Strategies for Obtaining Healthier Foods

José Manual Lorenzo Rodríguez
Francisco Javier Carballo García
Editors

Food Science and Technology

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STRATEGIES FOR OBTAINING HEALTHIER FOODS

JOSÉ MANUEL LORENZO RODRIGUEZ
AND
FRANCISCO JAVIER CARBALLO GARCÍA
EDITORS

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Chapter 5

ANTIBACTERIAL SUBSTANCES AS BIOPRESERVATIVES IN FOODS

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ABSTRACT

Food biopreservation aims at extending shelf-life and ensuring safety of our food supply by means of antimicrobials which are naturally present in food. This chapter describes the current-state-of-the-art of natural antimicrobials of microbial, animal and plant origin that play a current role in food preservation as well as others which are less explored but with promising prospects. Bacteriocins produced by lactic acid bacteria have been widely studied in such a way that classification, mechanisms of action, strategies for food application and their use in different foods have been approached. Another antimicrobial metabolite of bacterial origin is reuterin, produced by Lactobacillus reuteri, which is characterized by its broad inhibitory spectrum. Lysozyme, a protein usually isolated from hen egg-white, has successfully contributed to control the late-blowing in cheeses for decades, but its use as food preservative could decline as it can provoke allergic reactions. An overview of the essential oils from plant origin and their feasibility as food preservatives is also provided. Finally, the lytic enzymes (endolysins) of bacteriophages, the natural enemies of bacteria, are shown as one of developing strategies in food preservation.

Keywords: biopreservation, antimicrobial, bacteriocins, reuterin, lysozyme, essential oils, bacteriophages

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INTRODUCTION

The increasing consumers’ demand for safe food along with their concern regarding synthetic chemical additives have led the scientific community along with the food industry to seek for alternative preservatives with a wide spectrum of antimicrobial activity. In this regard, biopreservation continues gaining interest as means to provide less-processed food while keeping the organoleptic properties and nutritional value without compromising safety. Biopreservation is defined as the use of natural or controlled microbiota or naturally occurring antimicrobials with the purpose of preserving foods and beverages and extending their shelf-life [1]. By using antimicrobials which are naturally present in food, biopreservation is perceived as healthy and environmentally-friendly. Furthermore, it may be applied from “farm-to-fork” and may be combined with other preserving strategies as an additional hurdle to inhibit the growth of undesirable microorganisms and prevent food spoilage. On the other hand, the use of natural antimicrobials has to be implemented in a case-by-case basis as their efficacy may be compromised by food composition.

One of the oldest methods of biopreservation is fermentation based on the growth of some microorganisms that create an unfavorable environment for the development of spoilage and pathogenic microorganisms, while providing foodstuffs with enhanced organoleptic properties [2].

Lactic acid bacteria (LAB) are one of the most widely used microorganisms involved in fermented processes. Indeed, they are used as starters and protective cultures for the manufacture of a great variety of dairy, vegetables and meat fermented products. Their protective ability rely on the production of a number of antimicrobial metabolites (organic acids such as lactic, acetic, and formic acid, bacteriocins, ethanol, H2O2, CO2, diacetyl, reuterin, reuterocyclin, and even other inhibitory substances with antifungal activities [3]. Other natural antimicrobials of animal origin (e.g., lysozyme) and plants (e.g., essential oils) have also found a niche of application as biopreservatives in food. Finally, bacteriophages encoded lytic enzymes (endolysins and virion-associated hydrolases) that are able to lyse bacteria by degrading the bacterial cell wall peptidoglycan when added externally becoming an interesting tool in food biopreservation. In this context, this chapter describes the biochemistry and mode of action of these natural antimicrobials and reviews their current and prospective uses in food.

