Can natural preservatives serve as a new line of protective technology against bacterial pathogens in meat and meat products?

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Abstract

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Contamination of meats and meat products by pathogenic microorganisms is responsible for significant percentage of outbreaks of foodborne illness. There are also concerns over the carcinogenic potential of dietary nitrate and nitrite in the processed meat products. The past few decades have seen extensive search for novel technologies alternative to synthetic chemical preservatives to reduce the level of contamination of foods by pathogenic and spoilage microbes. This review provides general overview of natural preservatives with potential applications in the meat industry, including phages and their endolysins, bacteriocins, microbial lipopeptides, antimicrobial peptides of plant or insect origin, and essential oils or extracts of plant origins. Instead of providing summary data from the published literature, we attempted to elaborate the challenges facing the development of novel natural preservatives as the antimicrobial hurdles, taking into consideration of the fact of sharp contrast between extensive studies in this particular field and very limited industrial use. More specifically, we emphasized the great importance of having streamlined approaches and methodological guidelines in research and development of natural preservatives so that the journey to their industrial use for safer meats and meat products could be shortened or made easier.

Keywords: Meat and meat products; safety; natural preservatives; hurdle technology

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Introduction

Meat is a nutrient-dense food as a significant source of human nutrition. This is reflected on steady increase of world meat production, reaching 337 million tons in 2020, up 45 percent as compared with 2000 (FAO, 2022), although there are arguments in recent years of wide spread reduction of meat consumption in highincoming countries for concerns of unsustainability of current animal farming (Henchion et al., 2021; Leroy et al., 2023). Because meats and meat products are prone to microbial contamination during slaughtering and/or subsequent processing, and serve as good substrates for microbial growth, they are important sources of foodborne infections in humans (Nørrung et al., 2009; Warmate and Onarinde, 2023), contributing in substantial part to estimated loss of 33 million years of healthy life globally from approximate 600 million cases of foodborne illness and 420,000 deaths each year (Havelaar et al., 2015; WHO, 2015). This review is attempted to give an overview of microbial pathogens in meats and meat products and introduce natural preservatives to mitigate bacterial pathogens in these products for their potential as part of the antimicrobial hurdles in the production process. Emphasis is placed on major challenges in this particular field and on the need to develop technical and methodological guidelines or protocols for development of novel preservatives with improved efficacy towards potential industrial applications.

Overview of microbial pathogens in meats and meat products

Contamination of meats and meat products by pathogenic microorganisms is responsible for significant percentage of outbreaks of foodborne illness, about 39% (90/229) in China in 2020 (Li *et al.*, 2021), ca. 22% (77/355) in the EU in 2021 (European Food Safety *et al.*, 2022), and around 30% (380/1281) in the US over the 6-year period from 2009-2015 (Dewey-Mattia *et al.*, 2018). In the African continent where there were no outbreak data from regional authorities, food surveillance data from some African countries indicate that meats or meat products accounted for 46% (93/201) of the bacterial recoveries of major pathogens in the 15-year period from 2000 to 2015 (Paudyal *et al.*, 2017). While these African data were factual, there was bias towards meats because 39% of the studies (45 out of 116 included in this meta-analysis) focused on meat with some studies reporting multiple organisms in meats (Paudyal *et al.*, 2017).

Data from governmental agencies have indicated that the major foodborne pathogens implicated in foodborne illness may vary among different continents (top five in descending order of numbers of cases/infections): *Campylobacter*, *Salmonella*, *Yersinia*, Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* in the European Union in 2021 (European Food Safety *et al.*, 2022); *Campylobacter*, *Salmonella*, Shiga toxin-producing *E. coli*, *Shigella* and *Yersinia* in the US in 2022 (Delahoy *et al.*, 2023); and *Salmonella*, *Vibrio parahaemolyticus*, pathogenic *E. coli*, *Staphylococcus aureus* and *Bacillus cereus* in China in 2020 (Li *et al.*, 2021). Presence of *L. monocytogenes*, particularly the ST87 strains in ready-to-eat foods, is also a risk factor in Chinese food system (Cheng *et al.*, 2022).

In addition to data on the main pathogens involved or confirmed in general foodborne outbreaks from individual years, two recent review articles reported on bacterial foodborne outbreaks specifically related to red meat and meat products (Omer et al., 2018; Warmate and Onarinde, 2023). In one of the papers based on peerreviewed journal articles in the period 1980–2015, the authors reported that most of the outbreaks were attributed to verotoxigenic E. coli and Salmonella, causing 33 and 21 outbreaks, respectively, mostly in Europe and the United States, and that the implicated food items included beef, lamb, pork, and meat products (Omer et al., 2018). The other review reported their findings of major foodborne outbreaks linked to red meat and its products based on 1729 reports, 101 from peer-reviewed journals and 1628 from two official websites - the US Centers for Disease Control and Prevention and the European Centers for Disease Control and Prevention (Warmate and Onarinde, 2023). They found that most of the outbreaks were caused by Salmonella (469 or 27.1%), followed by E. coli (414 or 23.9%), and then Clostridium (294 or 17%.). S. aureus and L. monocytogenes accounted for 134 (7.8%) and 120 (6.9%) of the outbreaks, respectively. Absence of *Campylobacter* in these lists is apparently due to exclusion from their analyses of the white meat (poultry meat) which is considered as a major contributor to human campylobacteriosis (Hermans et al., 2012; Chlebicz and Slizewska, 2018; European Food Safety et al., 2022). In some African countries, the major pathogens in the food systems included pathogenic E. coli, S. aureus, Salmonella, Bacillus and L. monocytogenes according to the metaanalytical data of publications from 2000 to 2015 (Paudyal et al., 2017). It is obvious that Campylobacter was not targeted in the surveyed African studies.

By putting both the red and white meats together, the major bacterial pathogens in meats and meat products could be typified as *Salmonella*, *Campylobacter*, pathogenic *E. coli*, *L. monocytogenes* and *Clostridium*. This is quite similar to a review published a decade ago titled as "Food-borne diseases — The challenges of 20 years ago still persist while new ones continue to emerge" (Newell *et al.*, 2010). It is clear to us that the Gram-positive pathogens, such as *L. monocytogenes*, *Clostridium*, etc., continue to pose threat to public health in addition to the three well-recognized pathogenic bacteria (*Salmonella*, *Campylobacter* and *E. coli*) that persist throughout 1990s and until today. All these indicate that the meat industry should continue to take more strict measures or introduce novel technologies to prevent their products from bacterial contamination, or reduce the bacterial load or suppress their growth, if contamination deems unavoidable.

Natural preservatives as novel technologies for meat industry

In addition to the public health risk of major foodborne pathogens in meat or meat products as discussed above, there are also concerns over (1) the carcinogenic potential of dietary nitrate and nitrite in the processed meat products (Crowe *et al.*, 2019; Said Abasse *et al.*, 2022), (2) the spoilage-related loss of animal-sourced foods, particularly the meats that are considered as having the highest level of gas emissions per kilogram of food (Lipinski, 2020), and (3) the causal relation between the presence of antimicrobial resistance determinants within the foodborne microbial reservoirs and their transmission to human pathogens (Zinno *et al.*, 2023). Because of the growing societal demand for safe and naturally processed meats and meat products, the meat industry continues to search for novel technologies alternative to synthetic chemical preservatives to mitigate bacterial contamination by suppressing their growth in meat and meat products while implementing high standard hygiene procedures, including appropriate use of disinfectants along the production line.

There are quite a number of review articles dealing with potential applications of novel technologies, non-thermal and non-synthetic chemicals, in the meat industry, such as bacteriophages and their lysins (Ge *et al.*, 2022; Elois *et al.*, 2023), bacteriocins (Bhattacharya *et al.*, 2022), plant extracts (including essential oils from plants) (Kalogianni *et al.*, 2020; Yu *et al.*, 2021), cold atmospheric plasma (Paulsen *et al.*, 2022; Zhang *et al.*, 2023), supercritical carbon dioxide (Soares *et al.*, 2019; Buszewski *et al.*, 2022), etc. Here we provide the mechanistic aspects, pros and cons of phages, antimicrobial peptides (AMPs, including phage endolysins, bacteriocins, lipopeptides and plant-sourced antimicrobial peptides), and essential oils and extracts of plant origins proper to their application in meats and meat products as part of the antimicrobial hurdles. The readers are encouraged to refer to these recent articles for more detailed information on non-thermal and physical or physicochemical technologies.

Phages for mitigation of specific foodborne bacteria

Brief overview of bacteriophages in control of foodborne pathogens in

meats

Phages, also known as bacteriophages, are the viruses that infect and replicate only in bacterial cells. They are the most abundant biological entities on earth with estimated numbers of 10^{31} and can be found in almost every environment (Batinovic *et al.*, 2019). Phages generally have narrow host range and may be species- or even strain-specific, infecting only a single bacterial species or specific strains within a species (Koskella and Meaden, 2013), though there could be some phages showing broad lytic activity, such as the phage SS3e from *Salmonella* Enteritidis active against other enteric bacteria (Kim *et al.*, 2018) and phage PS5 infecting *S*. Enteritidis, *S*. Typhimurium, and *E. coli* O157:H7 (Duc *et al.*, 2020).

