis. Endolysins possess modular structures, with enzymatically active domains and cell wall binding domains enabling specific recognition and breakdown of peptidoglycan bonds in bacteria, particularly Gram-positive species. Endolysins have evolved over millions of years, exhibiting high efficiency in bacterial lysis. In the context of the poultry industry, antibiotics play a crucial role in disease control and growth promotion, but their overuse contributes to antibiotic resistance and poses risks of zoonotic infections. Bacteriophage endolysins represent a promising alternative for combating multidrug-resistant (MDR) bacterial infections and eradicating bacterial biofilms associated with poultry pathogens. However, challenges including delivery stability and efficacy in poultry environments need to be addressed. Encapsulation into liposomes or nanoparticles enhances endolysin stability and delivery, while spray-dried formulations can preserve bioactivity and enhance stability for potential poultry applications. Endolysins offer a viable alternative to antibiotics for combating bacterial resistance, although further research is needed to optimize their application and overcome limitations. This review describes bacteriophage endolysins as a sustainable alternative to antibiotics in poultry farming.

INTRODUCTION

Bacteriophages are viruses that infect bacteria and use bacterial cells for replication. Lytic bacteriophages employ a multi-component lysis system that ultimately disrupts the bacterial cell wall, leading to lysis. This process releases multiple progeny phage particles [1, 2].

At the end of the lytic cycle, a phage protein known as holin form pores in the cytoplasmic membrane. This action facilitates the entry of the endolysins into the peptidoglycan layer of the host bacterial cell. The endolysins then rapidly degrade the peptidoglycan, causing osmotic lysis of the bacterial cell [3].

Phages that infect Gram-positive bacteria express endolysin genes characterized by a modular structure. These endolysins typically feature at least two distinct functional domains: an N-terminal enzymatically active domain (EAD) and a C-terminal cell wall binding domain (CBD). The CBDs possess specific recognition capabilities, allowing them to bind to ligands present on the bacterial cell wall. Meanwhile, the EADs catalyze the breakdown of peptidoglycan bonds, ultimately leading to bacterial cell lysis [4, 5].

Phage endolysins are highly efficient murein hydrolases that have evolved over millions of years to rapidly lyse bacterial cells. While bacteriophages are viable antibacterials, endolysins offer several advantages over phage particles, making them superior candidates as alternatives to antibiotics [6, 7].

According to the World Health Organization (WHO), the use of antibiotics in animal production should be controlled and restricted due to the risk of selecting resistant bacteria in different environments when these drugs are used indiscriminately. Therefore, the European

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Union (EU) prohibited the use of antibiotics as growth promoters in poultry and other livestock in 2006 as a precaution and the EU ban forced other countries to comply with these new regulations in order to maintain access to this major economic market [8]. This issue is a major public health concern due to the potential for antibiotic resistance to transfer to the human population through the consumption of poultry meat, although the exact mechanisms and extent of this transfer are not fully understood (Figure 1) [9]. This review highlights bacteriophage endolysins as a highly promising and sustainable alternative to antibiotics in the poultry industry, with minimal risk of resistance development.