BACTERIOCINS

Bacteriocins are antimicrobial peptides synthesized by bacteria. As such, they belong to the wide family of antimicrobial peptides which are produced by virtually all forms of life and, despite of their huge structural diversity, they are characterized by their cationic and amphipathic properties that promote interaction with the cytoplasmic membrane of sensitive bacteria. The early use of nisin, a bacteriocin synthesized by Lactococcus lactis, to inhibit the growth of Clostridium tyrobutyricum and prevent late blowing during cheese ripening has fueled research on the isolation and characterization of several bacteriocins to be exploited, mostly as food biopreservatives, but also as processing aids, in animal health and sanitation (Figure 1).
Due to the extensive use of lactic acid bacteria (LAB) in food fermentations, most of the current knowledge on bacteriocins and their use as natural antimicrobials in food has been attained through the study of their bacteriocins. Bacteriocin production is a wide trait among LAB and producers have been isolated from all representative genera including industrially relevant strains from *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus thermophilus* which are commonly used as food starters. LAB bacteriocins are typically small positively charged peptides (30-60 residues), amphipathic and unstructured in aqueous solutions. Due to these properties, they withstand the harsh conditions found during food processing (temperature, salts, pH). Notably, they are not toxic and do not alter the organoleptic properties of food products. LAB bacteriocins have been traditionally classified into two major classes \([4, 5]\). Class I or lantibiotics are post-translationally modified peptides characterized by the presence of lanthionine and β-methyl lanthionine that introduce intramolecular thioether bonds. Nisin and lactacin 3147 are two examples of this class. Class II encompasses non-modified or marginally modified peptides which are further subdivided. Class IIa or pediocin-like bacteriocins are characterized by a conserved N-terminal signature YGNGV and stand out because of their strong anti-*Listeria* activity. Class IIb require the concerted action of two peptides that function as one antimicrobial unit as lactococcin G. Class IIc includes circular bacteriocins with their N- and C-terminus covalently linked (e.g., the enterocin AS-48). Finally, miscellaneous bacteriocins such as lactococcin A, lactococcin
972 and leaderless bacteriocins that do not fit in any of the other subclasses are compiled in class IId. Nonetheless, novel structures and biosynthetic machineries are being discovered by in silico mining of bacterial genomes and new families are proposed, further supporting the high biodiversity among bacteriocins [6]. The biosynthetic machinery of bacteriocins may be located in the chromosome and in mobile genetic elements and genes encoding for the structural peptide and immunity functions are often clustered with others involved in transport and processing as well as with those involved in the dehydration and cyclation reactions in the case of lantibiotics.

The antimicrobial activity of LAB bacteriocins relies mostly on their interaction with the negatively charged bacterial surface and their ability to form pores that destabilizes the selective permeability of the cytoplasmic membrane [7]. However, it is becoming widely recognized that many LAB bacteriocins interact with docking molecules or receptors that facilitate pore formation increasing their antimicrobial potency because, besides pore formation, other synthetic and metabolic pathways are inhibited. This is the case of nisin and other lantibiotics that specifically bind to lipid II, a membrane anchored cell wall precursor. Consequently, cell wall synthesis is inhibited and, together with pore formation, these bacteriocins are able to kill at very low concentrations, i.e., within the nanomolar range [8]. Several class II bacteriocins interact with components of the mannose phospho-transferase system (man-PTS) [9] and an increasing number of bacterial receptors, mostly membrane located, have been shown to be somehow involved in the killing activities of other non-modified bacteriocins such as the undecaprenyl pyrophosphate phosphatase Upp used by lactococcin G, the metalloprotease YvjB by the class IId LsbB, and the maltose ABC transporter by the circular garvicin ML [10, 11, 12] Only a few LAB bacteriocins have been described that do not disrupt membrane permeability. Through binding to lipid II, Lcn972 and likely garvicin A inhibit the formation of the septum between daughter cells during cell division [13]. Resistance to bacteriocins has not become an issue yet likely due to their generalized action on bacterial membranes and the fitness costs associated to resistance. However, bacteriocins that bind to non-essential receptors (e.g., sugar transporters) are prone to easily develop highly resistant mutants. For example, loss of the man-PTS renders Listeria monocytogenes resistant to pediocin-like bacteriocins [14]. Reduced bacteriocin binding by an altered bacterial surface, activation of ABC pumps, orphan immunity proteins and production of specific degrading enzymes are other mechanisms by which otherwise sensitive Gram positive bacteria become resistant to bacteriocins [15]. The external membrane of Gram negative bacteria acts as a physical barrier and, unless its integrity is compromised, Gram negative bacteria are intrinsically resistant to LAB bacteriocins.