Phages utilize one of the two lifecycle strategies: lytic or lysogenic (Ofir and Sorek, 2018). During a lytic replication cycle, a phage attaches to the specific target bacterium, introduces its genome into the host cell cytoplasm, and utilizes the ribosomes of the host to manufacture its proteins or even other enzymes or cell components for assembly into multiple copies of the original phage. At the end of the lytic infection cycle, progeny phage particles emerge from the cell in a process that usually involves cell lysis by phage proteins. The new phages are released to infect another host cell. In the lysogenic replication cycle, the phage also attaches to the susceptible bacterium and introduces its genome into the bacterial cell cytoplasm. Nonetheless, the phage genome is integrated into the bacterial cell chromosome or maintained as an episomal element. Its genome is then replicated and passed on to daughter bacterial cells without killing them. Such integrated phage genomes are called prophages.

It is the lytic phages that are targeted for screening against susceptible foodborne pathogens of interest. A good candidate phage should have a number of critical features, lytic, polyvalent (i.e., multiple susceptible bacterial species or serovars), thermo-stable, and lack of genes related to microbial resistance or virulence. If polyvalent phages are not available, a cocktail of phage strains have to be used for effective mitigation to cope with diverse strains of the same target bacterial species from different backgrounds: types of foods, sources or localities, etc. It appears that research has been extensive to examine the roles of phages in controlling both Grampositive and -negative foodborne pathogens in foods, such as *L. monocytogenes*, *S.*

aureus, Salmonella, E. coli O157:H7, *C. jejuni, Y. enterocolitica*, etc. (Ge *et al.*, 2022; Elois *et al.*, 2023; Kocot *et al.*, 2023).

Here we give some latest examples used in meats, or elaborate those not clearly shown in these review articles. Park et al (2023) characterized a Salmonella phage MSP1 that could strongly infect S. Thompson and S. Mbandaka isolates and other serovars, including Dublin, Enteritidis, Heidelberg, Paratyphi, and Typhimurium (Park et al., 2023). MSP1 could withstand wide ranges of pH (4-12) and temperature (30-60°C), and no genes associated with antibiotic resistance and virulence were found in its genome. MSP1 significantly reduced S. Thompson on chicken meat below the detection limit at 3 h post-treatment (hpt) when used at 10⁸ PFU/piece or about 1.5 to 2 logs reduction of colony-forming units (CFU) when used at 10^7 PFU/piece. The Escherichia phage Tequatrovirus EP01 exhibited broad host range against 31 E. coli isolates (out of a total of 59 tested) and one of the four tested Salmonella strains (Zhou et al., 2022). EP01 possesses moderate pH stability (4-10) and thermal tolerance (30-80°C), and does not have genes related to virulence and drug resistance. EP01 at 1 MOI (multiplicity of infection) significantly reduced CFU of the strains E. coli O157:H7, O114:K90 (B90), and O142:K86 (B) on meat (unspecified) by about 0.5-2 log at 24 hpt. A polyvalent phage PS5 was found to reduce S. Enteritidis, S. Typhimurium, and E. coli O157:H7 (≥1.2 log CFU/piece) in raw chicken skin or raw beef (Duc et al., 2020). List-Shield bacteriophage (a cocktail of six bacteriophages) was effective in reducing L. monocytogenes numbers in experimentally contaminated beef samples (about 2.3 logs reduction) during 15 days storage (Ishaq et al., 2020).

Challenges and opportunities in application of phages in combating foodborne pathogens in meats

From what have reported so far, there are quite a number of challenges in using phage for food safety even if there are some good attributes of using phages as biocontrol technology, such as non-chemical (or green) and targeted/specific antimicrobial activity. There are issues of efficacy and stability, as well as biosafety that needs to be solved in the screening stage such that there is absence of genes related to antimicrobial resistance or virulence to avoid lateral gene transfer (Lee *et al.*, 2022).

The first thing that the food industry or the regulatory authority concerns is sufficient efficacy. (1) It appears that the efficacy of phage treatment on pathogen reduction is limited to some degree, mostly falling in the range from 0.5 to 2 log reduction, not full elimination (Moye *et al.*, 2018; Kocot *et al.*, 2023), although there are some reports of up to 3-5 log reduction (Leverentz *et al.*, 2004; Islam *et al.*, 2020;

Park et al., 2023). Here "reduction" is meant by comparison with the initial bacterial inoculum (initial natural contamination level), not with the bacterial numbers in the mock-treated control samples at the same time points post-treatment as some reports used (Islam et al., 2020; Park et al., 2023). Lytic phage is, theoretically, considered as bactericidal. In reality, however, the phage activity is often compromised as reviewed elsewhere, such as superinfection, bacterial mutations, etc. (Abdelsattar et al., 2021; Elois et al., 2023). (2) Also there is substantial heterogeneity in the population of even a single bacterial strain, not to mention the different strains of the same species, in a particular matrix with different degree of susceptibility to phage infection (Chibani-Chennoufi et al., 2004; Denes and Wiedmann, 2014). This suggests that some bacterial cells in the population could be at non-receptive stage of their growth, hence countable in a particular test time point or shown as regrowth at later time points (Hudson et al., 2015; Fister et al., 2016; Moye et al., 2018). (3) Alternatively, there might be low chances of contact between phages and bacterial cells, especially when the bacterial density (level of contamination) is low and present on the surface of meats or meat products, and the phage preparation applied is relatively of low MOI (Moye et al., 2018).

Secondarily, more detailed Standard Operating Procedures (SOP) or guidelines targeting specific pathogen-food combinations are required before phages or their cocktails with proven efficacy are to be incorporated in the food industry for some particular products for which current control strategies are not effective. (1) To address the problem of regrowth due either to phage resistance, or to inappropriate ratio of phage particles to bacterial cells (i.e., too low MOI), or to low chances of phage-bacterium contact on the surface of meats or meat products (in case of low contamination level), a cocktail of several different phages (preferably covering majority of the target bacterial strains in the country or region) in sufficient concentration are highly recommended, and so is the timing and way or condition (such as food factors like salt or pH in the fermented meat products) of application (Leverentz et al., 2004; Moye et al., 2018; Lee et al., 2022). (2) Microbiological criteria (zero tolerance or permissive levels per 25 gram (or mL) of target food products (e.g., Commission Regulation (EC) No. 2073/2005) should be considered to optimize the phage-mediated mitigation either for eradication or for control below the regulatory level within specified shelf-life so that the treatment is effective but not costly, acceptable by the industry. (3) Another option to increase the efficacy or to inhibit regrowth is to combine with other antibacterial components. A phage cocktail in combination with nisin and/or polylysine showed greater antibacterial effects against bacterial cocktail of S. Typhimurium, S. Enteritidis, and E. coli O157:H7 at 4°C, 24°C and 37°C (Duc et al., 2023). The endolysin LysSA97 (376 nM) and

carvacrol (3.33 mM) showed 0.8-1.0 log reduction in *S. aureus* cells, while combination of both at the same concentrations led to enhanced reduction by 4.5 log (Chang *et al.*, 2017). Additionally, phages coding polysaccharide depolymerase enzymes could be considered to increase efficacy via biofilm removal and broaden the antimicrobial spectrum (Gutierrez *et al.*, 2016).

Thirdly, there are technical issues to be addressed in phage screening and preparations during R&D for food products in general, and in particular the fermented meat products with low pH, high salinity and low probability of phage-bacterium contact on the solid surface (Moye et al., 2018; Shannon et al., 2020). (1) It would be excellent if there are commonly acceptable protocols based on the category of foods, let's say for liquid versus solid foods, or for plant- versus animal-sourced foods, etc., so that the results could be evaluated or extrapolated with reasonable level of confidence. These include, but not limited to, (a) a reference susceptible host strain of known sources and publicly available; (b) artificial contamination range $(10^2, 10^4)$ and 10^{6} CFU/g or mL, covering the possible range of natural contamination); (c) phage concentration range (e.g., 0.1, 1 and 10 MOI for liquid foods; and 10, 100 and 1000 MOI for solid foods; (d) incubation temperature range (5°C, 15°C and 25°C, simulation of temperature abuse); (e) food matrix factors (liquid vs solid, low pH in fermented food, high salinity in fermented meat products, etc.) (Shannon et al., 2020); (f) processing factors, such as temperature of product processing if the phage is to be used prior to thermal treatment. (2) Another important aspect is the methodology with which the phage treatment efficacy is evaluated. According to the methods in majority of the publications, the phage-treated food samples were homogenized and centrifuged, and the bacterial pellets (and their dilutions) are directly plated on the agar plates. This would have confounding effects on the final outcome due to carryover of the phage particles either attached to the bacterial surfaces or residing within the host cells in low numbers not enough to lyse the host cells, but could start replication to sufficient numbers to break the host cells during the pre-counting incubation at temperature optimal for bacterial growth and phage replication therein. This means that there could be overestimation of the phage-mediated bacterial reduction (or efficacy), unless the authors could provide evidence, let's say, by qPCR to exclude the presence of phage genes in the homogenized samples with or without enrichment in broth media. Therefore, more accurate methods need to be explored to provide convincing efficacy data. Because there is no such a method available to selectively inactivate phages without harming the host bacteria for accurate plate counting, one approach would be to detect the transcriptional levels of metabolismrelated genes as markers of bacterial viability at the end of phage treatment.