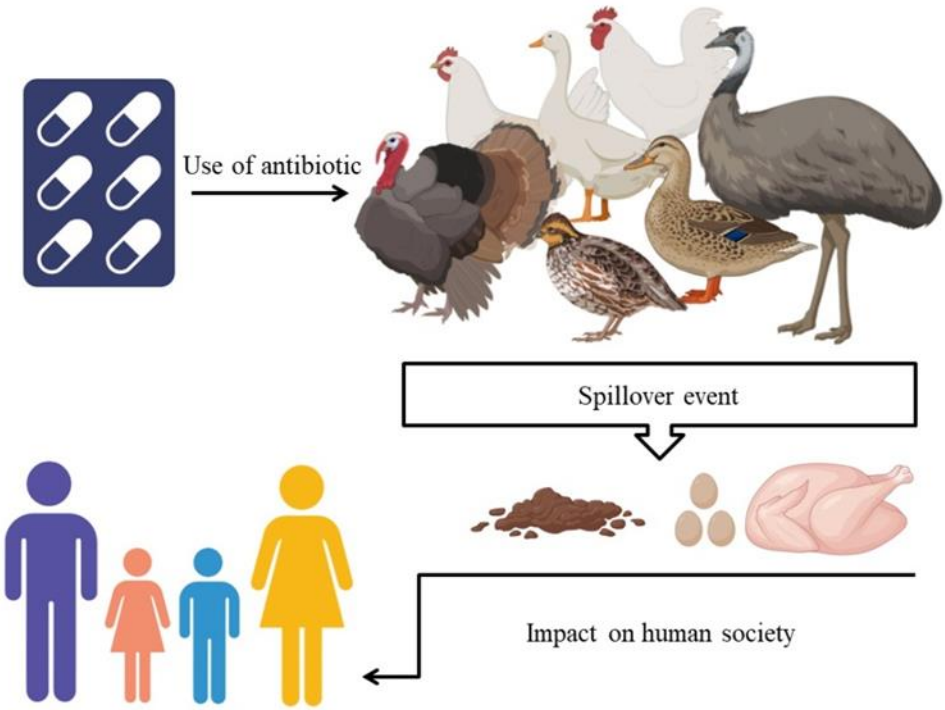

Fig. 1. Antibiotic use in poultry farming and its entry into food, the environment, and human health systems (Created with BioRender.com)

Antibiotics in the poultry industry

Antibiotics play a crucial role in the poultry industry, combating disease outbreaks, treating illnesses, and promoting poultry growth [10]. However, broad-spectrum activity (targeting multiple bacterial types) can disrupt the balance of beneficial microbiota [11].

A variety of Gram-negative bacteria, including *Salmonella enterica* serovars, *Campylobacter jejuni*/*coli*, and *Escherichia coli*, as well as Gram-positive bacteria such as *Enterococcus* spp., *Staphylococcus* spp., and *Clostridium perfringens*, have been identified in poultry carrying multiple antimicrobial resistance (AMR) determinants [12].

Poultry products pose a significant risk to human health due to foodborne pathogens, notably salmonellosis and campylobacteriosis. Yet, effectively controlling *Salmonella* spp. and *Campylobacter* spp. during poultry processing and production poses significant challenges due to multiple sources of pathogen contamination, such as contaminated feed, water, and surfaces within poultry enterprises. Additionally, the close and frequent interaction between humans and birds further complicates disease management efforts [13].

The use of antibiotics in animal feed as growth promoters is a primary contributor to antibiotic resistance and a significant stressor for the poultry gut epithelium, leading to dysbiosis [14], and the emergence of MDR bacteria that pose a critical threat to both human and animal health [15, 16]. Antibiotic overuse in animal husbandry exerts strong selection pressure that drives the proliferation and spread of antibiotic-resistant pathogens.

Among antibiotic families, tetracycline is commonly used in the treatment and control of various animal diseases, particularly in poultry farming. Tetracycline offers the advantage of oral administration with manageable side effects in poultry. It is extensively employed in treating infections in both humans and animals. Additionally, tetracycline is among the most cost-effective classes of antimicrobials available [17, 18].

Therefore, there is an urgent need to develop novel techniques for controlling foodborne bacteria. Bacteriophage endolysins have emerged as a highly

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specific, effective, and environmentally friendly alternative for controlling antibiotic-resistant foodborne pathogens, including in poultry processing environments [19].

The antibacterial activity of bacteriophage endolysins

Bacteriophage endolysins exhibit potent antibacterial activity by hydrolyzing specific bonds in the peptidoglycan layer, thereby causing rapid osmotic lysis of the bacterial cell. Endolysins typically exhibit high specificity at the species or genus level, although several naturally broad-spectrum endolysins have been identified [20]. Their unique "outside-in" mode of action**,** which does not require bacterial metabolic activity, virtually eliminates the development of resistance and enables bactericidal effects within minutes to hours. Table 1 provides a list of several examples of bacteriophage endolysins demonstrating antibacterial activity.

