



Enzybiotics, a Promising Era in Confronting Bacteria in the Poultry Industry

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Abstract

Since the discovery of antibiotics, they have been used widely for disease control. Antibiotics were also found to be useful in growth promotion in the poultry industry. However, their overuse and misuse have led to bacterial resistance against them. Antibiotic resistance is a global issue that results in considerable health and economic losses. Marked antibiotic resistance against various antibiotics has been observed in poultry infections. To counteract the burdens of antibiotic resistance, various alternatives for antibiotics are being studied. These alternative approaches have also been subjects of interest in the poultry industry, as poultry infections result in dramatic economic loss, and in cases of zoonotic infections, the transmission of infection from chicken leads to dramatic health burdens in humans. Phage therapy, probiotics, and anti-microbial peptides administration are some examples of these alternative approaches. Another antibiotic alternative approach is called “enzybiotics”. In enzybiotics, peptidoglycan hydrolases (endolysins) are used to degrade bacterial cell walls. These enzymes are mostly found in bacteriophages’ genomes because bacteriophages have to degrade the peptidoglycan layers of bacteria both to enter and exit their bacterial host. Bacterial genomes also contain some regions with peptidoglycan hydrolase properties which help bacteria in growth and division. The properties of various peptidoglycan hydrolases have been studied to find the more potent and applicable ones for future uses. Due to their advantages, endolysins are promising antibiotic alternatives. In this review, we will discuss the role of enzybiotics in the poultry industry. Also, endolysin advantages and limitations of their administration are discussed here.

Keywords: *Enzybiotics, Endolysin, Peptidoglycan hydrolase, Clostridium perfringens, poultry, Antibiotic resistance*

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Introduction

Bacterial infections are among the major causes of global health burden (Antimicrobial Resistance Collaborators, 2022). In 2019, 13.7 million deaths were linked to bacterial infections (Antimicrobial Resistance Collaborators, 2022). Besides their role in human diseases, bacteria also cause dramatic effects on the veterinary industry. Approximately 50% of the mortality rate of the broilers in the first week of their lives seems to be caused by bacterial infections. Bacterial diseases contribute to half of the non-outbreak-related broilers' mortality rate. Additionally, bacteria-induced outbreaks increase broilers' mortality dramatically (Thøfner & Christensen 2021). Antibiotic discovery has provided remarkable benefits to societies, both in saving human lives and in decreasing economic losses (Hegemann *et al.*, 2023; Ogwuche *et al.*, 2021). Moreover, antibiotics benefited us in the field of veterinary due to their usage as animal growth promoters (Dibner & Richards 2005). Unfortunately, antibiotic overuse and misuse have contributed to a notable surge in antibiotic resistance (Organization World Health, 2017). About 5 million deaths in 2019 were found to be associated with bacterial resistance (Antimicrobial Resistance Collaborators, 2022). In the poultry industry, besides the treatment failure-caused economic losses, antibiotic resistance is considered a threat to human health, due to resistant zoonotic pathogen transfer (Nhung *et al.*, 2017). Regarding the dangers of antibiotic resistance, the USA, Europe, and China banned antibiotic usage as animal growth promoters in 2017, 2006, and 2020, respectively (Dibner & Richards 2005; Centner, 2016; Hu & Cowling, 2020). The application of an antibiotic alternative is another solution to combat bacterial infections without increasing antibiotic resistance (Murray *et al.*, 2021). Some antibiotic alternatives are probiotics, antimicrobial peptides, phage therapy, and enzybiotics (Łojewska & Sakowicz 2021; Murray *et al.*, 2021).

Enzybiotics

Enzybiotics definition consists of two words, antibiotics and enzymes. This category of antibacterials is comprised of enzymes with the ability to kill bacteria. Peptidoglycan hydrolases are a subtype of enzybiotics. Peptidoglycan hydrolases degrade bacterial cell walls by targeting the

peptidoglycan layer of the bacteria. These enzymes are named endolysins and are mostly found in the bacteriophage genome. Bacteriophages use endolysins both for entering their host bacteria and exiting their progeny phages after a lytic cycle (Danis-Włodarczyk *et al.*, 2021). Another source of peptidoglycan hydrolases is the prophage region of the bacterial genomes (Evseev *et al.*, 2023). Some sequences with hypothetical peptidoglycan hydrolase properties are found in the prophage region of many bacterial genomes. Peptidoglycan hydrolases are also needed for cell wall remodeling and division. These peptidoglycan hydrolases are named autolysins (Leonard *et al.*, 2023). According to the site of action of the peptidoglycan hydrolases on their target peptidoglycan layer, scientists divide these enzymes into 5 groups, including muramidase, glucosaminidase, endopeptidase, amidase, and lytic transglycosylase (Sekiya *et al.*, 2021a).