Temperature definitely has significant impact on the activity of phages that depend on the growth or activity of the host bacteria (Denes and Wiedmann, 2014). An interesting phenomenon is that phage treatment seems to be equally effective at low and higher temperatures, over 3 logs reduction with phage LPST94 on two Salmonella serovars at 4°C and 25°C in milk, apple juice, chicken breast, and lettuce (Islam et al., 2020), about 1 log reduction in phage EP1-treated E. coli O157:H7 on meat or Salmonella Enteritidis in milk at both temperatures 4°C and 25°C. With L. *monocytogenes*, phage P100 was found even more effective at $(4^{\circ}C)$ than at 10°C or 20°C (Fister *et al.*, 2016). From the bacterial counting methods used there, phage carry-over from the treated samples is apparent. Thus, it is difficult to make the conclusion. In the report by Huang et al. (2018), the phage LPST10 (MOI=100) had marginal effect (<1 log reduction, as compared with the initial inocula) on S. Typhimurium on sausage at 4°C or 28°C, while in milk it seemed to be more effective at 4°C than at 28°C. In both cases, the phage titers remained largely unchanged at 4°C during the 6-h incubation with the host bacteria, but increased by 1 log at 28°C. It is difficult to believe that there would be active replication of the phages with significant bacterial lysis if the temperature used does not allow growth of their host bacteria. Tokman et al. (2016) showed that plaquing efficiency (bacterial lysis) was significantly affected by both strain and temperature in testing a Listeria phage LP-048, and phage-infected L. monocytogenes cells did show sufficient plaquing upon extended incubation for 7 days at 4°C (Tokman et al., 2016). This is easily understandable because L. monocytogenes is known to grows slowly at refrigeration temperature. These instances are good examples of the potential, actually factual, phage carry-over effects that may compromise proper interpretation of the data.

Phage endolysins

Phage endolysins and their potential in mitigating foodborne pathogens

Phage endolysins are peptidoglycan hydrolases encoded by double-stranded DNA (dsDNA) phages that are dependent on host bacteria for synthesis and produced late in the lytic cycle (Loessner, 2005). Most phages utilize endolysins to hydrolyze the peptidoglycan on the bacterial cell wall (Young *et al.*, 2000). The first steps in phage lysis involve a temporally controlled permeabilization of the cytoplasmic membrane by holins which is followed by enzymatic degradation of the peptidoglycan. The hydrolytic breakdown of the host cell wall would lead to bacterial lysis. Holins, either canonical holins or pinholins, are small transmembrane proteins that cooperate with endolysins to achieve bacterial lysis, thereby releasing the phage progeny into the

extracellular environment. In both Gram-positive and -negative bacteria, holins are required to fully sensitize cells to the lytic action of canonical endolysins (Fernandes and Sao-Jose, 2016; Cahill and Young, 2019).

However, Gram-positive and -negative bacteria have different cell envelope structures. Gram-negative bacterial cell envelope has a thinner peptidoglycan layer, a proteinaceous outer membrane, and bacterial capsule covering the outer membrane, rendering it more difficult for endolysins to lyse Gram-negative bacteria than the Gram-positive ones from outside unless the endolysins are genetically modified to cleave the outer membrane proteins (OMPs). Recently a third functional class of lysis proteins, the spanins, is shown to be required for outer membrane disruption for Gram-negative bacteria (Cahill and Young, 2019), suggesting that phage-mediated lysis of Gram-negative bacteria is a stepwise process involving holins, endolysins and spanins in succession. Added to these multiple barriers are another layer outside the bacterial cells, the biofilm. Biofilm formation is part of a survival strategy for an organism to resist suboptimal environmental conditions such as limited nutrient availability, lethal concentrations of antibiotics or disinfectants (Gloag et al., 2020), or even antimicrobial peptides including phage endolysins (Zhang QY et al., 2021). Therefore, endolysins could be less effective or ineffective on Gram-negative bacteria or even on Gram-positive bacteria encircled by a layer of biofilm. Proper strategies should be sought to screen for endolysins having anti-biofilm activity or engineered for anti-biofilm activity (Liu et al., 2023) or for outer membrane permeabilization activity (Kocot et al., 2023).

Gram-positive endolysins have modular structure in which catalytic activity and substrate recognition are performed by two different types of structural domains called CBDs (C-terminal cell wall-binding domain) and enzymatically active domains (EADs) at the N-terminus, respectively. EAD cleaves specific bonds within the bacterial peptidoglycan. However, phage endolysins from Gram-negative bacteria usually do not have a specific CBD module, or their EADs are generally located at the C-terminus, whereas the CBDs, if present, are located at the N-terminal end (Liu *et al.*, 2023), such as the endolysins of *Pseudomonas aeruginosa* phages, KZ144 and EL188, with modular structures of N-terminal CBD and C-terminal EAD (Briers *et al.*, 2007).

It is generally recognized that endolysins are effective on foodborne pathogens (Lee *et al.*, 2022; Kocot *et al.*, 2023) or even their biofilm (Liu *et al.*, 2023). Other than those described in these reviews, there are some latest publications in this area. An endolysin LysCP28, encoded by orf28 from *Cl. perfringens* bacteriophage BG3P, has an N-terminal glycosyl-hydrolase domain (lysozyme) and a C-terminal SH3

domain. It exhibited lytic activity against *Cl. perfringens* strains (77 of 96 or 80.2%), including A, B, C, and D types, showed antibiofilm effect, and had nearly 3 logs reduction of *Cl. perfringens* contaminated on duck meat (at 50 µg/mL concentration) in 48 h at 4 °C (Lu *et al.*, 2023). The endolysin rlysJNwz, from bacteriophage JNwz02, had bactericidal activities against *Salmonella* of several serovars and a number of *E. coli* strains, and its combination with EDTA displayed about 86% reduction of viable *Salmonella* on contaminated eggs or lettuce (Shen *et al.*, 2023). Lysin EN4, a Gram-negative bacterial peptidoglycan-degrading enzyme, was effective in reducing *S*. Enteritidis and *S*. Typhimurium in raw chicken meat (Abhisingha *et al.*, 2023).

Approaches for more effective endolysins

Endolysins generally have limited antibacterial activity with only about 1 log reduction as shown above or have variable antibacterial efficiency depending on the intrinsic food components (proteins, carbohydrates, fat, etc.) and biochemical factors (temperature, pH, and ionic strength) in foods (Shannon *et al.*, 2020). Therefore, strategies for more effective endolysins should focus on screening those able to remove biofilms, endolysin engineering for permeabilization of Gram-negative outer membrane or potentiation of the catalytic activity of EADs.

Endolysins with anti-biofilm or outer membrane permeabilization activity

The foodborne pathogens are prone to forming biofilm in foods or food processing plants, making them more difficult to eradicate. Although physical and chemical treatments are often used to control biofilm formation, these treatments can have significant drawbacks (Galie *et al.*, 2018). Therefore, endolysins could be good alternatives for biocontrol of foodborne pathogens if they are endowed with antibiofilm activity. The LysCP28 from *Cl. perfringens* phage BG3P was found to possess anti-biofilm activity in addition to its inhibition of *Cl. perfringens* strains (Lu *et al.*, 2023). The phage DW-EC specific for *E. coli* of different pathotypes was effective in controlling pathogenic *E. coli* and showed anti-biofilm activity on polystyrene and stainless-steel surfaces. Different from the phage Youna2 that has a narrow host range, infecting only certain strains of *Weizmannia coagulans*, its endolysin PlyYouna2 exhibited a broad antimicrobial spectrum against Gramnegative foodborne pathogens such as *E. coli*, *Y. enterocolitica*, *Cronobacter sakazakii*, etc., suggesting that the endolysin could be active to destabilize bacterial outer membrane (Son *et al.*, 2023).