Table 1. Antibacterial activity of endolysins against target bacteria

Bacteriophage endolysin	Bacterial target	Reference
phiSM101	*Clostridium perfringens*	[21]
Ply3626	*Clostridium perfringens*	[22]
PlyGVE2CpCWB	*Clostridium perfringens*	[23]
CP25L	*Clostridium perfringens*	[24]
LysCP28	*Clostridium perfringens*	[25]
LysSE24	*Salmonella* strains	[26, 27]
Innolysin Cj1	*Campylobacter jejuni*	[28]
LysO78	*Escherichia coli*	[29]
LysZ5	*Listeria monocytogenes, Listeria innocua and Listeria welshimeri*	[30]
CS74L	*Clostridium acetobutylicum and Clostridium tyrobutyricum*	[31, 32]
Gp110	*Salmonella* and other Gram-negative bacteria	[33]
CP25L	*Lactobacillus johnsonii*	[34]
LysAm24, LysAp22, LysECD7, LysSi3[1]	*Klebsiella pneumoniae*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, and *Enterobacter* spp.	[35]
LysAB1245	*Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*	[20]

Note: Each endolysin was tested individually.

Biofilm removal efficacy of bacteriophage endolysin

Biofilms exhibit high tolerance to antimicrobial agents owing to their intricate microbial structure and matrix composition. The extracellular polymeric substances (EPS), particularly polysaccharides, are prime targets for phage-encoded depolymerases, while exposed peptidoglycan is targeted by endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) [36]. Phages encode various enzymes, including VAPGHs, endolysins, and depolymerases, to engage with and eliminate their host bacteria. Several of these enzymes are already harnessed as recombinant proteins, providing a potent antibacterial strategy for combating biofilms [36].

Bacteriophage endolysins disrupt bacterial biofilms by lysing embedded cells via peptidoglycan degradation, breaking down the biofilm structure and making bacteria more susceptible to clearance. Studies have shown that bacteriophage endolysins can effectively penetrate and degrade biofilms formed by various bacterial species, including poultry-relevant pathogens such as *Salmonella* spp. and *C. perfringens*. This supports the development of new therapeutic strategies to combat biofilm-associated infections in medical, veterinary, and poultry processing settings. The mechanism and efficacy of bacteriophage endolysins in biofilm disruption and bacterial cell lysis are illustrated in Figure 2.

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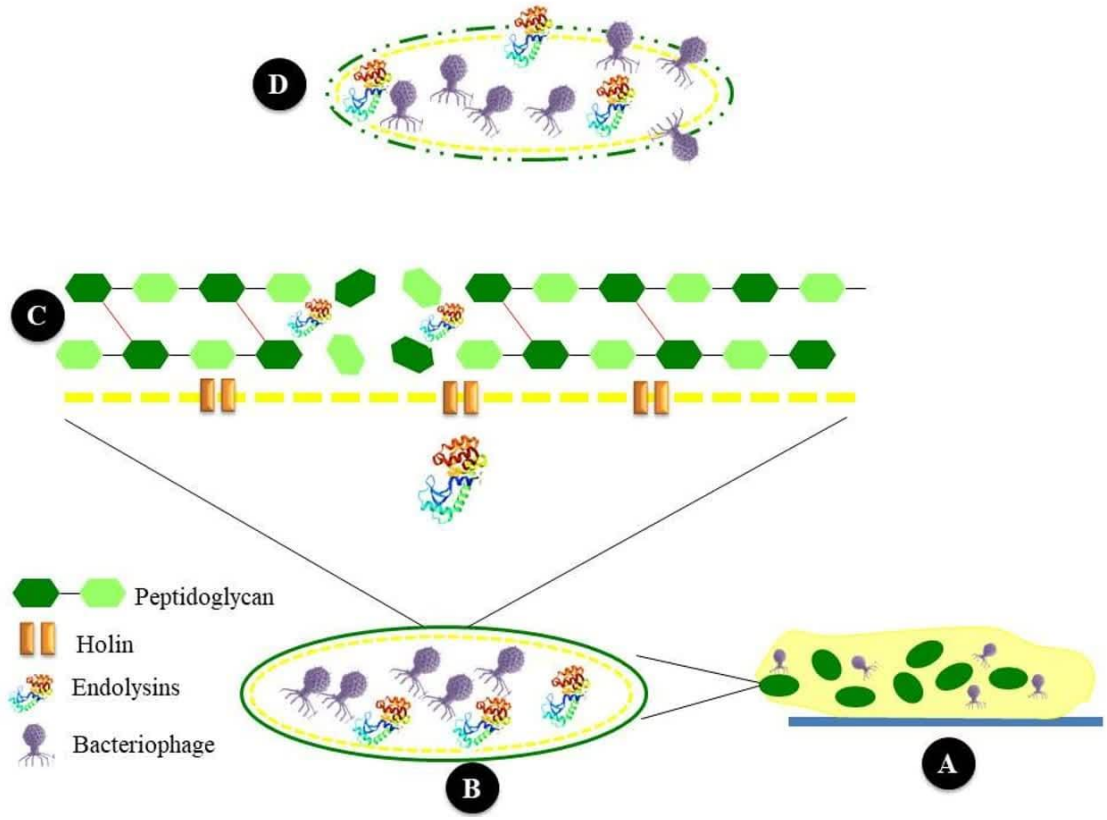

