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Phage lytic proteins: a natural approach to agro-food safety

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ABSTRACT

Sustainability is a leading trend in the context of food production. Additionally, consumers increasingly demand safer and less-processed products. Among the different technologies used to maintain the quality and extend the shelf-life of fresh and minimally-processed food, natural antimicrobial agents offer a promising strategy to replace conventional compounds. In this regard, phage lytic proteins or lysins, such as endolysins and virion-associated peptidoglycan hydrolases (VAPGHs), have been proposed as a viable option for the avoidance and elimination of undesirable bacteria within the food production chain. Even when applied exogenously, these proteins can degrade the bacterial cell wall maintaining their lytic activity. This feature, alongside their modular structure, which can be exploited for bioengineering, provides significant biotechnological potential. However, despite the promising properties of lysins, the main obstacle for their commercialization is the limited legal information regulating their use. This challenge underscores the need to navigate complex regulatory pathways. The primary objective of this review is to address this crucial gap and summarize the many prospective applications of endolysins during the different stages of food production. By doing so, we aim to provide clarity and insight into the regulatory challenges that must be overcome for the successful utilization of lysins.

1. Introduction

The global demand for food has been steadily rising in parallel to the increase in the world population. At the same time, climate change is turning the sustainable use of natural resources for food production into a priority. In this context, agro-food policies need to improve food security in order to supply adequate food to an ever-changing society where food safety plays an important role in providing consumers with safe, nutritious products. Under these circumstances, the United Nations Sustainable Development Goals (SDGs) focus on achieving food security and sustainable agriculture by 2030[1]. The establishment of indicators measuring food security have helped to improve and report progress in this aspect, especially food safety indicators that can help reveal the issues of food control systems. However, safety is still a matter of concern due to the increasing number of foodborne disease outbreaks. In 2021, 4 005 food outbreaks, affecting 32 543 people, were reported in 27 European Union Member States (EU MSs) and the United Kingdom according to the latest data published by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control[2]. Numbers in the United States are quite similar, where approximately 56 000 people are hospitalized each year due to foodborne diseases[3]. Globally, the World Health Organization (WHO) estimated that unsafe food is responsible for 600 million cases of foodborne diseases per year causing 420 000 deaths worldwide[4]. Most outbreaks are caused by *Campylobacter*, *Salmonella* and toxin-producing pathogens such as *Staphylococcus aureus*. These data highlight the urgent need to improve our antimicrobial

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repertoire to combat food poisoning.

Bearing in mind all the above, food safety research must be integrated into a sustainability framework to address the current challenges towards achieving food security. In addition, consumers are demanding eco-friendly practices while keeping food properties intact. Traditional antimicrobial methods such as thermal treatment, high pressure or chemical treatment can affect the organoleptic qualities or even the nutritional value of foods[5]. Also, chemical compounds pose an environmental problem due to the released residues along the agro-food production chain. In fact, it has been demonstrated that disinfectants can enhance antibiotic resistance, with several examples in literature reporting cross-resistance between antibiotics and disinfectants[6-8]. Thus, some conventional biocidal agents can persist in the environment, representing a constant source of stress for bacteria, which leads to adaptations that sometimes result in antibiotic resistance. This ultimately represents a threat for human health. Moreover, the enormous volume of antibiotics used in farming practices has aggravated this problem, as most of the antibiotics end up excreted in waste, harming the environment at different trophic levels. The general use of these compounds has led to a constant exposure of ecosystems to antimicrobial residues, sometimes interfering with plant physiology[9]. Therefore, it is clear that the use of chemical antimicrobials should be limited or restricted giving preference to others that do not have harmful effects on the environment, such as natural antimicrobial agents. These natural agents fall under the umbrella of biological treatment, which is based on the use of substances derived from organisms. These compounds, however, can occur naturally or be produced in the laboratory by chemical synthesis and cloning techniques.

In the context of this search for sustainable and natural solutions to prevent food contamination with bacterial pathogens, the specific characteristics of phage lytic proteins (lysins) make them a promising alternative. These proteins are peptidoglycan-degrading enzymes encoded by most bacteriophages. Some of them, called endolysins, are produced at the end of the lytic cycle to facilitate the release of the new viral progeny due to bacterial cell wall disruption (lysis from within) (Fig. 1A). Others, the virion-associated peptidoglycan hydrolases (VAPGHs), partially degrade the cell wall after phage adsorption in order to facilitate injection of the viral genome into the host cell. More specifically, lysins target different bonds found within peptidoglycan, which is the major structural component of the bacterial cell wall. When purified and exposed to this structure, particularly in Gram-positive bacteria, these enzymes can cause "lysis from without" (Fig. 1B). Lysins display a rapid bacteriolytic activity even when they are applied exogenously as recombinant proteins, eliminating specific pathogens without affecting the beneficial microbiota[10]. The limited host range of lysins is convenient in food environments because each particular product harbours its own microbial ecosystem. Among their many advantages, the low chance of developing bacterial resistance and their ability to destroy biofilms are suitable characteristics that advocate their use along the food production chain. In addition, bioengineering strategies can enhance the possibilities offered by these proteins and improve their use as antimicrobials, biosensors to detect bacteria or even to create new enzymes. In the case of phage