Genetic engineering for more potent endolysins

Endolysins have innate insufficient efficacy, as discussed above, when used from the "outside" because of the barriers of biofilm and outer membrane. Ideally, effective endolysins for use in the food industry should be capable of penetrating the two or three consecutive barriers of biofilm, outer membrane and peptidoglycan layers, have more potent enzymatic activity and are resistant to environmental conditions related to food processing. Also, screening for natural endolysins having multiple functions could be of low efficiency. With the expansion of relevant databases, discovery of novel endolysins by genetic engineering could provide great opportunities.

One approach could be to identify the putative candidate endolysins of interests based on the systemic bioinformatic analysis from retrieval of genetic information and selection of endolysin candidates to evaluation of protein solubility and potency of antibacterial and antibiofilm activities. Kim et al. (2023) found 114 putative endolysins against methicillin-resistant *S. aureus* (MRSA) which were divided into three groups based on their combinations of conserved domains. One candidate endolysin LyJH1892 was expressed in *E. coli* with good solubility and showed potent lytic activity against both general *S. aureus* and MRSA strains.

Another approach is to generate hybrid or chimeric endolysins by swapping EAD and CBD domains of different endolysins of interest or by fusing with antibacterial peptides, such as ceropin A, or with bacteriocins able to translocate the outer membrane such as colistin A that targets E. coli vitamin B12 transporter (BtuB), or with phage receptor binding protein (RBP). (1) One of the novel chimeric endolysins ClyC, screened from a hybrid library of 12 natural staphylococcal endolysins, showed enhanced antibacterial activity against S. aureus in milk and blood, and had antibiofilm property (Lee et al., 2021). A chimeric random fusion endolysin library was constructed by inserting oligonucleotide of 20 repeated NNK codons upstream of the endolysin gene Bp7e to screen for engineered artificial-Bp7e (Art-Bp7e) endolysins (Sui et al., 2023). A representative protein, Art-Bp7e6, exhibited broad antibacterial activity against E. coli, S. Enteritidis and P. aeruginosa. (2) Generation of hybrid endolysins fused with antibacterial peptides, bacteriocins or phage RBPs is another approach. The endolysin ST01 with low activity against Gram-negative bacteria exhibited enhanced activity against S. Typhimurium, E. coli, Acinetobacter baumannii, etc. when fused with cecropin A (CecA) to the N-terminus (Lim et al., 2022). Endolysins 10-24(13), PBEC30 and PBEC56 N-terminally fused with cecropin A showed increased antibacterial activity against E. coli and several other Gramnegative species (Jeong et al., 2023). Colicin-Lysep3 is a fusion protein of the

translocation and receptor binding domains of colicin A and phage lysin Lysep3 that was bactericidal against about 70% of the tested *E. coli* strains (22/32) (Yan *et al.*, 2017). Of the 228 novel innolysins from fusing 23 endolysins with RBP Pb5, innolysin Ec21 was found to be the best antibacterial candidate, leading to 2.2 logs CFU reduction of *E. coli* counts (Zampara *et al.*, 2020). Similar strategy was used to fuse the phage T5 endolysin with the H-fiber (a RBP of *C. jejuni* prophage). Innolysin Cj1 exerted antibacterial activity against diverse *C. jejuni* strains and led to 1.6 log reduction on chicken skin (Zampara *et al.*, 2021).

Bacteriocins as biopreservatives

Bacteriocins are active antibacterial peptides produced by Gram-positive and negative bacteria that contain relevant genes encoded in the plasmids or chromosomes, and majority of the bacteriocins are cationic and hydrophobic in nature (Choi *et al.*, 2023). Lactic acid bacteria (LAB) are prolific in bacteriocin production and they themselves have also been used as protective cultures (Webb *et al.*, 2022; Fischer and Titgemeyer, 2023) (not included here due to space limitation). Bacteriocins exhibit inhibitory activity against phylogenetically related species and even distant species from the producer and some of them are used in the food industry as biopreservatives (Gálvez *et al.*, 2007; Bhattacharya *et al.*, 2022; Todorov *et al.*, 2022).

Classification of bacteriocins and modes of actions

Bacteriocins are classified by molecular weight, intrinsic function, structural feature of amino acids, etc. They have been subdivided into four categories: class I, II, III, and IV as well as subgroups of classes (Klaenhammer, 1988; 1993; Cotter *et al.*, 2005; Choi *et al.*, 2023). Class I bacteriocins have molecular masses <5kDa with a leader peptide and are post-translationally modified and heat-stable. They are further divided into 6 subclasses by their distinct physiochemical nature (Ia to If): Ia (lantibiotics or lanthipeptides containing thioether amino acids), Ib (head-to-tail circular peptides), Ic (sactibiotics or sactipeptides), Id (linear azole- or azoline-containing peptides), Ie (glycosylated bacteriocins or glycocin composed of N-acetylglucosamine or N-acetylhexosamine residue), and If (lasso peptides) (Alvarez-Sieiro *et al.*, 2016; Choi *et al.*, 2023). Class II bacteriocins are heat-stable and unmodified bacteriocins with 30-70 amino acids (<10 kDa). They can be divided into 5 subclasses based on structural features: IIa (pediocin (PA-1) like peptides containing a YGNGV motif in which N represents any amino acid), IIb (two-peptide bacteriocins), IIc (circular bacteriocins), IId (unmodified, linear, non- pediocin- like,

single- peptide bacteriocins that do not belong to other subclasses), and IIe (microcin E492-like bacteriocins) (Cotter *et al.*, 2013; Alvarez-Sieiro *et al.*, 2016). Class III bacteriocins are large molecular weight and heat-labile antibacterial proteins, and those of class IV are a combination of proteins with a lipid or carbohydrate moiety for full activity (Choi *et al.*, 2023).

Bacteriocins have many distinct mechanisms of action that differ from those of antibiotics. Electrostatic interactions between cationic bacteriocins and the negatively charged components of the bacterial membrane (phospholipids and teichoic acids of Gram-positive bacteria or lipopolysaccharide of Gram-negative bacteria) could be the first mechanistic step in mounting their activity (Soltani et al., 2021). As with phage endolysins, the outer membrane of Gram-negative bacteria acts as an effective barrier against cationic bacteriocins produced by Gram-positive bacteria, making Gramnegative bacteria more resistant to these bacteriocins (Cao-Hoang et al., 2008). The antibacterial mechanisms of bacteriocins can be broadly divided into those that function primarily at the cell envelope and those that are active primarily within the cell, affecting gene expression and protein production (Cotter et al., 2013). A couple of recent reviews summarized all known mechanisms of bacteriocin action (Lozo et al. 2021) and the modes of action of ribosomally synthesized and post-translationally modified peptides, such as binding to membranes, receptors, enzymes, lipids, RNA, and metals as well as use as cofactors and signaling molecules, thus having a wide range of biological activities (Ongpipattanakul et al., 2022).

Applications of bacteriocins in meat and meat products

Nisin is the only bacteriocin licensed as a biopreservative with authorization in the European Union in 1983 (Directive 83/463/EEC), and approval by Food and Drug Administration for use in the USA in 1988 (FDA 21CFR184.1538) and more recently by Health Canada (NOP/ADP-0028) in 2017 (Soltani *et al.*, 2021). Although there have been numerous investigations for novel bacteriocins since then, nisin is still the only one used in food, including meat products, in different commercial preparations, such as Nisaplin® (Danisco, Copenhagen, Denmark), Chrisin® (Chris Hansen, Horsholm, Denmark) and Delvo®Nis (DSM, Delft, Netherlands) (Soltani *et al.*, 2021). By looking back to the initial data of antilisterial activity of nisin, a concentration of 10^4 IU of nisin did show significant reduction of nearly 2-2.5 logs CFU as compared with original inoculum on meat in the first few days (up to day 7) of storage at 5°C. However, it was not effective against *L. monocytogenes* and *S. aureus* on meat incubated at room temperature shown as regrowth of residual populations after initial reduction of 2 logs at day 0 (Chung *et al.*, 1989). These results

clearly indicate that nisin is bacteriostatic and its application should be combined with refrigeration storage.

Of the numerous studies on the exploration of novel bacteriocins for their potential use in the meat or food industry as reviewed in early years (Gálvez et al., 2007) or recently (Bhattacharya et al., 2022; Todorov et al., 2022; Bodie et al., 2023), we take a recent report as an example, not only because it was not in these reviews, but also because they used purified proteins expressed in the plant Nicotiana benthamiana for evaluation of their biopreservative effect in chicken meat contaminated with a cocktail of seven pathogenic serovars, a well-conducted experiment involving multiple strains in the artificial challenge studies (Schneider et al., 2018). They found that SalE1a and SalE1b, two of the five salmocins (colicin analogues effective on Salmonella spp.) possessed broad antimicrobial activity against all 99 major Salmonella pathovars, showing remarkably high potency (>10⁶ AU/ μ g recombinant protein, or $>10^3$ higher than colicins). Treatment of poultry meat confirmed the reasonable efficacy (inhibitory in the beginning and regrew to the initial level after 72-h storage at 10°C) when four of them were used in combination at higher concentrations (3mg+1mg+1mg+1mg/kg meat). However, when SalE1a was used alone at 3 mg/kg meat, initial 1.5 log reduction at 1 h after treatment was followed by regrowth to about 1 log CFU above the initial inoculum level. These findings also suggest that SalE1a is bacteriostatic on poultry meat.