Fig. 2. Efficacy of bacteriophage endolysin for biofilm removal. Sequential steps: A) Bacterial biofilm formation; B) Bacteriophage propagation and endolysins production; C) Degradation of the bacterial cell wall; D) Bacterial lysis (Created with BioRender.com)

DISCUSSION

Ongoing research explores the potential applications of bacteriophage endolysins for biofilm control. In a recent study, researchers isolated and characterized a bacteriophage named PBEF129, which infects *Enterococcus faecalis*, a Gram-positive pathogen that colonizes the human intestinal tract and forms biofilms, rendering it highly resistant to numerous antibiotics. When this endolysin was applied to bacterial biofilms on the surface of human intestinal cells cultured *in vitro*, the results indicated that it had comparable or slightly lower biofilm removal efficacy than its parent phage and cefotaxime, mainly due to limited matrix penetration [37].

In another study, the lytic capabilities of the endolysin LysH5 was investigated against biofilms formed by *Staphylococcus aureus* and *Staphylococcus epidermidis*. Staphylococcal biofilms pose significant challenges in both clinical and food-related environments due to their role as a primary source of contamination. Their findings confirm that LysH5 effectively reduced the population of staphylococcal sessile (biofilm-embedded) cells by 1–3 $\log_{10}$ units compared to untreated controls. Furthermore, they assessed the lytic potential of LysH5 against specific subpopulations, including *S. aureus* exponential cultures and persister cells induced by rifampicin and ciprofloxacin treatment. Their results indicated that LysH5 not only exhibits remarkable activity against staphylococcal biofilms but also inhibits persister cells, suggesting its potential as an adjunct therapy alongside certain antibiotics [38].

Other studies investigating the efficacy of bacteriophage endolysins in removing biofilms include the following:

Cha *et al*. (2019) demonstrated a reduction of *S. aureus* biofilm mass by 80–90% on various food contact surfaces, such as polystyrene, stainless steel, and glass, using the LysCSA13 endolysin [39].

Shen *et al*. (2013) utilized PlyC endolysin to rapidly disrupt the biofilm matrices of *Streptococcus pyogenes*, whose biofilm-embedded cells exhibit high tolerance to conventional antibiotics [40].

Oliveira *et al*. (2014) found that the combination of Lys68 endolysin with malic or citric acid resulted in a synergistic ≈1.5–2 $\log_{10}$ CFU reduction of *Salmonella* biofilms [41].

The LysPA26 endolysin demonstrated the ability to reduce viable biofilms of *P. aeruginosa* 8327 by 2–3 $\log_{10}$ CFU/cm² after 48 h on polystyrene surfaces [42]. Simmons *et al*. (2012) reported that Ply511 endolysin disrupted *Listeria monocytogenes* biofilms and showed

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strong synergy when combined with proteinase K [43]. Collectively, these studies demonstrate the high potential of endolysins, alone or in combination with other agents, as next-generation antibiofilm therapeutics.