The inhibitory spectrum of LAB bacteriocins is usually restricted to Gram positive bacteria closely related to the producer strain which can be regarded as a reflection of the competitive advantage that bacteriocin producers have over others competing for the same niche. However, several LAB bacteriocins such as nisin are able to inhibit relevant pathogenic and food spoilage bacteria and on this basis, many bacteriocins active against L. monocytogenes, Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus and C. tyrobutyricum, among others, have been isolated and selected for food biopreservation.

There are three main strategies to apply LAB bacteriocins in food. They can be added in a (semi-) purified form as an additive and, accordingly, their use in food has to comply with all in-force regulatory issues in each country. So far, nisin is the only authorized bacteriocin worldwide as a food additive. Alternative, more complex and undefined fermentates prepared
from food-compatible substrates (e.g., milk whey) that have been fermented by one or more bacteriocin producers and thermally treated are commercialized in US and Canada [16]. Finally, taking advantage that many LAB are currently used as starter cultures in the food industry, bacteriocin producers have been included in starter formulations and are expected to produce the bacteriocin in situ.

Examples of potential applications of bacteriocins in the food chain have been extensively revised in the literature [17, 18, 19]. Broad spectrum lantibiotics such as nisin and lacticin 3147, the circular bacteriocin AS-48 and the class IIa pediocin PA-1 have been by far the most studied that have been applied in a wide range of food products including dairy, meat, vegetables, cereals, beverages and canned food. On the contrary, narrow spectrum bacteriocins have found their niche of application in food bioprocessing rather than food biopreservation. For example, introduction of a bacteriocin producer as adjunct culture in food fermentations has shown to control the adventitious microbiota in fermented products as well as to promote lysis of starter cells and accelerate cheese ripening [20].

Recent trends of bacteriocin applications in food encompass their use in combination with other antimicrobials and preservation methods, i.e., in hurdle technology. This strategy exploits synergistic (or additive) antimicrobial activities and helps to reduce the severity of the treatments required to ensure safety while maintaining the nutritional value, fresher taste and, importantly, a reduction of the production costs [18, 19]. An elegant example is the combined used of chelating agents or organic acids with bacteriocins to target Gram negative bacteria [21]. Bacteriocins also increase microbial inactivation by nonthermal processes, such as high hydrostatic pressure (HHP), pulsed electric fields (PEFs), ionizing radiation and ultrasonication [22]. Active packaging incorporating bacteriocins into edible films has been explored as well with promising results [23].

There are currently other fields of application of bacteriocins that remain rather underexplored but worth mentioning as they directly or indirectly may contribute to a safer food supply. Preliminary in vitro tests have demonstrated the anti-biofilm activities of some potent bacteriocins such as AS-48 and nisin which may be useful in sanitation of industrial surfaces [24, 25]. Moreover, bacteriocins are putative candidates to decrease the pathogen load in animal production and fresh produce as well as to reduce the use of antibiotics as growth promoters by the use of bacteriocinogenic probiotics [26]. Other primary production systems such as aquaculture may also benefit from the antimicrobial activity of bacteriocins. Araújo et al. [27] recently showcased the active role of nisin produced by Lactococcus cremoris WA2-67 against Lactococcus garvieae infection in rainbow trout. In vivo experiments demonstrated that only the nisin producer exerted protection compared with an isogenic nisin knock-out strain. These and other examples in the literature are encouraging and support the enormous potential that bacteriocins have along the food chain.