Genetic engineering of bacteriocins for improved efficacy

The narrow antibacterial spectrum generally displayed by bacteriocins from lactic acid bacteria represents an important limitation for their application as food biopreservatives. Efforts have been made to yield hybrid bacteriocins of broader antibacterial spectra. One novel hybrid peptide Ent35-MccV was obtained by combining enterocin CRL35 (effective only on Gram-positive bacteria) and microcin V (effective only on Gram-negative *E. coli*) (Acuna *et al.*, 2012). Ent35-MccV showed potent antimicrobial activity against 12 of the 14 tested *Listeria* spp. strains isolated from contaminated foods and against almost all food *E. coli* isolates tested, including the O157:H7 strains. With ground beef patties as a model food for inoculation with *L. monocytogenes* or *E. coli*, Ent35-MccV at 125 AU/g led to about 0.5 log reduction for both bacteria on day 1 after treatment and growth of both bacteria was inhibited throughout the 10-day storage at 4°C (Acuña *et al.*, 2015). The group also tried to further improve its activity by mutating amino acids in the hinge region between enterocin CRL35 and microcin V. One mutant bearing a tyrosine in the central region of the hinge (Ent35-GYG-MccV) is 2-fold more active against *E*.

coli and 4-fold more active against *Listeria* (Navarro *et al.*, 2019). This approach fosters the rational design of peptides with enhanced antimicrobial activity (Navarro *et al.*, 2020).

Tiwari et al. (2015) constructed two hybrid bacteriocins, enterocin E50-52/pediocin PA-1 (EP) and pediocin PA-1/enterocin E50-52 (PE) by combining the N terminus of enterocin E50-52 and the C terminus of pediocin PA-1 in reverse order. Both hybrid bacteriocins showed reduced MIC (minimum inhibitory concentration) compared to those of their natural counterparts. The hybrids PE and EP were active against tested Gram-positive and Gram-negative bacteria, such as *Micrococcus luteus*, *S*. Enteritidis, and *E. coli* O157:H7. The MICs of hybrid PE and EP were 64- and 32fold lower, respectively, than those of pediocin PA-1, and 8- and 4-fold lower, respectively, than those of enterocin E50-52. Fathizadeh et al. (2020) reported another recombinant bacteriocin (Ent A-Col E1) by fusing enterocin A with colicin E1. Its MIC was 10 µg/ml for *S. aureus* and *E. coli* and 20 µg/ml for *P. aeruginosa* and *E. faecalis*. The fusion protein, when used at 1 or 2 times its MIC, could resulted in 99% to 99.9% reduction of these bacterial inocula in broth media. However, further research is required to see if these hybrid bacteriocins could be used as biopreservatives for meat or meat products.

Other antibacterial peptides as potential preservatives

Besides bacteriocins and endolysins described above, there are also antimicrobial peptides (AMPs) of medical importance with potential application in the food industry to combat foodborne pathogens. These include lipopeptides from prokaryotic and eukaryotic microbes, and antimicrobial peptides from plants and insects (Meena and Kanwar, 2015; Ioannou *et al.*, 2023).

Lipopeptides (LPs) are a group of biosurfactants containing a core hydrophilic peptide chain linked to a hydrophobic fatty acid chain, leading to their amphiphilic nature (Pilz *et al.*, 2023). The length, composition and structure, e. g. cyclic or linear, of the peptide chain and lipid moiety, differ extensively, dictating the physiochemical properties and biological activities (Götze and Stallforth, 2020). LPs are produced by various microorganisms, including bacteria, fungi and algae. The majority of LPs are of bacterial origin, specifically of the *Bacilli* class, and synthesized via non-ribosomal peptide synthases, and the most described families of non-ribosomal LPs (NRLPs) in literature include iturin, surfactin, fengycin/plipastatins and kurstakin (Théatre *et al.*, 2022; Pilz *et al.*, 2023). Novel NRLPs are continuously reported (Clements-Decker *et al.*, 2022; Zhang *et al.*, 2023). The lipolanthine MicA is the first LP of ribosomal origin (RiLP) having anti-staphylococcal activity (Wiebach *et al.*, 2018). Recently

reported RiLPs include goadvionins (Kozakai *et al.*, 2020), albopeptins (Oikawa *et al.*, 2022), selidamides (Hubrich *et al.*, 2022), and others (Pilz *et al.*, 2023).

Plant AMPs act as the first line of defense against phytopathogens, widespread in the plant kingdom and can be found in all plant organs (Lima *et al.*, 2022; Sharma *et al.*, 2022). Plant AMPs are ribosomally derived and share several common characteristics with those from microbes, insects and animals, such as their molecular forms, positive charge and amphipathic nature (Tam *et al.*, 2015). They are structurally diverse and can be divided into families based on their sequence similarity, cysteine (Cys) motifs, and distinctive disulfide bond patterns (i.e., characteristic Cys pattern with a defined number of non-Cys residues between the two neighboring Cys) which, in turn, determine their tertiary structure (Tam *et al.*, 2015). Plant AMP families include thionins, defensins, hevein-like peptides, knottins, stable-like peptides, lipid transfer proteins, snakins and cyclotides (Lima *et al.*, 2022).

Insect AMPs play an important role in the humoral immune system. In holometabolous species AMPs are biosynthesized mainly in the fat body and transferred into the hemolymph, while in heterometabolous species they are produced by hemocytes and secreted into the hemolymph following infection (Bulet and Stöcklin, 2005; Marmaras *et al.*, 2009). More than 200 AMPs have been identified from insects to date (Erdem Büyükkiraz and Kesmen, 2022). Insect AMPs are divided into three groups based on their amino acid sequence and structures: (1) cecropins, the linear peptides with α -helix but lack Cys residues; (2) defensins with 6-8 conserved Cys residues and a stabilizing array of 3 or 4 disulfide bridges as well as 3 domains consisting in a flexible amino-terminal loop; and (3) proline and/or glycine-rich peptides (Wu *et al.*, 2018). The most explored insect AMPs are cecropins, drosocin, attacins, diptericins, defensins, ponericins, drosomycin and metchnikowin (Mylonakis *et al.*, 2016; Wu *et al.*, 2018). More new peptides can still be discovered by combined use of mass spectrometric techniques, antimicrobial assays and RNA-seq (Lin *et al.*, 2022; Scieuzo *et al.*, 2023).

LPs and AMPs from plants and insects possess antibacterial properties against fungal and bacterial pathogens, including those foodborne (Wu *et al.*, 2018; Kourmentza *et al.*, 2020; Lin *et al.*, 2022; Sharma *et al.*, 2022; Pilze *et al.*, 2023; Wang *et al.*, 2023). Mechanistically, the negatively charged bacterial cell membrane is the primary target for electrostatic interaction with the positively charged antimicrobial peptide residues, and the hydrophobic property, e.g., of tryptophan, leucine, etc., aids in insertion of LPs into the lipid bilayer, leading to depolarization of bacterial cell membrane and eventual bacterial lysis (Mylonakis *et al.*, 2016; Lima *et al.*, 2022; Pilz *et al.*, 2023). Some AMPs function via non-membrane target (i.e.,

intracellular processes): they first translocate into bacterial cells without perturbing the cell membrane and then prevent critical cellular processes by interacting with intracellular targets, leading to inhibition of protein and nucleic acid synthesis, and degradation of enzymes and proteins (Erdem Büyükkiraz and Kesmen, 2022; Sharma *et al.*, 2022).

Irrespective of extensive research, there are only few AMPs so far approved for clinical use in medicine (Zhang et al., 2021). One such antibacterial lipopeptide is daptomycin from Streptomyces roseosporus approved by the US Food and Drug Administration (FDA) in 2003 for the treatment of complicated skin infections. Daptomycin has rapid, concentration-dependent bactericidal activity against most clinically significant Gram-positive pathogens and exhibits excellent safety profile (Eisenstein, 2004). Natamycin, produced by *Streptomyces natalensis* and effective against almost all foodborne yeasts and molds, was the one (other than nisin) approved by US FDA (21 CFR §172.155, 2000) as preservative for cheese (Davidson et al., 2013; Elsser-Gravesen, 2014). Natamycin was also tested as antimicrobial coating for shelf-life extension of cheese (Azhdari & Moradi, 2022). In the food industry, lipopeptides were only tested as preservatives for its antioxidant activity, but not as antibacterial preservatives thus far. Direct incorporation of lipopeptides in ground beef patties at a concentration of 0.5% (w/w) was found to be more effective than gelatin film enriched with lipopeptides as a coating, in inhibiting lipid oxidation (Jemil et al., 2020). There is also paucity of information on the potential use of LPs and AMPs from plant and insect origins as antibacterial preservatives (Sharma et al., 2022; Ioannou et al., 2023).