Salmonellosis remains a major foodborne threat to human health, making the control of Salmonella biofilms in poultry production as a critical priority. Salmonellosis is commonly associated with the consumption of contaminated poultry meat, eggs, and derived products. The efficacy of bacteriophage endolysins and whole phages against *Salmonella* biofilms is summarized in Table 2.

Table 2. The application of bacteriophage on inhibition of the *Salmonella* biofilms

Phage	*Salmonella* spp.	Effect	Biofilm Reduction	Reference
LysSTG2 endolysin *	*Salmonella Typhimurium*	A 100 μg/mL LysSTG2 treatment (1 hour) reduced 72-hour *S. Typhimurium* biofilms by 13%, while combination with hypochlorous water (40 mg/L (40 ppm) available chlorine) achieved a 99% reduction.	0.7 log (13%) → 2.0 log (99%)**	[44]
Phage SE2	*Salmonella enterica serovar Enteritidis*	Biofilm studies showed a 97% reduction in viable cells.	1.5 log (97%)	[45]
Phage P22	*Salmonella enterica* subsp. enterica serovar *Typhimurium* strain DMC4 (abbreviated as *S. Typhimurium*)	The ability of *S. Typhimurium* to form biofilms was significantly diminished at higher phage concentrations (10^6 PFU/mL and above). Effective at 10^4-10^8 PFU/mL	1.0-3.0 log (90-99.9%)***	[46]
Phage P22 + EDTA + nisin	*Salmonella Typhimurium*	The combination of the three agents inhibited *S. Typhimurium* biofilm formation by 93% at low phage titer concentrations, but only resulted in a 70% reduction in mature biofilms	1.1 log (93%) prevention; 0.5 log (70%) removal	[47]
Phage cocktail (4.8 dominant)	Different *Salmonella* serotypes	Phage 4.8 was most effective overall (53%), especially against *Enteritidis* (60%). *Typhimurium* was highly susceptible (100%), while *Mbandaka/Senftenberg* showed low susceptibility (0–17%). Phage 5.1 had the weakest efficacy (31%), despite 60% success against *Heidelberg*.	0.3-2.0 log (17-100%)	[48]
Phage PVP-SE2	*Salmonella Enteritidis*	Phage PVP-SE2 effectively controlled *Salmonella Enteritidis* biofilms, showing: • 1.5-5.1 log CFU/cm² reductions on polystyrene at 22°C (optimal at MOI 0.1) • 1.4-1.9 log reductions on stainless steel • Minimal efficacy (0.5 log) at 4°C • Better performance against younger biofilms (24h *vs* 48h) • Modest but significant 0.5-1 log reductions on refrigerated poultry skin	0.5-5.1 log (68-99.9%)	[49]
Phage cocktail (vB_SenM-1/-2/-3)	Various serovars and strains of *Salmonella enterica*	All tested Salmonella strains (2 *Typhimurium* + 2 *Enteritidis*) showed ≥1-log reduction with at least one phage across all temperatures (25–42°C) and MOIs (0.1–1), confirmed by CFU/mL.	≥1.0 log (90%)	[50]
Phage cocktail (BP 1369/1370)	*Salmonella spp.*	• 3.0, 2.0, and 3.0 log CFU/cm² reductions on stainless steel, rubber, and MBEC surfaces • >1.0 log CFU/cm² reduction on lettuce-adhered cells	1.0-3.0 log (90-99.9%)	[51]

*LysSTG2 is a phage-derived endolysin targeting *Salmonella enterica* serovar Typhimurium biofilms. It is not a bacteriophage itself but a lytic enzyme encoded by phages.

**Log_{10} reductions were calculated as $-log_{10}$ (1 - percentage reduction/100)

*** Range estimated from concentration-response relationship

Enhancing delivery formulations of endolysins for improved control of MDR infections

Therapeutic use of endolysins is an emerging approach in combating MDR infections. However, interdisciplinary efforts aimed at optimizing these therapies are increasingly evident in current literature. Enhancing the stability of these endolysins can be achieved through encapsulation or other methods, such as chemical modification, or encapsulation within liposomes or nanoparticles, thereby preserving their full activity within the body [52]. The exogenous application of recombinant endolysin is primarily effective against Gram-positive bacteria due to the inability of endolysins to penetrate the outer membrane of Gram-negative bacteria. In a study using protein engineering, researchers succeeded in producing Artilysins (engineered endolysins), which were capable of lysing Gram-negative bacteria [53]. In other words, if endolysins can access the peptidoglycan of Gram-negative bacteria, they can break it down. However, researchers have developed a novel approach utilizing liposome-mediated endolysin encapsulation to overcome this limitation.