Endolysin structure

The endolysins that target gram-positive bacterial cell walls are composed of at least an enzymatically active domain (EAD) with peptidoglycan hydrolase properties in the N-terminal region, and a cell wall binding domain (CBD) in the C-terminal region, which helps in recognition of the target bacteria (Figure 1a) (Schmelcher *et al.*, 2012). In contrast, gram-negative bacterial endolysins don't contain CBD; however, the majority of them contain a C-terminal portion rich in charged amino acid residues (Ghose & Euler 2020). These charged portions help the endolysin in destabilizing the target bacteria's lipopolysaccharide (LPS) layer, thus facilitating the arrival of the endolysins to their site/place of action (Figure 1b) (Gutiérrez & Briers 2021).

Endolysin advantages

Both bacteriophages and endolysins have narrow host ranges, so commensal bacteria will be immune from unwanted eradication (Murray *et al.*, 2021). Bacteriophages are only capable of destroying their host bacterial strain; whereas, the bacteriophage endolysin host range is broader to some extent. Bacteriophages are only capable of destroying their host bacterial strain; whereas, the bacteriophage endolysin host range is broader to some extent (Wang *et al.*, 2022).



Figure 1. Endolysin structure. (a) Endolysin structure in gram-positive bacteria. (b) Endolysin structure in gram-negative bacteria.

Endolysins are preferred to phages because endolysins are usually less immunogenic. Moreover, some endolysins are capable of penetrating eukaryotic cells (Liu *et al.*, 2023a). So, unlike phages, endolysins can be used against intracellular bacteria (e.g. *Salmonella* species) (Liu *et al.*, 2023a; Diacovich *et al.*, 2017). There is a low risk for the development of resistance against endolysins (Rahman *et al.*, 2021). Although endolysins are promising factors to combat bacterial infections, they need much more investigation to reach the market (Schmelcher & Loessner 2021).

Bacterial infections in the poultry industry

The poultry industry is a widespread food industry, as more than 90 billion tons of chicken meat are produced annually (FAO, 2020). The average global amount of poultry meat consumption in 2011 was 14.5 kg per capita with an increasing trend over the years (Wahyono & Utami 2018). In Iran, the average poultry meat consumption was estimated to be 23 kg per capita (Ranaei *et al.*, 2021). One reason for their considerable production rate is that poultry industry costs are relatively reasonable. But it is notable that due to the improper use of antibiotics in poultry farming, antibacterial resistance has grown to a dangerous point (Nhung *et al.*, 2017). The most burdensome bacterial infections in poultry are caused by *Salmonella* species, Avian Pathogenic *Escherichia coli* (APEC), *Campylobacter jejuni*, *Clostridium perfringens*, *Clostridium botulinum*, *Pasteurella multocida*, and *Mycoplasma gallisepticum* (El-Saadony *et al.*, 2022; Kemmett *et al.*, 2014; Al Hakeem *et al.*, 2022; Ali & Islam 2021; Sato *et al.*, 2016; Li *et al.*, 2020; Awad *et al.*, 2022). The

resistant bacteria increase poultry production costs because antibiotic usage becomes ineffective against them and they increase mortality rates in poultry. Antibiotic resistance in Avian Pathogenic *Escherichia coli* against amoxicillin, ampicillin, and tetracycline was more than 80% in several studies (Nhung *et al.*, 2017). In our previous study, we examined the antibiotic resistance of *Escherichia coli* extracted from yolk sac infections. We found that antibiotic resistance against ceftiofur and gentamycin was 42.5% and 40%, respectively. Additionally, 70% antibiotic resistance against colistin and phosphomycin was observed in *Escherichia coli*; whereas, its resistance against sultrim, fluorophenicol, and erythromycin was 90% (Habibi & Ziyaii 2021). *Salmonella* isolates that were found in raw poultry meat showed 21.44-32.6% resistance against the cephalosporin family (Castro-Vargas *et al.*, 2020). *Campylobacter jejuni* strains' resistance against ciprofloxacin, nalidixic acid, and tetracycline was 92.5%, 88.9%, and 68.4%, respectively (Wieczorek *et al.*, 2018). Resistance of *Clostridium perfringens* isolates against doxycycline and oxytetracycline was 98% and 71%, respectively (Osman & Elhariri 2013).