Essential oils or extracts from plants

Plants as herbal medicines with "heat-clearing" and detoxifying activities has long been used in traditional Chinese medicine for treatment of infectious diseases. Such kinds of plants are found to be antibacterial, such as *Taraxacum officinale*, *Coptis chinensis* (Rhizome), and *Scutellaria baicalensis* as elaborated by Chen *et al.* (2021). Essential oils (EOs) and plant extracts have been considered good alternatives to synthetic additives for preservation of foods including meats and meat products (Aguiar Campolina *et al.*, 2023). Phenolic compounds are abundant in plants and can be found in herbs, spices, vegetables, fruits, wine, essential oils, olive oil and oil seeds, and have been utilized by the food industry for their antioxidant and antimicrobial functions (Kalogianni *et al.*, 2020; Yu *et al.*, 2021).

Major chemical components and possible antibacterial mechanisms

Plant-derived polyphenols can be classified as: phenolic acids (caffeic acid, rosmarinic acid, gallic acid, ellagic acid, cinnamic acid), flavones (luteolin, apigenin, chrysoeriol), flavanols (catechin, epicatechin, epigallocatechin, gallocatechin, and their gallate derivatives), flavanones (hesperidin, hesperetin, heridictyol, naringenin), flavonols (quercetin, kaempferol, myricetin), isoflavones (geinstein, daidzin, formononetin), coumarins (coumarin, warfarin, 7-hydroxycourmarin), anthocyanins (pelagonidin, delphinidin, cyanidin, malvidin), quinones (naphthoquinones, hypericin), alkaloids (caffeine, berberine, harmane), and terpenoids (menthol, thymol, lycopene, capsaicin, linalool) (Yu *et al.*, 2021).

EOs from aromatic plants, including bark, leaves, flowers and seeds, are composed of a mixture of several phenolic compounds as the basic active ingredients (terpenes, terpenoids, and phenylpropanoids) and can contain between 20 and 60 compounds, often with two or three at higher concentrations (20 to 70%) compared to the rest of the constituents. For instance, origanum EO contains 30% of carvacrol and 27% of thymol as the principal components (Bakkali *et al.*, 2008). EOs are rich in phenolic compounds and can be prepared from oregano (carvacrol, thymol, p-cymene, γ -terpinene), clove (eugenol), coriander (linalool), ginger (α -pinene, cineole, borneol, geraniol, α -curcumene, camphene and eucalyptol), rosemary (carnosic acid, carnosol, rosmadial, genkwanin, rosmarinic acid, 1,8-cineole, α -pinene, limonene and camphor), sage (α -thujone, β -thujone, camphor, 1,8-cineole, borneol, viridiflorol), thyme (thymol, carvacrol, ρ -cymene, γ -terpinene, linalool), and mint (menthol) (Kalogianni *et al.*, 2020).

Polyphenols have been recognized for their effective antimicrobial properties. The antimicrobial effect of EOs is reportedly due to the phenolic compounds they contain (Chouhan *et al.*, 2017). However, the mechanisms, though not yet clearly elucidated, may be complex because plants and plant extracts contain a diverse range of bioactive molecules that are different in their chemical structure and that might function in synergy/antagonism with each other. There may be several possible mechanisms of action to impact the bacterial cells, such as increased membrane permeability, decreased proton force, leakage of metabolites and ions, effects on enzymes, changes in membrane fatty acids, and so forth (Gyawali *et al.*, 2015; Aguiar Campolina *et al.*, 2023). The effect of EOs may vary with bacterial species. The Gram-positive bacterial cell wall structure allows hydrophobic molecules to easily penetrate the cells and act on the cell wall and within the cytoplasm, while Gram-negative bacteria are generally more resistant (Nazzaro *et al.*, 2013), possibly due to

the lipopolysaccharide layer present in their cell wall that limits the diffusion of hydrophobic compounds, such as essential oils (Danilović *et al.*, 2021).

Challenges in using essential oils and plant extracts in meats or meat

products

Although EOs and plant extracts do possess antibacterial and preservative effects via different mechanisms as reported over the past decade and recently reviewed (Kalogianni *et al.*, 2020; Pateiro *et al.*, 2021; Yu *et al.*, 2021; Aguiar Campolina *et al.*, 2023; Bodie *et al.*, 2023; Ji *et al.*, 2023) (please refer to these reviews for more details regarding their effects in tabulated forms), limitations regarding the application of such natural extracts in meat and meat products are also apparent: efficacy, safety to consumers, negative sensory effects on foods, production cost, standardization, etc.

The first issue lies on the efficacy. Majority of the studies have reported that plant extracts are mostly inhibitory, i.e., preventing further growth as compared with untreated control samples, not necessarily bactericidal (significant decrease as compared with the initial inoculum). For instances, oregano and thyme essential oils (1-2 times MIC) was not able to suppress *L. monocytogenes* growth in minced pork during storage at 4°C for 4 days although the authors reported significant reduction as compared with the untreated controls (Vidakovic Knezevic *et al.*, 2023). Mānuka oil, with MIC at 0.04%, suppressed growth of *L. monocytogenes* and *S. aureus* at 2.5% concentration in beef samples stored at 4°C for 16 days (Kaur *et al.*, 2023). When the mixture of rosemary and licorice extracts (2.5, 5 and 10 mg/mL) were sprayed on fresh pork chops or ham slices inoculated with *L. monocytogenes* (about 5.2 logs CFU/g or cm²), the effect was mainly inhibitory (no further growth observed) at 4°C for 7 days, but exhibited slight increase to about 5.2-5.8 logs CFU) during further storage until day 28, though the authors reported 2.5- to 3-logs reduction when compared with the untreated samples (Zhang *et al.*, 2009).

Meat-based foods have complex compositions, such as proteins and lipids that may interact with natural compounds, thus affecting the antibacterial activity. Other food factors, such as water activity (a_w) and pH, can also affect the performance of natural compounds (da Silva *et al.*, 2021). Therefore, food applications may require concentrations up to 100 times greater than those used in *in vitro* experiments (Aguiar Campolina *et al.*, 2023). For example, the MIC of 10% cinnamon EO on *L. monocytogenes* was 1:128. However, complete inhibition was seen only at 10% EO concentration in dry-cured ham with a_w 0.95 stored at 7°C for 7 days (<1 log CFU from the initial inoculum at 4 logs CFU), while no significant reduction was seen at aw 0.93 (3.7 logs CFU, compared to initial 4 logs) (Dos Santos et al., 2022). High concentrations of plant extracts could have undesirable sensory characteristics in the final products and potential toxicity for humans (Boskovic et al., 2017; Pateiro et al., 2021; Ji et al., 2023). Danilović et al. (2021) emphasized that EOs and extracts can cause changes in odor and flavor, and they should be used in the lowest possible concentration. Therefore, optimization is required if the plant extracts, including EOs, are to be used for meat products, such as by means of encapsulation, nanoemulsification, active packaging in the form of films and coatings, as well as combined use with other natural preservatives, such as bacteriocins and endolysins, or with physical treatments (refrigeration, gamma irradiation, high pressure processing, etc.) (Yu et al., 2021; Ojeda-Piedra et al., 2022; Ji et al., 2023). However, the efficacy does not seem satisfactory (only reduced CFU compared with untreated controls rather than reduction from initial inoculum) in the case of the alginate-based edible coating with oregano essential oil on chicken meat (Yang et al., 2023) or of alginate edible coating containing cinnamon essential oil nanocapsules and nisin on beef slices (Zhang et al., 2022). Until the combinatorial agents or techniques have proven efficacy with good sensory quality attributes in meat and meat products, safety to consumers should be rigidly evaluated for regulatory purposes, so are the standardization of the components and estimation of production cost to the food industry.

Combination of antibacterial hurdles for improved efficacy

Several intrinsic and extrinsic factors associated with foods serve to promote preservation. Water activity, temperature (low or high), preservatives (i.e., bacteriocins, endolysins, plant EOs, etc.), acidity (pH), competitive microorganisms (i.e., lactic acid bacteria) and redox potential (Eh) are among the most important factors. These extrinsic and intrinsic factors have limited antibacterial activity when applied singly. However, when combined with one another in a sequence or applied simultaneously, the activity of each is considerably enhanced. The result is synergistic food preservation referred to as a hurdle effect (Gragg & Brashears, 2014). With increased knowledge of the critical limits for growth, survival, and death of the most significant microorganisms associated with the supply chain of particular foods, it is possible to design effective food preservation strategies from a wide range of increasingly available technologies, thermal, non-thermal and natural. The ultimate goal is for the hurdles to control the naturally occurring microbial population to acceptable levels either by inhibition of their growth or even inactivation. In another word, hurdle technology represents the intentional combination of hurdles to preserve foods within defined shelf-lives. Hurdles can be strategically combined such that it is

possible for a food to become increasingly economical, have improved microbial safety and stability, as well as enhanced nutritional and sensory characteristics (Gálvez *et al.*, 2007; Gragg & Brashears, 2014; Karbowiak *et al.*, 2023). It is also important for the food producers to understand that any pre-harvest hygiene measures or those during slaughtering or processing are also hurdles aimed at reducing microbial loads on the raw meats or processed meat products. Here we provide a brief overview of the natural antibacterial agents as hurdle factors that may be combined themselves.