**REUTERIN**

Reuterin is a low-molecular mass antimicrobial substance produced by strains of Lactobacillus reuteri from glycerol under anaerobic conditions [28]. Reuterin is also known as HPA (3-hydroxypropionaldehyde) system because the active compound is a mixture of 3-HPA, HPA-hydrate, and HPA-dimer [29]. Its broad inhibitory activity includes Gram positive
(L. monocytogenes, S. aureus, Bacillus subtilis), Gram negative bacteria (Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa), yeasts (Candida, Torulopsis, Saccharomyces), fungi (Aspergillus and Fusarium) and protozoa (Trypanosoma brucei) [30].

The activity of reuterin is displayed in a wide range of pH (2-8) [31].

The mode of action of the HPA system has been described by [32]. According to these authors, the antimicrobial system provokes the depletion of free SH groups in reduced glutathione (GSH) and proteins that results in imbalance of the cellular redox potential, and consequently, in cell death.

Reuterin has been successfully assayed in dairy and meat products against pathogenic Gram positive and Gram negative bacteria. Particularly relevant is the synergistic effect of reuterin combined with nisin and lactoperoxidase against L. monocytogenes and S. aureus in cuajada, a Spanish dairy product [33]. Instead of using reuterin as food additive, the use of producing-strains of L. reuteri as adjunct starter in combination with glycerol (E422) is not affected by legal regulations. Indeed, cheese late blowing during 60 d ripening was prevented by using this approach [34]. In addition, the in situ production did not affect the odour and aroma quality of a semi-hard ewe milk cheese [35]. Reuterin has been also assessed for decontaminating the surface of cooked pork deliberately inoculated with either E. coli O157:H7 or L. monocytogenes. A 24 h exposure resulted in a decrease by 2.7 log CFU/cm² and 0.65 log CFU/cm², respectively [36].

Fernández-Cruz et al. [37] have recently approached a preliminary study of the toxicity of reuterin to support its suitability as food biopreservative. The assays were performed in the human hepatoma cell line HPG2 as the detoxification of a great variety of absorbed compound takes place in the liver. The results indicated a moderate toxicity on this cell line. The potential interaction of reuterin and prescription drugs was discarded by measuring the activity of cytochromes P450 enzymes in human liver microsomes.

LYSOZYME

Lysozyme is an antimicrobial protein mainly detected in hen egg-white and mammalians secretions (milks, saliva, tears). It is particularly effective against Gram positive bacteria by cleaving the β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in the bacterial wall. The hen egg-white lysozyme is one of the natural biopreservatives (E1105) which is commercially available. It has received the GRAS status by FDA (1998) and authorized by EU for use in cheeses. The ability of lysozyme to control the butyric fermentation in cheese was described by Lodi [38] among other authors. According to the Scientific Panel on Dietetic Products, Nutrition and Allergies, a dosage of 25-35 mg/kg of milk is used to avoid cheese late-blowing caused by C. tyrobutyricum that leads to the presence of lysozyme in concentrations ranging from 250 to 400 mg/kg in cheese, depending of the cheese type [39]. Lysozyme is also effective in control of L. monocytogenes growth of seafood (raw minced tuna and salmon products) [40]. With the purpose of reducing the use of sulfur dioxide dosage that can cause health problems in sulfite-sensitive consumers [41], lysozyme has been also incorporated to the winemaking process to control the growth of lactic acid bacteria such as Lactobacillus sp. that can produce unwanted volatile compounds.
during the alcoholic fermentation [42]. Likewise, lysozyme is suitable to prevent the growth of spoilage in unpasteurized beer without negatively affecting its sensory characteristics [43].

Its combination with cation chelating agents (e.g., EDTA) improves its effectiveness against Gram negative bacteria as they destabilize the outer cell membrane [44]. This system has allowed extending the Mozarella cheese shelf life by inhibiting coliforms and *Pseudomonas* sp. during storage [45].