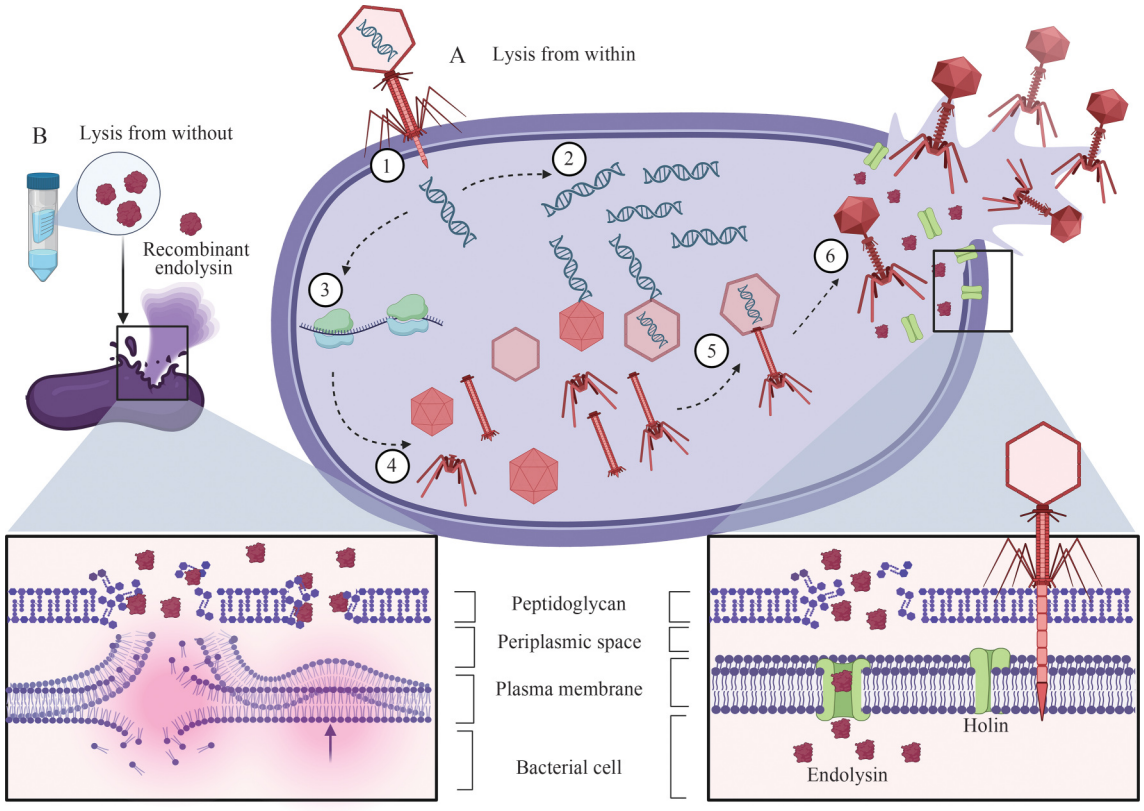

Fig. 1 Mechanism of action of endolysins from within and without. (A) Virulent phages enter their natural life cycle (lytic cycle) through attachment to (1) specific receptors, (2) replicate their genome and (3) synthetize viral proteins to (4 and 5) assemble new virions, (6) Lysis of the host cell by endolysins occurs from within the host cell with the help of holins (phage-encoded transmembrane proteins) to release the viral progeny. (B) When endolysins are purified and applied externally, they can reach the peptidoglycan without the need of holins and lysis occurs from without. Created with BioRender.com.

lytic proteins against Gram-positive bacteria, engineering can be easily performed because of their modular structure, as they consist of 2 kinds of domains: one non-catalytic domain, the so-called cell wall binding domain (CBD), involved in substrate recognition, and one or more catalytic domains called enzymatically active domains (EAD) (Fig. 2A). Depending on the type of bond they cleave in bacterial peptidoglycan, EADs can be categorized into different classes: glucosaminidases, lytic transglycosylases, lysozymes or muramidases, amidases, and endopeptidases (Fig. 2B). The last one can also be subclassified into interpeptide bridge endopeptidases and *L*-alanoyl-*D*-glutamate endopeptidases[11]. This structural organization allows domain swapping or deletion resulting in novel lysins with different specificity and bacteriolytic activity. In fact, it has been shown that some engineered lysins display a higher lytic potential than their parent protein[12-16].

The structure of lysins against Gram-negative bacteria differs from that of Gram-positive microorganisms, as they typically are globular proteins with a single domain acting as EAD. To overcome the barrier of the outer membrane present in Gram-negative bacteria, lysins have been engineered by adding cationic peptides (artilysins), phage receptor binding proteins (innolysins) or colicin-like bacteriocins (lysocins). Another approach is the use of membrane permeabilizers prior to endolysin application, such as pre-treatment using chelators (EDTA and organic acids) or polycationic agents (colistin, peptides) that destabilize the Gram-negative outer membrane[17]. Surprisingly, some native endolysins act exogenously against Gram-negative bacteria without the need of a permeabilizing agent[18-20]. In some cases, this ability has been associated with the presence of segments of the protein with an overall positive charge that can act as membrane permeabilizers[21]. Another feature of Gram-negative lytic proteins is their broader range of activity, being effective against different species, which is likely due to the absence of a CBD[18].

The diverse range of lysin architectures provides a rich source of information for data mining, enabling the discovery of novel functions, structure-function relationships, and the development of new tools and applications in multiple fields. In this scenario, and using the generic databases available, it is challenging to gain a holistic view of this vast diversity. Therefore, a dedicated database of phage lytic proteins is needed to provide a centralized repository for storing and organizing this information, allowing the integration of knowledge. This is the case of PhaLP, an open portal that offers a thoughtfully curated assortment of phage lytic proteins to guide in the development of novel endolysin-based products[22]. This kind of databases can expedite the screening and validation process to help in identifying candidate lysins for further experimental evaluation, saving time and resources in the initial stages of product development.

In the context of the rapidly evolving landscape of antimicrobial solutions for food safety and public health, it is crucial to assess the various options available from a multifaceted perspective. To aid in this assessment, we present a comparative analysis of phage lytic proteins and traditional antimicrobial methods, each with its own set of characteristics (Fig. 3). Based on their interesting properties, lysins have been tested as food antimicrobials and numerous articles support their potential in food safety. This review summarizes the current status of lysins as food safety agents and their potential applications in biocontrol, biosanitization, biopreservation and biodetection approaches.