Over the past 40 years since the approval of nisin application in foods by EU in 1983, no other single natural AMP, either microbial (endolysins or bacteriocins) or plant-sourced, except several phage preparations including the listeria-specific bacteriophage preparation first approved by FDA in 2006 (FDA, 2006; Moye et al., 2018), was formally authorized for use in the food industry irrespective of numerous studies (Yu et al., 2021; Bhattacharya et al., 2022; Todorov et al., 2022). From what have been described above or in these reviews, the major problem in applying natural AMPs and plant EOs or extracts is efficacy in controlling major pathogens in foods including meats and meat products. The AMPs are mostly bacteriostatic rather than bactericidal, i.e., possessing weak and limited antimicrobial activity unless used at high concentrations (Juneja et al., 2012), and their activities are usually compromised by food matrixes (Shannon et al., 2020). In addition to continuing search for novel natural antibacterial agents from the huge repository of phages and bacteria that contain genes encoding endolysins or bacteriocins, and for more potent and broadspectrum endolysins or bacteriocins by genetic engineering (Lee et al., 2022; Kocot et al., 2023), it is also important to consider different combinatorial hurdles of natural preservatives or individual integration of natural preservatives with non-thermal physical methods (for the later, the readers are directed to papers by Aaliya et al., 2021; Barbhuiya et al., 2021; Bigi et al., 2023).

Combination of EO at 0.6% and nisin at 500 or 1000 IU/g showed synergistic activity against *L. monocytogenes* and *E. coli* O157:H7 (though nisin does not inhibit *E. coli*) with EO at 0.6% plus nisin at 1000 IU/g being most effective by decreasing *L. monocytogenes* below the EU limit (set at 2 log CFU/g during 12-day storage at 4°C) in minced beef (Solomakos *et al.*, 2008a). The EO-nisin combination also showed an additive effect against *E. coli* O157:H7, decreased by about 1.0 log from the initial inoculum and no regrowth during storage at 4°C (Solomakos *et al.*, 2008b). The group further examined the combination of oregano essential oil (containing carvacrol 80.2% and thymol 4.8%) with nisin against *S*. Enteritidis in minced sheep meat. Oregano EO at 0.6% or 0.9% was effective enough to inhibit *S*. Enteritidis in the meat

during storage at 4° for 12 days while nisin had very limited additive effect (Govaris *et al.*, 2010). These results suggest that synergistic effect could be realized when both components are effective on the target bacteria.

Combination of LysSA97 (1.88 mM) and carvacrol (6.66 mM) exhibited synergistic effect against *S. aureus* in pasteurized skim milk, reducing more than 4 logs CFU in 3 h. However, this effect was abrogated in whole milk. When the same amount of the combinations was tested in beef, the bactericidal activity was not clear. However, combination of LysSA97 (18.8 mM, 10-fold increase) and carvacrol (6.66 mM) showed synergism in beef, reduced by $2.1\pm0.5 \log \text{CFU/cm}^2$ within 3 h at room temperature, suggesting that food matrix effect is pronounced with beef or whole milk (Chang *et al.*, 2017). Unfortunately, this study only examined short-term effect at room temperature, a scenario that does not allow extrapolation to refrigeration-stored meats with designated shelf-life.

Dipping solutions containing bacteriocins produced by *L. curvatus* CRL705 and *L. sakei* CRL1862 (Bact705/1862), nisin and organic acids (lactic acid, LA; acetic acid, AA) were tested alone or in combination against *L. monocytogenes* inoculated by immersion on vacuum-packaged frankfurters stored at 10°C during 36 days. Semipurified Bact705/1862 prevented *L. monocytogenes* growth, while nisin was not able to avoid bacterial regrowth after 20 days. Combination of Bact705/1862 with LA/AA was the most effective approach for pathogen reduction below detection level from day 6 to final storage. Frankfurters treated with Bact705/1862 pus LA/AA did not show significant differences in flavor, juiciness, color intensity and overall preference at 5°C for 22 days, as compared to fresh-purchased samples (Castellano *et al.*, 2018). With this combination, further optimization may be required to see if combination of nisin with acids could have similar efficacy to Bact705/1862 and LA/AA combination or if single acid at lower concentration could have equivalent effect. In a recent study by Shrestha *et al.* (2023), lactate at 2% did not potentiate nisin activity against *L. monocytogenes* in a RTE egg product, while acetate did even at 1% concentration.

The efficacy of mixtures of natural antimicrobial compounds, reuterin and microcin J25 in combination with peracetic or lactic acids was evaluated for inhibition of *S*. Enteritidis and total aerobes on broiler chicken carcasses. The MICs of reuterin, lactic acid, and microcin J25 against *S*. Enteritidis were 2 mM, 0.31% and 0.03 μ M, respectively. The combinations of reuterin + lactic acid or reuterin + microcin J25 were synergic in broth media, making these compounds effective at four times lower concentrations than those used alone. Spray of reuterin + lactic acid mixture onto chilled chicken carcasses reduced *Salmonella* spp. counts by 2.02 logs CFU/g at 4°C for 24 h, whereas reuterin + microcin J25 and peracetic acid reduced *Salmonella* by

0.83 and 1.13 log CFU/g, respectively (Zhang *et al.*, 2021). The synergy of reuterin with lactic acid or microcin J25 as inhibitors of bacterial growth was significant.

The above cases are just a few examples on the possible combinatorial hurdles using natural compounds. More information on this aspect is available in the reviews by Gálvez *et al.* (2007) and Karbowiak *et al.* (2023). There are also reviews on combined use of bacteriocins and bacteriophages as food biopreservatives (Rendueles *et al.*, 2023), non-thermal techniques (Roobab *et al.*, 2022) or their combination with natural preservatives for enhanced efficacy (Kaur *et al.*, 2022).

Challenges and future prospects

Challenges

Significant progress has been made over the past few decades in search for natural food preservatives to minimize the use of synthetic chemicals for production of safe meats or meat products. However, an interesting, but important or even critical, question requires an answer: while there are a large number of AMPs, including bacteriocins and endolysins, and natural components from plants investigated so far, why are there so few natural preservatives being approved for use in the food industry? too strict regulatory scrutiny, lack of impetus from the food industry, insufficient consumer demand to drive the process, and lack of consistency or shortage of perseverance from the research side? We suppose that this could be a multifactorial issue.

Apart from the challenges described above on application of phages, AMPs, essential oils and plant extracts, there are still a number of major issues to be addressed. The top one, we believe, would be the difficultness to compare one study from the other to properly evaluate the efficacy because of the lack of community (i.e., scientific and industrial community) acceptable experimental protocols or guidelines for investigation of the natural preservatives of any category. The investigation should be application-guided starting somewhere from the initial screening of candidates. Therefore, we propose to set up guidelines in collaboration with the experts from the food industry for the researchers, especially of the young generation, to follow in order to shorten the journey from paper publication to potential industry application. The guidelines could include the general one and those specific to particular food categories considering the fact that different food matrixes have significant impacts on efficacy. The major factors to include, but not limited to, 1) preparation of model foods simulating the products of interest, e.g., identical or similar food composition, a_w, pH, etc.; 2) preparation of the challenge inocula and their application. The inoculum should consist of a panel of five strains of the same target bacterial species isolated from food and be evenly distributed on the surface or within the food matrix; 3) storage time and temperature according to the target food products with the temperature abuse factor considered; 4) reproducibility of the results by at least three independent repeated experiments, each in triplicate; 5) the bacterial quantification methods should be accompanied with its limit of detection (lowest bacterial counts detectable with the particular method used), avoiding or minimizing the carry-over effects when bacteriophages are involved as mentioned above; 6) looking for synergistic effect, not the additive effect, when the preservation techniques are used in combination or in succession. That is to calculate the fractional inhibitory concentration (FIC) index, the lower, the better, according to Turgis et al. (2012). Where possible, mixture design methodology and predictive modeling could be incorporated (Mahmud et al., 2023); 7) reporting the efficacy data in terms of CFU reduction by comparing with the initial inoculum at time zero, not the reduction as compared with the untreated control samples at the same storage time point. The untreated control could only serve as "positive" control indicating that the target bacterium could grow in the specific model food under defined growth conditions.