Clostridium perfringens-induced necrotizing enteritis in poultry costs \$6 billion each year (Yuan *et al.*, 2022). In addition to infection-related increased mortality rates in poultry, zoonotic pathogens contribute to foodborne diseases in humans *Campylobacter jejuni*-induced foodborne diseases cost \$6.9 billion each year. Additionally, \$2.8 billion is spent on combating foodborne infections caused by *Salmonella* species (Scharff, 2020).

Endolysins against bacterial infections in poultry

Here, we explain some of the endolysins which were studied against burdensome bacterial infections in the poultry industry.

Clostridium perfringens endolysins

The effects of various endolysins against *Clostridium perfringens* have been studied so far. The list of *Clostridium perfringens* endolysins and their properties are shown in Table 1. The optimal temperature and pH level for their activity varies between the endolysins. Hence, each endolysin reaches its highest activity in a specific condition (Jeong *et al.*, 2023). For example, an endolysin that is designed to be eaten by chicken must resist low pH levels of the gizzard (pH~3) and intestine (pH~6-6.8) (Swift *et al.*, 2015). Here, we mention some examples of reasonable endolysins for each condition. NaCl concentration in raw meat and fish is approximately 10 mM, so ZP173 can be used as a food preservative in them. The pH level of most meat-based foods is below 7; as a result, PlyCP390 and CP25L are not good preservative options in such a situation. Till now, only one antibacterial agent (nisin) has been approved by U.S. Food and Drug Administration (FDA) to be used as a food preservative. Two endolysins (Psm and ZP173) were found to have stronger antibacterial effects than nisin; therefore, they are promising agents for being used as food additives in the future (Kazanavičiūtė *et al.*, 2018).

Besides the existence of natural endolysins, scientists are using biotechnological approaches (e.g. chimeragenesis and mutagenesis) to make endolysins with improved functions (Heselpoth *et al.*, 2021). For oral use of the endolysins in the poultry industry, a plant-based endolysin expression system can be used instead of *E. coli*-based systems (Kazanavičiūtė *et al.*, 2018). Unlike *E. coli*-based systems, plant-based systems don't need endolysin purification and they work more efficiently in expressing endolysins (Hammond *et al.*, 2019; Kazanavičiūtė *et al.*, 2018). Using a gut-colonizing bacteria which is genetically engineered to express endolysin is another way to bring endolysins to the gastrointestinal tract. The expression system must be strong and work continuously. A previous study used an engineered *Lactobacillus johnsonii* F19785 which expressed CP25L (Gervasi *et al.*, 2014).

In addition to their role in killing bacteria, endolysins can be used for diagnosing bacteria in contaminated foods (Ha *et al.*, 2018).

Other endolysins

Salmonella spp. endolysins seem to lyse a broader range of bacterial species. Outer membrane permeabilizers (OMPs) (e.g. malic acid, EDTA, and citric acid) help *Salmonella* species endolysins in the elimination of bacteria. In the same conditions, adding citric acid to Lys68 resulted in a 2.89 ± 0.27 Log reduction of *Salmonella Typhimurium* LT2; whereas, using Lys68 (without OMP) contributed to 0.14 ± 0.16 log reduction of *Salmonella Typhimurium* LT2 (Oliveira *et al.*, 2014).

During *Salmonella pullorum* infection of poultry, LySP2 administration reduced the mortality rate significantly compared to the control group (Deng *et al.*, 2023). For combating *Campylobacter jejuni*, Zampara *et al.* made innolysins, by fusing an endolysin to a phage receptor binding protein (RBP) (Zampara *et al.*, 2021).

Conclusion

As antibiotic resistance is growing fast, antibiotic alternatives are receiving special attention (Murray *et al.*, 2021; Organization World Health, 2017). Nowadays, various types of antibacterials (e.g. endolysin administration) are being tested to find a solution for counteracting the burdens of antibiotic resistance (Murray *et al.*, 2021). Some endolysin features like their "narrow host range", "low immunogenicity", and "low probability of bacterial resistance development against them" are among the factors that make them potentially applicable antibacterials for the future (Murray *et al.*, 2021; Rahman *et al.*, 2021; Liu *et al.*, 2023b). Endolysin administration also faces some limitations, for example, in gram-negative bacterial species, endolysin causes a release of the LPS layer into the bloodstream and might contribute to cytokine storm (Briers *et al.*, 2011; Meng & Lowell 1997).