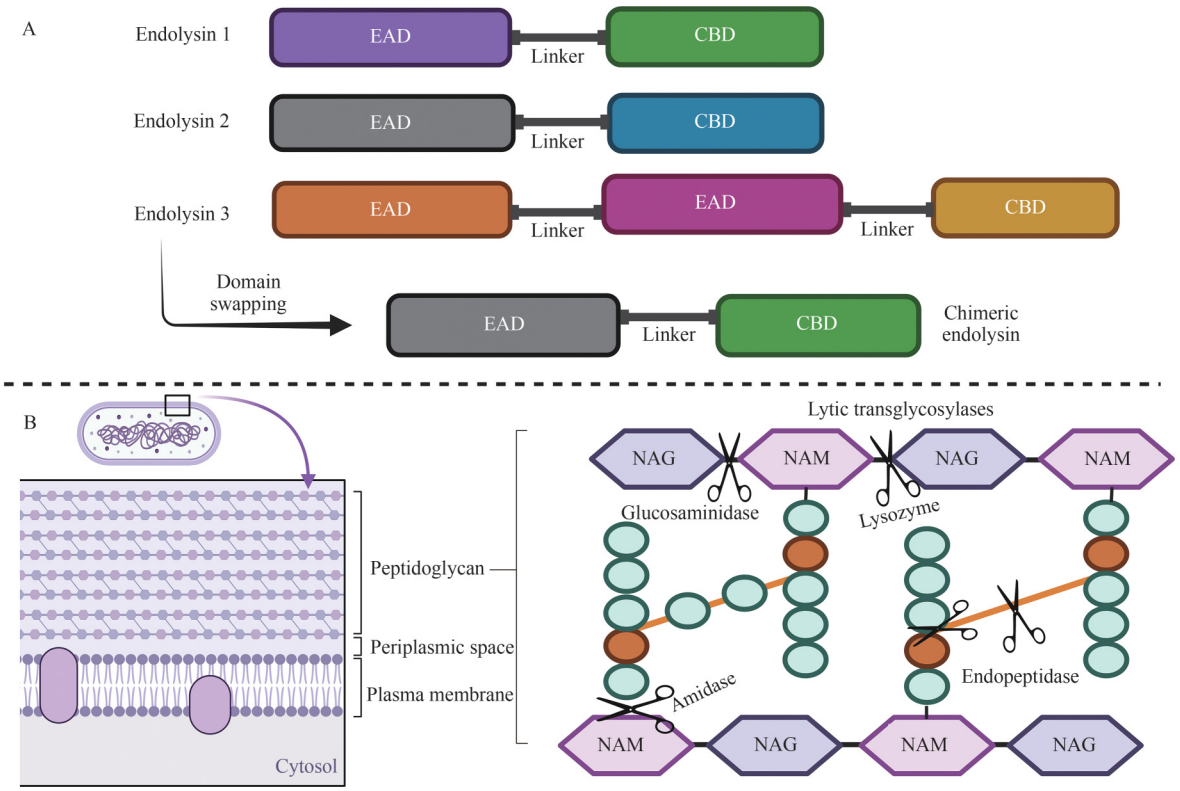

Fig. 2 (A) Schematic representation of the endolysin structure of Gram-positive bacteria. Natural endolysins have a modular structure with one or more catalytic domains (EAD) and one cell wall-binding domain. Engineering of chimeric lysins involves the linking of different domains to create novel lysins. (B) Diagram of the different peptidoglycan bonds that each kind of endolysin can cleave. Created with BioRender.com.

Features	Phage lytic proteins	Antimicrobial biocides	Antibiotics
Resistance	Low likelihood	May lead to antimicrobial resistance due to overuse	High and rapid occurrence due to overuse
Specificity	High specificity without potential impact on another species	Effective against a wide range of bacteria with potential impact on non-target species	Broad-spectrum is more common than narrow-spectrum
Production cost	High for large-scale production	Low	Low
Environmental impact	Biodegradable and minimal accumulation	Release of residues along the production chain	Overuse and improper disposal contribute to antimicrobial resistance
Human health	Less likely to induce allergies and no side-effects on microbiome	Limited potential for human health risks (toxicity)	Potential side effects, including allergic reactions and microbiome disruption
Discovery process	Simple	Difficult due to requirements for safety, and environmental impact	Difficult for novel antibiotics

Fig. 3 Comparative analysis of phage lytic proteins and traditional antimicrobial methods.

2. Biocontrol in primary production

Biological control of bacteria or bacterial biocontrol focuses on limiting pathogen growth in animals and plants using natural agents. Despite their widespread use, chemical antimicrobials are controversial due to their negative impact on the environment and human health[23-25]. By contrast, the use of lytic proteins as biocontrol agents is an eco-friendly and sustainable option due to their natural origin. Primary production encompasses the initial stages along the food production chain "from farm to fork", including plant and animal growth, which is crucial for the next stages. In this context, lysins can be applied in both organisms to treat bacterial infections without harming soil biodiversity or animal microbiota.

In agriculture, preharvest and postharvest application of lysins to crops may prevent the colonization of bacteria that are detrimental to plant health. In terms of economic losses, plant diseases are a huge worldwide problem, posing a threat to the food supply of a growing world population. The most consumed food crops, such as potato, tomato, olive, banana or citrus fruits, are affected by bacterial infections that represent a concern to global food security. Moreover, the use of traditional chemicals, like biocides or antibiotics may affect soil microbiota and be harmful to humans, as well as promote the selection and spread of drug resistance amongst bacterial populations, making it necessary to search for alternatives. Phage-based biocontrol has been already applied in several studies using the whole virion, but the application of lytic proteins is relatively new[26]. Although research regarding lysin application in plants is still in its infancy, there are some examples regarding the successful use of these enzymes to control phytopathogenic bacteria. For example, endolysins from phages Atu_ph02 and Atu_ph03 successfully killed *Agrobacterium tumefaciens*, a soil-borne bacterium responsible for significant economic losses in stone fruit and nut production[27]. Similar results were achieved even against Gram-negative bacteria, where a combination of endolysin LysPN09 with EDTA was effective against *Pseudomonas syringae* pv. *actinidiae* (Psa), the pathogen that causes bacterial bleeding canker in kiwifruit[28]. Another approach is the production of transgenic plants that express endolysins to prevent infection by phytopathogenic bacteria. Düring et al.[29] obtained transgenic potato plants expressing phage T4 endolysin, which were resistant to *Erwinia carotovora*, the causative agent of soft rot. In another study, CMP1 and CN77 endolysins expressed by transgenic tomato plants prevented infection by *Clavibacter michiganensis*[30]. The integration of phage lysins into genetically modified crops offers potential advantages for their convenient implementation and help to overcome production challenges. This concept can also be applied to use plants as bioreactors to produce endolysins at a large scale at a very low cost[31].

Since lysins are natural agents that occur in the environment, they can be marketed as biopesticides or biophytosanitary products, making them suitable for organic farming. Unlike conventional chemical pesticides, biopesticides are derived from natural materials such as microorganisms or other biological sources. In the European Union, the placing of plant protection products (PPPs) on the market is regulated by European Regulation (EC) 1107/2009, and requires prior authorization of the active substance based on a risk assessment by the EFSA in order to authorize the PPP final product[32]. The main gap in the European Regulation seems to lie in the lack of a "biopesticide" category, as it only differentiates between PPPs based on chemicals or microorganisms. The regulatory framework for pesticides in the USA is determined by 2 central authorities: the Environmental Protection Agency (EPA), which assesses the active substance registration, and the Food and Drug Administration (FDA), responsible for establishing the maximum residue levels. The EPA evaluates active substances under 2 federal statutes: the Federal Food, Drug, and Cosmetic Act (FFDCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Taking into count that successful pesticide registration depends on the potential effects on the environment and that this regulation is accustomed to biopesticides, lysins are suitable biopesticide candidates in the United States. Data requirements are listed on Title 40 of the Code of Federal Regulations (CFR) Part 158, which differentiates biochemical from chemical pesticides. Thus, lytic proteins could be registered as

biochemical pesticides as they are considered to be naturally occurring substances that control pests by non-toxic mechanisms.