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Encapsulation into liposomes. In a study, the endolysin BSP16Lys was encapsulated into cationic liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and hexadecylamine. The encapsulation efficiency of BSP16Lys into the liposome was determined to be 35.27%. When tested against *Salmonella Typhimurium* and *E. coli*, cells treated with BSP16Lys-encapsulated liposomes exhibited reductions in viable cell numbers by 2.2-log CFU/mL and 1.6-log CFU/mL, respectively. The experiments were conducted using log-phase (actively growing) cultures of *S. Typhimurium* and *E. coli* with initial concentrations of ~10^7 CFU/mL. All measurements were taken after 1 hour of treatment using CFU/mL determinations from 1 mL bacterial suspensions. They observed complete eradication of *S. Typhimurium*, corresponding to a >6–7 $\log_{10}$ CFU/mL reduction (from ~10^7 CFU/mL to below the detection limit of 10 CFU/mL), and a 1.6-log reduction in *E. coli* (from ~10^7 to ~2.5×10^5 CFU/mL). Control experiments with lysozyme-containing liposomes (2.0 mM lipid) showed strain-dependent differences: a 1.5 $\log_{10}$ reduction in *S. Typhimurium* (from ~10^7 to ≈3.2×10^5 CFU/mL) and a 1.9 $\log_{10}$ reduction in *E. coli* (from ~10^7 to ≈1.3×10^5 CFU/mL). These findings demonstrated the potential of liposome-mediated delivery of endolysin for exogenous application against Gram-negative bacteria [52].

Nanoparticle formulations offer several advantages for delivering antimicrobial agents like endolysins in poultry applications, including improved stability, controlled release, enhanced bioavailability, and targeted delivery to specific sites of infection [54].

These benefits make nanoparticles an emerging area of research and development for endolysin delivery. Among various options, chitosan nanoparticles represent a particularly promising approach as they are biocompatible and non-toxic, making them suitable for food safety applications. For instance, the bactericidal activity of endolysin Cpl-1 remains intact when encapsulated in chitosan nanoparticles [55].

Gondil *et al*. (2020) demonstrated this by encapsulating Cpl-1, a full-length endolysin, in chitosan nanoparticles to enhance its *in vivo* half-life and bioavailability [55]. Chitosan nanoparticles have been extensively investigated as macromolecular delivery vehicles for various compounds, including peptides, proteins, nucleic acids, and plasmids, to improve their bioavailability and protect them from the biological environment, thereby extending their *in vivo* half-life [56-58]

These strategies could also address the potential issue of immune system-triggered production of specific antibodies, which might reduce the efficacy of bacteriophages or enzymes, or even induce immune responses [59]. Furthermore, chemical modifications such as PEGylation of bacteriophages (the covalent attachment of polyethylene glycol (PEG) chains to the surface of the phage particles) or their lytic enzymes can enhance their *in vitro* stability and prolong their shelf lives [60].