The enhancement of the antibacterial spectrum of lysozyme has been also obtaining by thermo-chemical modification of the enzyme. The modified protein showed a great activity against *Pseudomonas fragi*, *Pseudomonas fluorescens*, *E. coli* and *Proteus mirabilis* [46]. The fusion of a hydrophobic peptide able to permeabilize the outer membrane of Gram negative bacteria to the C-terminus of lysozyme has been another successful approach to improve the activity of lysozyme activity against *E. coli* [47].

The use of lysozyme in food preservation has, however, some drawbacks. In fact, allergic reactions have been reported in the last decade associated to the presence of egg-white lysozyme in foods [48]. This is the reason why its use as additive has to be declared on the ingredient label according to the EC food legislation (2166/2010/EC). To overcome the problem, lysozyme immobilization has been suggested for applications in winemaking as the enzyme could be recovered from the product before being consumed [49]. Alternatively, the use of human lysozyme would prevent the immunological problems to sensitive people because it is less antigenic than egg-white lysozyme [50]. Of note, the amino acid sequence of both proteins shows a 40% of difference. This results in functional differences such as different immunogenicity. An additional advantage of human lysozyme is its greater activity in hydrolyzing the bacterial cell wall in a broader pH range. Therefore, the production of human lysozyme has become a matter of interest for scientists. In this regard, recombinant human lysozyme has been expressed in bacteria, yeasts, animals and plants. The production attempts in *E. coli* and *B. subtilis* failed because the protein was inactive as a consequence of the formation of inappropriate disulfide bonds. However, *Saccharomyces cerevisiae* has been a successful host and up to 74.5 units /mL were obtained [51].

**Essential Oils**

Essential oils (EOs) (cilantro, cinnamon, oregano, rosemary, sage, clove) are obtained from plants (flowers, seeds, roots, bark, wood, leaves, fruits). They show antimicrobial activities against foodborne pathogens and spoilage microorganisms that rely on a wide diversity of organic components with low molecular weight [52]. They usually contain about 20-60 components of which 2-3 show the higher concentrations [53]. Several methods are used for extraction purposes, but the steam distillation is applied in the 93% of the EOs [54]. Other methods such as hydro-distillation used to extract non-water soluble compounds with high boiling point, and solvent extraction in which the solvent is mixed with the plant material and heated. To avoid any chemical alteration of the EOs, the supercritical CO₂ extraction is an alternative but expensive approach [55].

Their mode of action is based on their ability to disrupt the cell wall and the cytoplasmic membrane resulting in leakage of ions and intracellular macromolecules, inhibition of the proton motif force and cellular lysis [52]. Gram positive bacteria are usually more sensitive
than Gram negative to EO components activity due to the protective role of the outer membrane [56]. Most of them have GRAS status but their use as food preservatives is usually limited by their flavoring activity that can negatively affect the food organoleptic properties [57].

According to their chemical structure the active compounds of essential oils are divided in four groups: terpenes, terpenoids, phenylpropenes and “others” [58] (Figure 2).

Terpenes are synthesized in the cytoplasm of cell plants and have a hydrocarbon backbone usually arranged into cycle structures (e.g., \( \text{p-cymene}, \text{limonene}, \text{terpinene}, \text{sabinene}, \text{and pinene} \)). They are classified as mono- and sesquiterpenes according to the number of isoprene units (\( \text{C}_{5}\text{H}_{8} \)) and usually show a low effectiveness as antimicrobials when applied as single compounds [59].

Terpenoids are terpenes in which biochemical modifications occurred by the action of enzymes that add oxygen molecules. Esters (lynalyl acetate), ketones (carvone), aldehydes (citronellal, geranial), phenols (carvacrol, thymol), and alcohols (linalool, geraniol) take part of this group. Terpenoids show activity against a great spectrum of microorganisms, thymol and carvacrol being the most active.