In animal husbandry, antibiotics have been widely used not only to prevent or treat infections but also to promote animal growth, an overuse that has fostered the emergence of antibiotic-resistant strains. Infectious diseases are a common occurrence in livestock production, resulting in animal mortality, decreased fertility, and diminished quality of animal products, ultimately impacting profitability. In addition, several important zoonotic pathogens colonize farm animals from which they can be transmitted down the food chain, ultimately posing a threat to public health. For this reason, alternative products are needed and bacteriophage-derived products have already been considered a viable option in the latest European regulatory framework on veterinary medicines[33]. In fact, different studies have reported the effectiveness of lysins, both *in vitro* and *in vivo*, against different farm animal pathogens. While the name endolysin or lysin is derived from their role in the phage life cycle, they can be also be referred to as enzybiotics, which is a more inclusive term formed by blending "enzyme" and "antibiotic" that encompasses all enzymes with antibacterial activity. For instance, there is ample evidence regarding the potential of enzybiotics to treat mastitis, which is one of the most prevalent infectious diseases in livestock. Mastitis is an inflammation of the mammary gland that can be caused by a wide range of microorganisms. It is the costliest disease in animal husbandry, leading to severe losses in milk production and premature culling of animals. The main mastitis-causing pathogens are various streptococcal species, *S. aureus* and *Escherichia coli*, microorganisms that have been shown to be susceptible to lysins[34]. In a study by Schmelcher et al.[35], endolysins derived from streptococcal phages maintained their lytic activity under physico-chemical conditions mimicking those found in bovine milk, and were able to decrease the intramammary levels of different streptococcal species in a mouse model of bovine mastitis. In clinical application, Fan et al.[36] reduced *S. aureus* concentrations in cows diagnosed with staphylococcal mastitis after a 3-day treatment with 20 mg of endolysin trx-SA1 per day per animal. PlyC is another potent endolysin for the treatment of streptococcal infections, eliminating the infection at a low dose (1 μg/mL) without causing cytotoxicity to bovine blood cells[37]. This endolysin had previously been shown to reduce streptococcal infections in horses, being 1 000 times more effective than a common disinfectant routinely used in livestock[38]. Besides extracellular bacteria, endolysins can also be engineered with a cell-penetrating peptide to target intracellular bacteria, an approach that has been successfully tested in bovine mammary alveolar cells infected with *S. aureus*[39-41]. Additionally, the application of lysin-based products may be a powerful prophylactic strategy to prevent bacterial infections, as suggested by Gutiérrez et al.[42].

Regarding the regulatory framework applicable to the use of lytic proteins in farm animals, alternatives to antibiotics in the United States may be regulated as animal drugs, biologics or feed additives and demonstration of their safety and efficacy is required for FDA approval. In the EU, the requirements for veterinary drugs are laid down in regulation (EU) 2019/6, which recently replaced directive 2001/82/EC[33]. One of the main aims of this regulation is to reduce the risks of antimicrobial resistance development, and the use of phages and endolysins should fit very nicely within this goal. Within this framework, phage-derived products like lytic proteins might be applied in animals as novel veterinary medicinal products. Interestingly, the European Medicines Agency (EMA) has recently published a guideline addressing the use of bacteriophages in veterinary medicine, but not the potential application of phage-derived proteins[43]. In a veterinary context, lysins may also be used as disinfectants and be regulated under the Biocidal Products Regulation[44]. For this specific purpose, lysins would be classified as PT3 category for veterinary hygiene products among the different biocidal product types (PT).

3. Biosanitization

It is well known that disinfection is essential to maintain the agro-food industry free of unwanted microorganisms that can be transmitted to workers or consumers. Processing facilities, animals, food handlers and farmers may be potential reservoirs of pathogenic bacteria; therefore, they must follow adequate disinfection protocols. In particular, biofilms that develop on surfaces of food manufacturing environments are a recurring source of contamination compromising food safety. Due to the structure of biofilms, bacterial cells embedded in the extracellular matrix are difficult to reach by sanitizers, being a potential reservoir from which bacteria can come into contact with food handlers or even food products. Thus, complete removal of biofilms is not easy, especially because they typically have a greater percentage of persister cells that can survive treatment with many disinfectants and subsequently allow regrowth of the biofilm[45]. Since lysins are responsible for peptidoglycan breakdown, destroying an important structural component of the biofilm cells, they could be used as biological disinfectants to prevent colonization and avoid biofilm formation. Moreover, it is important to assess the potential of lysins as disinfectants in common materials used in cookware and utensils. For example, Chang et al.[46] evaluated the efficacy of endolysin LysSA11 on a polypropylene plastic cutting board and a stainless steel knife artificially contaminated with *S. aureus* achieving a complete decontamination within 30 min in both utensils. In a recent study, Żebrowska et al.[47] characterized a thermostable endolysin that also maintains its activity over a wide pH range (pH 4–10) and is effective against several foodborne pathogens such as *Enterobacter cloacae*, *Salmonella* sp. or *S. aureus*. This endolysin (TP84_28) is a promising candidate for applications along the food production chain as it can be used to clean food-processing surfaces that are exposed to high temperatures.

Lysins could play a crucial role in preharvest decontamination, ensuring the safety of the food supply and minimizing the risk of foodborne infections. These strategies are particularly relevant for various food processing facilities, including slaughterhouses, as they help reduce contamination of carcasses and local tissues during the slaughtering or washing processes. Although more research is still needed to establish the best conditions to apply lytic proteins as biosanitizers, there are already several studies that report biofilm disruption on food surfaces. Importantly, lysins have the ability to eliminate both actively dividing cells and non-dividing cells, irrespective of the bacterial metabolic state[48]. Moreover, compared to antibiotics, they exhibit enhanced penetration into the deeper layers of biofilms[49]. Achieving this can be facilitated by preventing bacterial surface attachment and by disrupting or destabilizing fully formed biofilms. In some cases, lysins were even able to prevent biofilm formation at subinhibitory doses, an effect that might be related to the fact that they inhibit expression of autolysin-encoding

genes in some bacteria, such as *S. aureus*[50]. A landmark example is provided by LysCSA13, a staphylococcal endolysin that achieved an 80%–90% biomass reduction on different materials including stainless steel, glass and polystyrene[51]. Several staphylococcal lytic proteins have been reported to exert antibiofilm activity such as LysH5, CHAP-SH3b, LysRODI and LysRODIΔAmi to name only a few[16,42,48,50,52]. Of note, LysH5 has been also found to be effective against persister cells.

Apart from staphylococcal biofilms, recombinant lysins have been shown to be effective against biofilms formed by a variety of food pathogens and spoilage microorganisms including both Gram-positive and Gram-negative bacteria. For example, endolysin 293-amidase showed anti-listerial activity preventing *L. monocytogenes* biofilm formation on abiotic surfaces[53]; LysPA26 achieved a 2−3 lg viable cell reduction on 48-h mature biofilms of *Pseudomonas aeruginosa*[54] and Lys68 displayed a synergistic effect in combination with citric or malic acid to remove *Salmonella* Typhymurium biofilms[55].