Naturally occurring AMPs, including endolysins and bacteriocins, usually have limited antibacterial activity or spectrum as described in the preceding sections, and low selectivity, which results in toxic effects on host cells, etc. (Table 1). Therefore, AMPs as natural preservatives were most likely tested and ended in laboratories without being rigorously evaluated in the real food systems, let alone any efforts of targeted modifications for improved efficacy. It was ever suggested that the major difficulty in developing AMPs isolated from natural sources could be that they were evaluated and then brought too quickly to preclinical studies without having been fully optimized (Fox, 2013). A long and expensive process thus ended, leaving them excluded from the industry development (Fox, 2013; Barreto-Santamaría *et al.*, 2019). Thus, there are still good chances to chase for natural AMPs of higher potency and spectrum from previous preliminary studies as the starting points for further improvement of their efficacy as food preservatives.

When the efficacy of the candidate natural preservative is confirmed, the next issues to address are safety to the humans and possible emergence of bacterial resistance to endolysins, bacteriocins and other AMPs on which the regulatory authority will focus. (1) There are a panel of widely accepted methods, both *in vitro* and *in vivo* in animal models to examine safety of natural preservatives, as elaborated by Todorov *et al.*, (2022). The initial approach could use intestinal epithelial cells of

human or animal origin to test the cytotoxicity or interference with the barrier function of the intestinal epithelial cell monolayers. This is because humans are exposed to such agents via eating and the intestinal walls are the sites of their primary effects. (2) Resistance to AMPs also requires consideration though it is not as common as resistance to conventional antibiotics. Once a new antimicrobial agent is proven to be safe and effective against pathogens, it is critical to examine the potential risks of resistance development upon prolonged exposure to it (Cotter et al., 2013). This also applies to bacteriocins that are used as natural preservatives in foods. The bacteriocins most studied with regard to the development of resistance are nisin. lacticin 3147, pediocin-like bacteriocins, etc., possibly because of their early uses and investigations (Bastos et al., 2015). Bacteriocin resistance may be either innate or acquired, arising at different frequencies (generally from 10^{-9} to 10^{-2}) and by different mechanisms, and these mechanisms generally involve changes in the bacterial cell envelope in Gram-positive bacteria, which lead to (a) reduction or loss of bacteriocin binding or insertion, (b) bacteriocin sequestering, (c) bacteriocin efflux pumping and (d) bacteriocin degradation, etc., according to Bastos et al. (2015). Resistance mechanisms to other AMPs include alteration of cell wall components, lipid composition, efflux pumps, presence of capsules and biofilms, and secretion of proteases found in Gram-positive species (Assoni et al., 2020). For instance, resistance to daptomycin emerged soon after its application by altered membrane composition of S. aureus and B. subtillis (Jones et al., 2008; Hachmann et al., 2009).

The next issue could be examination of the production costs of the natural preservative itself as well as added cost to the meat or meat products with the use of such preservatives, either used alone or in combination so that the food industry could find possible solutions to balance the safety of their meats and the cost incurred from using the novel preservatives as the hurdles. This is particularly important for production and purification (even partial purification) of engineered endolysins, bacteriocins and other antimicrobial peptides either the original version or the hybrid ones (Barreto-Santamaría et al., 2019; Wibowo and Zhao, 2019). It may not be necessary to remove the fusion tags which are used for purification purpose if such fusion does not have significant impact on the antimicrobial function and safety. This is because removal of such tags by different means would not only adds instrumental cost and procedural time, but also reduce the AMP yield by 3-50 folds (Wibowo and Zhao, 2019). Another parallel issue here is to avoid or reduce the negative effects of natural preservatives on sensory quality of the meats or meat products, especially those of plant origin, by combinatorial use to reduce their concentration, or by microencapsulation, nano-emulsion, etc. (Ojeda-Piedra et al., 2022).

Future Prospects

Decades of research have shown that there are some good candidates with similar or even superior potency and spectrum as compared with nisin as reviewed here and elsewhere. More importantly, there are databases of several categories of AMPs, including lipoproteins and phage lytic proteins: 3569 AMPs in the APD3 database (https://aps.unmc.edu, accessed September 24, 2023)(Wang, 2023), 1744 peptides in the database Norine (non-ribosomal peptides)(https://norine.univ-lille.fr/norine)(Flissi *et al.*, 2020), and 16,095 phage lytic proteins in the database PhaLP (https://www.phalp.org/) (Criel *et al.*, 2021). Now it is time and possible to develop more active and broad-spectrum AMPs as candidate food preservatives by combining the knowledge from such databases with our past experience on individual AMPs in laboratory settings. Computational tools can be used for rational design of AMPs related to sequence, charges, structure, hydrophobicity, and amphipathicity for improved potency and safety (Table 1, Figure 1). Computational methods and artificial intelligence could facilitate MIC-guided identification of such novel AMPs (Aguilera-Puga *et al.*, 2024).

Efficacy (antimicrobial activity and spectrum) and cost are the major concerns of the food industry. The academia should work closely with the food industry to examine if some of these novel candidates could be exploited, as individual preservatives of sufficient potency or as important components of antibacterial hurdles, from the industrial perspective in terms of production cost by cost-benefit analysis or efficacy validation in industrial settings with well-planned and acceptable experimental protocols or guidelines as elaborated in the initial paragraphs of this section. Such novel peptides could be produced by recombinant expression (not necessarily by chemical synthesis unless developed for medical purpose). For possible mass production of recombinant AMPs at low cost, a number of important factors should be taken into accounts, including expression systems (hosts), fusion tags, purification process (if deemed necessary for efficacy or safety reasons), scale-up process, etc. (Wibowo and Zhao, 2019).

We anticipate that the journey to industrial use of novel natural preservatives for safer meats and meat products would be shortened or made easier with the introduction of streamlined approaches with well-acceptable guidelines and methodologies from laboratory simulation to pilot studies in the production/processing line.

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Author contributions

CC and LJ contributed two parts each, XL and HS participated in the organization of sections and discussion; and WF involved in conceptualization and finalization of the manuscript.

Conflict of interest

There is no conflict of interest.

Accel

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Received

Figure

Figure 1. Discovery of novel antimicrobial peptides via rational design

Accepted Manuschi

Table 1. Main characteristics of natural antibacterial preservatives and possibility for

improvement for meat and meat products

Types	Major advantages	Major disadvantages		Possibility to improve	
Phages	Bactericidal and strain- specific	(1) I 1 1 2 (2) M 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 2 1 1 1 2 1 1 1 2 1 1 1 2 1 1 1 1 2 1 1 1 1 1 2 1 1 1 1 1 1 1 2 1 1 1 1 1 1 1 1 1 1 1 1 1	Effective only at strain level, not at species level, i.e., narrow antimicrobial spectrum; May carry antimicrobial resistance genes or virulence genes with possible lateral gene transfer to other host strains.	(1)	Using a cocktail of phages to cover more strains or to screen phages active against more strains or even different species; Exclude phages that contain genes related to resistance or virulence.
Endolysins	 Encoded in phage genes; More effective on Gram-positive bacteria by hydrolyzing the peptidoglycan cell wall. 	(1) I F (2) I (0) F (2) (1) (2) (1) (2) (1) (2) (2) (2) (2) (2) (2) (2) (2	Limited antibacterial potency or even bacteriostatic; Less effective on Gram-negative bacteria because of presence of outer membrane proteins (OMPs) or when the bacteria are covered with biofilm or capsules.	(1)	Rational design and genetic modification to develop more potent AMPs; Genetic engineering to make hybrid AMPs of higher potency by bleaching the
Bacteriocins	 Produced mainly by lactic acid bacteria; Cationic and hydrophobic; mostly active against phylogenetically related species. 	(1) 1 s t (2) 1 (2) 1 t t	Narrow antibacterial spectrum, bacteriostatic; Less effective on Gram-negative bacteria because of OMPs, lipopolysaccharides, biofilm, etc.	(3)	OMPs, capsule or even biofilm; Scale up production of recombinant AMPs by optimized batch culture conditions
Lipopeptides (LPs), and AMPs from plants and insects	 LPs could be produced by bacteria, fungi or algae; Antimicrobial with broader spectra as compared with bacteriocins. 	(1) ((2) S a B	Could be cytotoxic (safety concern); Structurally diverse and antibacterial potency varies.	(4)	for lower cost; Incorporation into the antimicrobial hurdles for better efficacy.
Essential oils and plant extracts	 Mostly bacteriostatic Antioxidant 	(1) A V S t (2) M C A C A C A C C C C C C C C C C C C C	Antibacterial activity varies with their sources, mostly bacteriostatic; Negative sensory effects on meat or meat products when used at concentrations as antibacterial preservatives.	(1)	Encapsulation or nano- emulsification, active packing in the form of films or coatings; Combination with other hurdle factors.