Most of the endolysin-related research is conducted *in vitro* (Murray *et al.*, 2021). Engineering methods are also an important part of making efficient endolysins. Collectively, it must be noted that we're just at the start of the road of endolysin administration and several additional studies are

Endolysin	Source	Predicted enzymatic activity	Additional information	References
LysCPAS15	Bacteriophage CPAS-15	Amidase	- Optimum pH = 2-10 - Optimum temperature = <50 °C - After adding a CBD to LysCPAS15, the chimera was stable in temperature <60 °C, and in pH range between 4-12	(Cho <i>et al.</i> , 2021)
Ply3626	Bacteriophage phi3626	Amidase	-	(Zimmer <i>et al.</i> , 2002)
CP25L	prophage region of <i>C. perfringens</i> 5416-97, vB_CpeS-CP51	Amidase	- Optimum pH = 7.5 - Optimum NaCl concentration = 200-500 mM - Optimum temperature = 4-25 °C (its activity unchanged for 2 days in 37 °C but 30 min incubation in 65 °C inactivated CP25L)	(Gervasi <i>et al.</i> , 2014; Kazanavičiūtė <i>et al.</i> , 2018)
Psm	Episomal phage phiSM101	Muramidase	- Optimum pH = 6.5-7 - Optimum NaCl concentration = 250 mM	(Nariya <i>et al.</i> , 2011)
PlyCP10	Prophage region of <i>C. perfringens</i> Cp10	Muramidase	- Optimum pH = 6 - Optimum temperature = 4-42 °C - Optimum NaCl concentration = 50-100 mM	(Swift <i>et al.</i> , 2018)
PlyCP41	Prophage region of <i>C. perfringens</i> Cp10	Muramidase	- Optimum pH = 6.5 - Optimum temperature = 4-42 °C (but it was more thermostable than PlyCP10) - Optimum NaCl concentration = 50-100 mM (but 60% of its activity was retained in 600 mM)	(Swift <i>et al.</i> , 2018)
PlyCM	Prophage region of <i>C. perfringens</i> ATCC 13124	Muramidase	- Optimum pH = 6.4 - Optimum temperature = 35-45 °C	(Schmitz <i>et al.</i> , 2011)
Acp	<i>C. perfringens</i> strain 13	<i>N</i> -acetylglucosaminidase	-	(Camiade <i>et al.</i> , 2010)
PlyCP390	<i>C. perfringens</i> phage phiCP39-O	Amidase	- Optimum pH = 8.2	(Simmons <i>et al.</i> , 2010)
PlyCPS2	Phage CPS2	Amidase	- Optimum pH = 7.5-10 - Optimum temperature = 25 °C - Optimum NaCl concentration = up to 500 mM	(Ha <i>et al.</i> , 2018)
PlyCP26F	<i>C. perfringens</i> phage phiCP26F	Amidase	- Optimum pH = 6.8	(Simmons <i>et al.</i> , 2010)
Psa	prophage region of <i>C. perfringens</i> st13	Amidase	- Optimum temperature = 37 °C - Optimum NaCl concentration = 300 mM	(Sekiya <i>et al.</i> , 2021b)
GVE2EAD-CP26FCBD	Chimeragenesis	Amidase	- Optimum temperature = 22 °C (95% of its activity retained after incubation in 50°C for 30 min) - Optimum pH = 8 - Optimum NaCl concentration = 10 mM	(Swift <i>et al.</i> , 2015; Swift <i>et al.</i> , 2019)
ZP278	<i>C. perfringens</i> CPE str.4969, prophage region	Muramidase	- Optimum pH = 4-8 - Optimum NaCl concentration = 500 mM	(Kazanavičiūtė <i>et al.</i> , 2018)
LysCP28	<i>C. perfringens</i> phage, vB_CpeS_BG3P	Muramidase	- Optimum temperature = 37-42 °C - Optimum pH = 7	(Lu <i>et al.</i> , 2023)
PlyCpAmi	<i>C. perfringens</i> 13124	Amidase	-	(Tillman <i>et al.</i> , 2013)
ZP173	<i>C. perfringens</i> CPE str.4969, prophage region	Muramidase	- Optimum temperature = 4 °C (More than 70% of its activity retained after 1 week in 37 °C) - Optimum pH = 5.2 - Optimum NaCl concentration = 50 mM and higher NaCl concentrations	(Kazanavičiūtė <i>et al.</i> , 2018)
LysCPD9	bacteriophage CPD9	Muramidase	- Optimum temperature = 25 °C - Optimum pH = 6 - Optimum NaCl concentration = 0 mM (50% of LysCPD9 activity was conserved in 500 mM)	(Choi <i>et al.</i> , 2023)
ClyY	Chimeragenesis	Muramidase	- Optimum temperature = up to 95 °C - Optimum pH = 5-9 (6) - Optimum NaCl concentration = 0-1000 mM	(Choi <i>et al.</i> , 2023)