The susceptibility of biofilms to antimicrobials largely depends on factors such as biofilm complexity, strain sensitivity, and the stage of biofilm maturation. Due to these complexities, completely eliminating the total biofilm biomass (including adhered cells and extracellular material) can be challenging. As a result, a combination approach involving phages, lysins, antibiotics, and other antimicrobials has been demonstrated as an effective means of achieving biofilm eradication. The use of phage and enzyme treatments in conjunction with other antimicrobial agents has shown promising results in combating biofilms. For example, Duarte et al.[52] reported the synergistic effects of combining a phage-derived lytic protein, CHAPSH3b, with the virulent bacteriophage phiIPLA-RODI when treating 24-h-old *S. aureus* biofilms. The combined treatment resulted in greater reductions in viable cell counts compared to individual treatments. The enzyme exhibited rapid antibacterial activity, significantly reducing the bacterial population within 7 h of exposure. Subsequently, the phage contributes by predation, preventing regrowth of the bacterial population. Of note, the lytic protein CHAPSH3b demonstrated effectiveness against at least 90% of bacteriophage insensitive mutants, thereby helping to prevent the development of phage resistance during treatment.

Commercial phage-based disinfectants are already available on the market, confirming their huge potential in this field by using the whole virion (PhageGuard Listex, SalmoFresh™, SalmoLyse™). However, lysin-based disinfectants are still not available as commercial products, primarily due to the substantial challenge of ensuring their stability and long-term effectiveness, along with the need to establish efficient and cost-effective manufacturing methods to facilitate their broader utilization. In this context, research efforts should focus on optimizing formulations to improve the stability and shelf-life of lysin-based disinfectants. In that sense, techniques such as encapsulation or the use of stabilizers and novel delivery methods can all contribute to enhanced performance. This may be due to the lack of authoritative guidelines in order to meet the specific criteria required for their approval as biocidal active substances. From a regulatory perspective, enzybiotics could be considered as disinfectants and regulated as such. In the USA, disinfectants are regulated by the same central authorities that regulate pesticides (EPA and FDA), following similar requirements about safety and efficacy but with different classification criteria. More specifically, lysins as agro-food disinfectants may be regulated by the EPA alone since the FDA is only responsible for clinical disinfectants. At the EU level, disinfectants are regulated by the Biocidal Products Regulation[41]. Biocidal products are classified into 22 PT divided in 4 main groups, where disinfectants can be classified in 5 categories. As biosanitizers, lytic proteins could be categorized in PT4 for disinfectants which may enter into contact with food or feed areas. Of note, food preservatives are excluded from this category and are evaluated by the EFSA.

4. Biopreservation

Biopreservation refers to the inhibition of foodborne pathogens and spoilage microbiota in order to increase the shelf-life of foods by using natural agents, thereby enhancing safety and preventing spoilage[56]. One of the main advantages of lysins as biopreservatives is their high specificity. Indeed, they can be used to target specific bacteria in edible products without affecting the microbial ecosystem of both food and consumers. At the same time, lytic proteins exhibit a broader range than bacteriophages, which facilitates their applicability. Although lysins have been reported to be effective in a variety of foods, their use in dairy products has probably been studied the most. Foodborne pathogens that produce enterotoxins such as *S. aureus* are the most targeted microorganisms in dairy products, because some enterotoxins are heat resistant and can persist in food even after bacteria are killed through heat treatment[57]. As a result, it is essential to control these microbes as early as possible, before they produce and release enterotoxins into the food matrix. Moreover, these enterotoxins can be found in cheese made from raw milk and other artisanal dairy products. In such cases, lysins can be a good strategy to prevent proliferation of enterotoxin-producing bacteria while leaving fermenting microorganisms untouched.

Some pathogens are able to resist food processing methods, an issue that can be solved by using active food packaging, which refers to a packaging system that interacts with food surfaces by releasing bioactive compounds[58]. In this context, for instance, phage lytic proteins may be added to the material's matrix to improve food safety as an antimicrobial packaging system. Once again, this approach has only been tested with bacteriophages by incorporating them into biopolymers to create antimicrobial films[59-61], but lysins are also a promising option. Furthermore, lytic proteins do not have some of the potential shortcomings of phages, especially concerning the development of resistance. In that sense, Weng et al.[61] highlighted the importance of choosing the optimal mode of administration for different antimicrobials on foodstuffs in order to maximize their effect.

Although the successful use of lysins has already been demonstrated at the laboratory scale, their potential application may vary depending on the food conditions. For instance, the efficiency of these enzymes depends on the food matrix and other parameters, including temperature, pH, salt concentration or storage duration[62]. Further research is needed to assess these parameters and characterize lytic activity in specific foods. To date, there are several reports about the applicability of lysin-based biopreservation in different food products, being dairy products the most widely studied (Table 1). Indeed, numerous endolysins have been successfully tested against *S. aureus* in milk. For example, LysH5 achieved an 8-lg reduction in 6 h and CHAP-SH3b could eliminate *S. aureus* in 15 min in different types of milk[13]. Similarly, CHAPK_CWT-LST and Ami2638A also displayed rapid antistaphylococcal activity in milk, and Lys109 achieved the same effect but required 45 min of incubation[63-64].

Table 1
Summary of the endolysins application in several food matrices against different foodborne pathogens.

Food applications	Target pathogen	Endolysin (+ additional hurdle)	Highlights
Cow milk	*S. aureus*	CHAP-SH3b	Reduction below detection levels within 15 min at 37 °C in different types of milk
		CHAPK_CWT-LST and Ami2638A	2.64-lg and 1.73-lg reduction, respectively, within 3 h; Ami2683A achieved a 3.5-lg reduction in raw milk
		Lys109	Reduction below detection levels within 45 min
		LysRODI	2-lg reduction after 24 h at 25 °C
		LysRODIΔAmi	Reduction below detection levels at different temperatures and times in milk; 7-lg reduction during fresh cheese production
		PlyP825 + HPP	Synergistic effect prior incubation with endolysin PlyP825 allows reduction of pressure treatment (200 MPa)
		LysH5	8-lg reduction in 6 h at 37 °C
		LysH5 + nisin	Synergistic effect in milk where nisin enhanced 8-fold the lytic activity of LysH5
		LysSA97 + carvacrol oil	Reduction below detection levels after 3 h at room temperature
Fresh cheese	*L. monocytogenes*	PlyP100	Prevented growth after cold storage for 3 weeks
		PlyP100 + nisin	4-lg CFU/g reduction after 28 days stored at 4 °C
Mozzarella	*L. monocytogenes*	PlyP825 + HPP	Synergistic effect prior incubation with endolysin PlyP825 to pressure treatment (400 MPa) during storage at 10 °C
Soya milk	*L. monocytogenes*	LysZ5	4-lg reduction after 3 h at 4 °C
Pears	*E. amylovora*	phiEa1	Inhibited growth after 5 days at 26 °C by direct application of cell lysates on immature pears
Lettuce	*L. innocua*	Ply500	4-lg reduction after 24 h
	S. aureus	SAP8 + nisin	Growth inhibition after 1 h at room temperature
Meat	*S. aureus*	LysSA11	3-lg reduction in ham within 15 min at 4 °C
		LysSA97 + carvacrol oil	2-lg reduction in beef after 3 h at room temperature
	Salmonella spp.	EN4 + $NaHCO_3$	1.3-lg reduction in raw chicken meat after 48 h at 4 °C and 1.6-lg reduction in thawed chicken meat after 48 h at 30 °C