Spray-dried endolysins for inhalable powder formulations. In a recent study by Wang *et al*. (2023), the feasibility of producing inhalable powders using spray-dried endolysins Cpl-1 and ClyJ-3 was investigated with various excipients. Each endolysin was individually tested with leucine and either lactose or trehalose for the spray drying process. While Cpl-1 maintained its bioactivity in the spray-dried powders, ClyJ-3 showed a complete loss of bioactivity after atomization of the liquid feed solution. Formulations of Cpl-1 with leucine and either lactose or trehalose exhibited promising physicochemical properties and aerosol performance, with fine particle fraction values exceeding 65%. These findings suggest that Cpl-1 can be successfully formulated as spray-dried powders suitable for inhalation delivery to the lungs, offering stable formulations for potential poultry applications [61]. Wang *et al*. (2023) demonstrated differential stability of endolysins upon spray-drying: Cpl-1 preserved full bioactivity, while ClyJ-3 completely lost antibacterial activity upon atomization. This difference arises due to inherent structural variations among the proteins. The chimeric instability of ClyJ-3 may be due to its altered Cell Wall-Binding Domain (CBD), Enzymatically Active Domain (EAD) and linker regions which would be susceptible to shear stress denaturation and air-liquid interface denaturation during atomization, as consistent with its previously known instability in nebulization. In contrast, the native phage-derived conformation of Cpl-1 confers excellent stability with preservation of structural integrity (established by unaltered pre- and post-spray drying spectra) and biological activity even with leucine/lactose or leucine/trehalose excipients. The high fine particle fraction (>65%) of stable Cpl-1 formulations suggests good pulmonary delivery potential, though poultry applications may favor water/feed delivery due to the technical constraints of stabilizing more sensitive endolysins like ClyJ-3. Future stabilization processes could explore nanoparticle encapsulation to stabilize sensitive proteins during processing [61].

Therefore, incorporating endolysins into feed additives or water treatments may be more feasible for poultry applications. Administering phage treatment through drinking water or feed not only reduced mortality rates but also enhanced weight gain and improved feed conversion ratios (FCR) in experimentally infected broiler chickens [62]. Nonetheless, further research and development would be necessary to evaluate the efficacy, safety, and practicality of such approaches in poultry production settings.

Advantages and disadvantages of the bacteriophage endolysin application

Overall, while endolysins offer several advantages for controlling bacterial infections, there are also challenges

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and limitations that need to be addressed for their successful implementation in various fields, including medicine, agriculture, and food safety (Table 3).

Table 3. Advantages and disadvantages of the bacteriophage endolysin application

Advantages	Reference	Disadvantages	Reference
High specificity	[63]	Narrow host range for some endolysins	[64]
Rapid action (within hours)	[63]	Potential immunogenicity, eliciting an immune response, may limit long-term efficacy or cause adverse host reactions	[59]
Low risk of resistance development	[63]	Large-scale production challenges	[65, 66]
Some endolysins have a broader spectrum of action compared to some phages	[67]	Formulation need and stability issues due to sensitivity under certain environmental conditions, such as extreme pH or temperature	[68]
Biodegradability, offering an environmentally friendly alternative to synthetic antimicrobials	[69]	Regulatory challenges due to varying international standards for biologic approval	[70]

CONCLUSION and FUTURE PERSPECTIVE

Endolysins are a sustainable alternative for managing MDR bacterial diseases in poultry farming, serving as an environmentally friendly alternative to conventional antibiotics. While their key advantages like target specificity and rapid action have been thoroughly reported (Table 3), a key advantage is their ability to provide targeted antimicrobial action with minimal impact on beneficial microbiota or resulting in resistance. Literature shows significant advances in overcoming formulation challenges using newer delivery systems such as nanoparticle encapsulation and spray-drying to allow for higher stability and bioavailability under conditions of the real world. Yet, their full potential depends on addressing key challenges: scaling up manufacture to the level demanded by farms, designing poultry-specific delivery modalities responsive to plural farm environments, and structuring regulatory frameworks to define such novel biologics. The path forward needs concerted efforts by researchers, industry stakeholders, and policymakers to translate these promising lab results into feasible, cost-effective, and scalable solutions that have the potential to revolutionize poultry health management in line with global efforts to reduce antimicrobial resistance.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest associated with this manuscript.

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AI DISCLOSURE

No AI tools were used in the preparation of this manuscript.

DATA AVAILABILITY

All data supporting the findings of this study are included within the manuscript.

AUTHORS' CONTRIBUTIONS

MK: Conceptualization; Supervision; Writing – Original Draft; Writing – Review & Editing. IRH, AJ, MG, MHA, MM, PG: Investigation; Writing – Review & Editing. All authors read and approved the final version of the manuscript.

ETHICS STATEMENT

This study is a review article that relies solely on the synthesis of previously published literature and does not involve original experimental research on human or animal subjects. Therefore, approval from an ethics committee was not required.

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