Table 1. *Clostridium perfringens* endolysins and their properties

needed to elucidate all aspects of their usage (Abdelrahman *et al.*, 2021). Till the time of the availability of these antibiotic alternatives on the market, we must consider some points to slower the antibiotic resistance progression. Some of these points are that antibiotics must be prescribed by the in-charge veterinarians, and only after the determination of the antibiogram tests of the bacterial strains (Darboe *et al.*, 2023). It is also beneficial to set a cut-off point for antibiotic residue in poultry tissues for their usage as important human foods. Antibiotic residue can be reduced if we leave a long time between poultry antibiotic consumption and slaughter. This lag (withdrawal time) varies among different antibiotics (Habibi, 2018).

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آنزای بیوتیک ها، راه حلی امیدوار کننده در مقابله با باکتری ها در صنعت طیور

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چکیده

از زمان کشف آنتی بیوتیک ها، آنها به طور گسترده ای برای کنترل بیماری ها استفاده شده اند. همچنین مشخص شد که آنتی بیوتیک ها در تقویت رشد در صنعت طیور مفید هستند. با این حال، استفاده بیش از حد و سوء استفاده از آنها منجر به ایجاد مقاومت باکتریایی در برابر آنها شده است. مقاومت به آنتی بیوتیک یک معضل جهانی است که منجر به خسارات بهداشتی و اقتصادی قابل توجهی می شود. مقاومت آنتی بیوتیکی در برابر آنتی بیوتیک های مختلف در عفونت های طیور نیز مشاهده شده است. برای مقابله با بار مقاومت آنتی بیوتیکی، جایگزین های مختلفی برای آنتی بیوتیک ها مورد مطالعه قرار گرفته است. این رویکردهای جایگزین در صنعت طیور نیز مورد توجه قرار گرفته اند؛ زیرا عفونت های طیور منجر به زیان اقتصادی چشمگیری می شوند و در موارد عفونت های مشترک بین انسان و طیور، انتقال عفونت از مرغ سلامت انسان را به مخاطره می اندازد. فاژ درمانی، استفاده از پروبیوتیک ها و تجویز پپتیدهای ضد میکروبی نمونه هایی از این رویکردهای جایگزین هستند. از دیگر روش های جایگزین آنتی بیوتیک به "آنزای بیوتیک" می توان اشاره کرد. در آنزیمیوتیک ها، پپتیدوگلیکان هیدرولازها (اندولیزین ها) برای تخریب دیواره سلولی باکتری استفاده می شود. این آنزیم ها بیشتر در ژنوم باکتریوفاژها یافت می شوند؛ زیرا باکتریوفاژها باید لایه های پپتیدوگلیکان باکتری ها را هم برای ورود و هم برای خروج از میزبان باکتریایی خود تجزیه کنند. ژنوم باکتری ها همچنین حاوی توالی هایی با خواص پپتیدوگلیکان هیدرولاز است که به رشد و تقسیم باکتری ها کمک می کند. خواص پپتیدوگلیکان هیدرولازهای مختلف برای یافتن انواع قوی تر و کاربردی برای استفاده در آینده مورد مطالعه قرار گرفته است. با توجه به مزایای آنها، اندولیزین ها جایگزین های آنتی بیوتیکی امیدوار کننده ای هستند. در این مقاله به نقش آنزای بیوتیک ها در صنعت طیور خواهیم پرداخت. همچنین، مزایا و محدودیت های تجویز اندولیزین ها مورد بحث قرار می گیرد.

واژه های کلیدی: آنزای بیوتیک ها، اندولیزین، پپتیدوگلیکان هیدرولاز، کلستریدایوم پرفرنجنس، طیور، مقاومت آنتی بیوتیکی

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