In a recent study, endolysin LysRODI and its derived variant LysRODIΔAmi showed good efficacy against *S. aureus* in milk and during lab-scale fresh cheese production[65]. Interestingly, the engineered lysin LysRODIΔAmi displayed a much higher lytic activity compared to its parent protein representing a promising candidate for biopreservation in dairy products. Other studies have focused on the potential of lytic proteins to eliminate *Listeria monocytogenes*, such as PlyP100, an endolysin that could resist high temperatures, high NaCl concentrations, low pH and long-term storage in fresh cheese[66]. Another good example concerning the control of *L. monocytogenes* is the use of endolysin LysZ5 in soya milk at refrigeration temperatures, where a 4 lg reduction was achieved within 3 h of treatment[67]. Regarding biopreservation in vegetables, endolysin phiEa1 inhibited *Erwinia amylovora* growth by direct application on unripe pears[68] and Ply500 achieved a 4 lg reduction of *Listeria innocua* in iceberg lettuce[69]. On the other hand, the application of endolysins in meat has also been tested against *S. aureus* by Chang et al.[43], achieving a 3-lg reduction in ham treated with LysSA11. Abhisingha et al.[70] successfully used lysin EN4 in combination with sodium bicarbonate ($NaHCO_3$) instead of EDTA to decontaminate *Salmonella* spp. in chilled and thawed chicken meat.

Lysins can also be used as part of a hurdle technology approach by combining them with other antimicrobials or physical treatments like high pressure processing (HPP). Since several food quality factors like texture, colour or flavour are influenced by HPP treatment, the use of lytic proteins allows a reduction in the pressure levels while maintaining optimal antimicrobial effects. For instance, van Nassau et al.[71] explored this approach by combining endolysins with HPP to kill *L. monocytogenes* resulting in more than a 5 lg CFU reduction, while each treatment alone only achieved a 0.2−0.3 (lg CFU) reduction. This synergy was also observed by Misiou et al.[72] when applying endolysin PlyP825 and HPP in different food products such as milk and mozzarella cheese. A long-term synergistic effect against *L. monocytogenes* was also achieved between endolysin PlyP100 and the bacteriocin nisin in fresh cheese stored at 4 °C[73]. Similarly, in a previous study, the combination of nisin and endolysin LysH5 showed a synergistic activity in milk against *S. aureus*[74]. More recently, Kim et al.[75] achieved a significant decrease in viable *S. aureus* cell counts by using a combination treatment consisting of endolysin SAP8 and nisin on lettuce. Another example was provided by Chang et al.[76], who applied endolysin LysSA97 with carvacrol oil against *S. aureus* in milk and beef. Although they found a synergistic effect between these 2 antimicrobials, it must be noted that this synergy appeared to be influenced by the lipid content of foods.

Application of phage lytic proteins as biopreservatives in the European food industry follows the EC regulation 1333/2008, in which all food additives must be demonstrated to have a useful purpose under their conditions of use, and undergo a safety evaluation by the EFSA Panel on Food additives and Flavourings[77]. Conversely, the biopreservative use of lysins in food packaging may be considered as a treated article and, therefore, follow the Biocidal Products Regulation. In the United States, food additives are regulated by the FDA according to the Food Additives Amendment to the FFDCA of 1958 that mainly focuses on assessing safety and efficacy. In particular, natural antimicrobials like phage lytic enzymes are considered biopreservatives, and their approval relies on the Generally Recognized as Safe (GRAS) assessment. Under the FDA and the Federal Food, Drug and Cosmetic Act, GRAS is a regulatory

classification term used to identify compounds with safety evidence that is generally available. As a result, substances with this recognition do not require premarket approval by the FDA as they are not considered food additives. Regarding lysin-containing antimicrobial packaging, approval will depend on the status of each individual substance. Considering all the aforementioned information, the pathway to lysin marketing in the USA may be relatively quick using the GRAS regulatory approval procedure.

5. Biodetection

Bacterial detection is key to preventing widespread contamination and facilitating the timely application of the most suitable treatment to avoid food poisoning outbreaks. The classical detection methods for foodborne pathogens are based on cultivation of microorganisms on selective media with agar, which are time-consuming and labour-intensive. To overcome this issue, biochemical technologies like PCR or ELISA have been used to detect specific foodborne pathogens or toxin-encoding genes, but these are expensive and/or laborious methods. In this context, it must be highlighted that lysins have evolved to specifically interact with molecules on the surface of bacterial cell walls, being natural biosensors for specific pathogens. This recognition relies on the CBD of lytic proteins, which can bind to peptidoglycan with a high specificity and affinity (similar to the antibody-antigen interaction). This specificity has been exploited for the detection of microorganisms in different environments such as foodborne pathogens along the food production chain. Thus, CBDs represent an opportunity to design highly sensitive and rapid pathogen detection systems that can be used in food matrices but also on abiotic surfaces, allowing early detection of dangerous microorganisms that otherwise would reach the consumer.

Fusion of endolysin CBDs with fluorescent proteins produces a bioprobe that can bind to the host cells and release a fluorescence signal. This strategy can simultaneously identify several pathogens in a single food sample by using different fluorescent proteins. In a study performed in milk and Camembert cheese samples, 3 CBDs tagged with different fluorescent markers were used to distinguish several *Listeria* strains[78]. Similarly, *Clostridium tyrobutyricum* was detected during cheese ripening by using the fluorescent CBD of endolysin CTP1L to check its evolution in cheeses[79]. This particular method provides a useful tool to monitor the growth of *C. tyrobutyricum*, which is a cheese spoilage bacterium capable of resisting industrial process. Other important foodborne pathogens such as *B. cereus*[80] and *C. perfringens*[81] were also successfully detected in food.

Additionally, CBDs have been exploited to quantify the concentration of pathogens by using a magnetic separation technique. This strategy, which involved coating magnetic beads with the CBD, could successfully detect and capture more than 90% of *L. monocytogenes* cells in different food samples[82]. This method was combined with reverse transcription PCR (RT-PCR) in raw milk to achieve a highly sensitive detection limit, more specifically between 10^2 and 10^3 CFU/mL[83]. Yu et al.[84] used these CBD-coated beads *via* immunomagnetic separation to detect up to 400 CFUs of *S. aureus* in milk. In a later study, Yi et al.[85] could decrease the detection limit of *S. aureus* cells to 78 CFU/mL using the same technique. In another work, the combination of CBD-coated beads and qPCR achieved an outstanding detection level of 2 CFU/mL in PBS and less than 10 CFU/mL in milk or beef extract[86].

Although there are several advantages to the use of CBDs for pathogen detection, such as high specificity, rapid detection and low probability of cross-reactivity, a major drawback is that this approach is not valid to detect Gram-negative pathogens due to the presence of an outer membrane.

6. Potential hurdles for the industrial implementation of phage lytic proteins

Phage lytic proteins have shown great potential to be applied in the food production chain, particularly for controlling bacterial pathogens that are resistant to traditional antimicrobial strategies. However, their application in the food industry faces several challenges that must be taken into consideration for future research such as stability and shelf-life, safety and cost-effectiveness.

Regarding their stability and shelf-life, the long-term efficacy of these enzymes can be influenced by diverse environmental factors like temperature and pH[87]. Although the optimal storage temperature of lysins can vary depending on the specific enzyme, storage at refrigeration temperatures (2–4 °C) is generally recommended to maintain their activity over time. Lytic proteins can also be stored at −20 °C or even −80 °C for long-term storage with the addition of stabilizing agents such as glycerol, but freeze-thawing cycles can negatively affect stability. Generally, phage lytic proteins are active and stable within a moderate temperature range typically around 20–45 °C. This could be due to the fact that the majority of studied phages are mesophilic, while their thermophilic counterparts are not well characterized. In some cases, lysins have been shown to retain activity after exposure to high temperatures, which may be beneficial for their application in various fields such as food processing.

There are various examples of thermostable endolysins in nature like the previously mentioned TP84_28, Lys68, PVP-SE1gp36 and Ph2119, some of which have been reported to withstand temperatures of up to 95 °C[55,88]. Future studies should focus on assessing and improving the thermal stability of lytic proteins, which is necessary for estimating their long-term storage and application. For example, Heselpoth et al.[89] successfully increased the stability of endolysin PlyC by thermostabilizing its CHAP domain through computational modelling. In particular, the authors found a mutation containing a thermally advantageous hydrogen bond that increased the denaturation temperature by 2.2 °C. Although this is a relatively modest increase, this specific mutation led to a substantial 16-fold enhancement in kinetic stability. Furthermore, when aiming to enhance the thermal stability of proteins, it is typically necessary to combine multiple amino acid mutations with individual, minor effects on stability. If each mutation employs a distinct mechanism of stabilization, the combination of these beneficial mutations can synergistically stabilize the protein.

Like temperature, pH changes can cause loss of lytic activity. In general, many lysins have been shown to exhibit optimal activity at a slightly acidic or neutral pH, typically between pH 6 and 7.5. Again, there are some phage lytic enzymes in nature that can resist a wider pH range such as LysZC1 endolysin that can work between pH 5–10[90]. Notably, LysRODI, demonstrated the highest activity at pH 7 but also exhibited activity within a range of pH 5–9[42]. This adaptability is a noteworthy characteristic that reflects the diversity in lysin behaviour. Thus, optimizing the pH of a potential formulation can help maintain the stability of the lysin. Another suitable approach may be to engineer these proteins in order to increase their pH range.

In addition to optimization of the amino acid sequence of lytic proteins, there are other strategies that can help improve stability in different environmental conditions. In that sense, artilysation, which we previously discussed regarding their enhanced efficacy, can also be used to improve the resistance of lysins to external conditions. For example, a study by Rodríguez-Rubio et al.[91] showed that the chimeric protein Art-240 could resist pH variations and higher NaCl concentrations better than its parent protein λSa2lys.

Another approach consists in encapsulating lysins in nanoparticles, liposomes, or other delivery systems to protect them from environmental factors and increase their shelf-life. This technique offers a controlled release of lytic proteins and allows minimizing their exposure to light, oxygen or other compounds. Various phage and lysin encapsulation studies have been performed but the encapsulation efficiencies obtained so far are quite low, approximately around 35% maximum[92]. Although the majority of these examples focused on bacteriophages, there are some examples of endolysin encapsulation. Portilla et al.[93] encapsulated endolysin LysRODI in liposomes, which are spherical structures composed of a lipid bilayer that can effectively deliver drugs to cells. The authors produced liposomes using 2 different types of lipids, which were chosen to create a pH-sensitive membrane that could release the endolysin in an acidic environment. Encapsulation of LysRODI increased its stability in the extracellular environment and the liposome-encapsulated LysRODI was able to effectively reduce *S. aureus* cells in both planktonic cultures and biofilms at pH 5.5. This approach was performed previously by Bai et al.[94] to evaluate the antimicrobial activity of endolysin-containing liposomes against Gram-negative bacteria, effectively killing *Salmonella* Typhimurium and *E. coli* without any additional treatment to destabilize the outer membrane. In this case, the authors used cationic liposomes comprised of dipalmitoyl-phosphatidylcholine (DPPC) as a backbone lipid due to its favourable stability and cholesterol to maintain the rigidity of liposomes. Because the study aimed to develop positively charged liposomes to interact effectively with Gram-negative bacteria, hexadecylamine (HDA) was included to impart a positive charge to the liposomes. Although this seems to be a successful strategy, potential limitations might include the specificity of this approach to certain Gram-negative bacterial species, the scalability and cost-effectiveness of producing such liposomes on a larger scale, or any ecological considerations regarding the disposal of these materials.

In a previous work conducted in 2017, researchers utilized a different approach for endolysin encapsulation, employing a thermal trigger for lysin release[95]. Specifically, they encapsulated a truncated endolysin called CHAPk and a bacteriocin called lysostaphin within poly (*N*-isopropylacrylamide) nanoparticles. Because the focus of the study was on treating skin infections caused by *S. aureus*, these nanoparticles were designed to release the encapsulated agents at a temperature of 37 ℃. Therefore, both antimicrobials would only be released within the infected area where the temperature is elevated, retaining their synergistic effect. As it has been shown, the specific type of delivery system used in each study may vary depending on the experimental design and goals. These studies suggest that encapsulation of phage lytic proteins may be a promising strategy that has potential applications also in food safety.

When transitioning from laboratory-scale to industrial-scale expression and purification, safety considerations come into play. These considerations encompass the entire production process, starting from selecting the appropriate expression host to the downstream processing steps involving cell harvesting and lysis, purification and endotoxin removal. Thus, the production method to provide the food industry with these proteins can influence their safety and, when they are produced by microorganisms, it is mandatory to guarantee a high level of purity. This limitation can compromise the production cost of lysins, as it can vary depending on the production method, purification process and scale of production. Some production methods, such as recombinant protein expression, can be expensive and may require significant investment in equipment and expertise. However, other purification methods, such as size exclusion chromatography and membrane filtration, could be more cost-effective and scalable.

The safety of lytic proteins can be influenced by multiple factors, including the specific enzyme, dose, route of administration, and potential interactions with other compounds. Therefore, the safety of these enzymes must be considered prior to their implementation in the food industry. For instance, the antimicrobial nature and specificity of lytic proteins reduce the potential for off-target effects and unwanted side effects. Moreover, these enzymes might be rapidly degraded by proteases in the human body, which would limit their persistence and potential for accumulation or long-term effects. In fact, several experimental studies have reported that lysins are innocuous for animals and humans when administered both topically and systemically. A study in 2014 looked at the safety of endolysin SAL-1 in 2 animal models (canines and rodents) by administering intravenously a phage endolysin-based drug namely SAL200 (containing SAL-1)[96]. After several weeks of administration, no signs of toxicity were found. In 2018, a study about the toxicity and preclinical safety of 2 endolysins (PaI and CpI-1) against *Streptococcus pneumoniae* showed no significant inflammatory gene expression, microbiome changes or cellular responses in cell lines[97]. Also in this study, the authors carried out an *in vivo* toxicity study in mice, and observed an increase in immunoglobulin G (IgG) levels without an impact on the lytic activity. Of note, neither Cp1-1 nor Pa1 generated an IgE response, which is generally linked to hypersensitivity and allergic reactions. When administered topically in humans with atopic dermatitis, Moreau et al.[98] reported the efficacy of a recombinant chimeric endolysin (Endobioma™) against *S. aureus*. A cream containing the endolysin was applied in children and adults for 2 weeks, improving skin sensitivity and atopic dermatitis symptoms in general. Taken together, the results of a growing body of studies about the innocuity of lysins support their implementation not only in the clinical field but also in food safety and food processing systems.

Overall, the cost-effectiveness of lytic proteins in the industry is an important consideration that depends on several factors, including production cost, efficacy and, ultimately, regulatory approval. This last factor can be very challenging, as each country has its own regulatory agencies and each commercial application its own policies and regulations. In Table 2, a concise comparative overview of regulatory agencies and their approaches to lysin regulation in the USA and Europe is presented. While these 2 regions are indeed prominent in the global regulatory landscape, it is crucial to recognize that a holistic perspective should encompass regulatory frameworks in other countries as well. The USA and Europe serve as valuable benchmarks for understanding the regulatory landscape, making them ideal starting points for companies seeking to introduce endolysin-based solutions into the international markets. Densely populated

Table 2
Overview of regulatory agencies and their approaches to regulating potential commercial endolysins applications in the USA and Europe.

Potential commercial applications	Regulatory agencies and regulations in the USA	Regulatory agencies and regulations in Europe
Biopreservatives	FDA: GRAS	EC, EFSA: Regulation No. 1333/2008
Biopesticides	EPA, FDA: FIFRA	EC: Regulation No. 1107/2009
Animal drugs	FDA: Center for Veterinary Medicine (CVM), New Animal Drug Application (NADA), Animal Drug User Fee Act	EU, EMA: Veterinary Medicinal Products Regulation No. 2019/6
Biocides	EPA: FIFRA	EU: Biocidal Products Regulation No. 528/2012

countries like India and China present a distinct set of challenges that necessitate a comprehensive examination of regulatory considerations for novel antimicrobials. These considerations are vital for addressing critical issues related to food safety, public health, and the sustainable practices of agriculture. In India, the regulatory authority entrusted with this responsibility is the Food Safety and Standards Authority of India (FSSAI). In China, a similar role is fulfilled by the China National Center for Food Safety Risk Assessment (CFSA), and the State Administration for Market Regulation (SAMR) plays a central role in regulating different kinds of products such as animal drugs and biocides.

Food safety regulations can affect the cost of producing these novel antimicrobials but also their approval process may be expensive and lengthy. In fact, applications can be under review for years and the average cost in the case of food additives can potentially run up to several thousands of dollars. For example, an estimated total cost for obtaining GRAS approval in the USA would require toxicological studies and safety evaluations, expert consultations and scientific data compilation, GRAS notification preparation, regulatory consulting and legal fees, as well as the corresponding administrative fees by the FDA. Addressing these challenges will require a multi-disciplinary approach involving collaboration between industry, regulatory bodies, and researchers to ensure the safe and effective use of lysins in the food industry.

7. Conclusion

The safety of our globalized food supply chain remains under constant threat from risks such as microbiological hazards. Identifying the key safety factors, as well as implementing smarter systems, is central to reducing its vulnerability to contamination. Despite all the progress that has been made over the decades, the complex nature of food systems requires constant research and updates to improve harmlessness and quality assurance.

Overall, it is quite clear that regulations to control the use of antimicrobials are essential in order to guarantee both environmental and human welfare. However, placing natural antimicrobial products on the market involves regulatory burdens that may delay the development of novel compounds. In the case of phage lytic proteins, this represents a concern given the urgent need to overcome the current antimicrobial resistance threat and to address global food security. In fact, out of all the promising lysins identified in the laboratory, only one has been commercialized so far (Staphefekt^{TM} as a medical device under Directive 93/42/EEC, for its use in skin care applications).

Furthermore, it is imperative to recognize that the landscape of regulations and laws governing the use of antimicrobials, including lysins, is far from uniform. Different countries and associations have distinct regulatory frameworks, approval processes, and standards in place. These variations can pose challenges for the global adoption of lysins in food safety. Navigating the diverse regulatory landscapes and ensuring compliance with regional requirements is a crucial consideration for the broader utilization of lysin-based antimicrobials. As we advance in the field of enzybiotics and lysin applications, it becomes increasingly important to harmonize regulatory approaches on a global scale, facilitating the development, approval, and commercial availability of these novel antimicrobial solutions while upholding the highest standards of safety and efficacy.

Nonetheless, challenges for the implementation of enzybiotics in the food industry are not limited to regulations. For example, it is imperative to achieve optimal stability of these proteins on the animal/surface/food to which they are to be applied, as well as guarantee lysin manufacture at a moderate cost in order to feasibly scale up their production at the industrial level.

Altogether, a significant body of research on the efficacy and safety of lytic proteins has opened new avenues for implementing these enzymes as antimicrobials to improve food safety. In this scenario, as long as lysins meet the corresponding requirements, the competent authorities should facilitate the endorsement of these novel products considering the existing scientific evidence.

Declaration of competing interests

The authors declare no competing interests.

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