Phenylpropenes show a six aromatic phenol group and a three-carbon propene tail of cinnamic acid. They constitute a relatively small part of essential oils, the most studied being eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde.

Overall, the antimicrobial activity of the essential oil compounds such as terpenoids and phenylpropenes are closely related with the presence of phenolic rings in their structure as this group of compounds shows the highest activity [60]. For instance, the exchange of the hydroxyl group in the phenolic ring of carvacrol by methyl ether affects the hydrophobicity and, consequently, the interaction with the bacterial membrane is negatively affected, resulting in a reduced activity [61]. In addition, other factors such as the culture medium composition, the selected microbial strains as potential targets, the method of extraction, the activity assay technique or the solubility of the essential oils can affect the antimicrobial activity [62]. It should be also noticed that sublethal concentrations of some essential oils provoke notorious changes in the membrane fatty acids profile of some bacterial species such as \( \text{E. coli} \) treated with limonene and cinnamaldehyde, or \( \text{Salmonella} \) in the presence of eugenol and carvacrol. This is suggested to be an adaptive mechanism to stress conditions. By contrast, no substantial changes were observed in \( \text{S. aureus} \) and \( \text{Pseudomonas} \) under similar treatment [63].

Other essential oils contain degradation products derived from terpenes, glycosides, lactones, unsaturated fatty acids and sulfur- and nitrogen-containing compounds [64]. Representative of the latter group are allicin and allyl isothiocyanates, respectively. Allicin (diallyl thiosulfinate) is responsible for the pungent smell of garlic and has antibacterial, antifungal, antiparasitic and antiviral properties. It derived from sulfoxide alliin by the action of the enzyme alliinase once the garlic tissues are damaged [65]. It is transported across the cell membrane and its \(-\text{S(O)}–\text{S}–\) group interacts with the free SH groups of the intracellular enzymes [66]. Isothiocyanates (mustard oils) are found in plants from Brassicaceae family (mustard, broccoli). These compounds are the result of the enzymatic cleavage of released glucosinolates from intracellular compartments of plants. Their antimicrobial activity relies on a general inhibition of enzymes and alteration of proteins by oxidative cleavage of disulfide bonds [67].
It has been observed that higher concentrations of EOs compounds have to be used in food systems to get the same antimicrobial effect than in vitro assays. Thus, the potential additive of synergistic effect of these compounds has been tested. In this regard, the synergistic effect of the combination of carvacrol and eugenol against undesirable microorganisms allows reducing the amount needed to exert antimicrobial effect and, thus, their combined use in food systems would allow to counteract the negative effect on the sensory attributes of food product [68]. Likewise, the synergistic effect of cinnamaldehyde and thymol allows to reduce up to 25% the applied concentration to be effective against *E. coli* [69] and similar result was observed against *Salmonella typhimurium* [70]. The interaction of essential oils or their components with food components such as fats, carbohydrates, proteins can reduce their effectiveness as food preservatives. Thus, before being added to food products, the evaluation of their effectiveness within food model systems must be assayed to determine any potential interaction that could results in a reduction of the antimicrobial activity [71].

Several examples of successful application in foods of EOs have been reported. Lemongrass oil and cinnamon oil inactivated *Salmonella Enteritidis, E. coli,* and *L. innocua* in apple and pear juices in a low concentration (1 µl/mL) [72]. Carvacrol and thymol combined with alkaline electrolyzed solutions of NaCl decreased the aerobic bacterial counts in carp fillets [73]. Cinnamon and clove oils added to skimmed milk were able to inhibit the growth of *Listeria monocytogenes* Scott A [74]. Thyme oil used at 0.6% (v/w) showed a strong inhibitory activity against *L. monocytogenes* Scott A in minced beef meat [75].

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Thymol improved the microbial stability of refrigerated pasta by inhibiting the growth of mesophilic and psychrotrophic bacteria, coliforms, *Staphylococcus* spp., coliforms, moulds and yeasts [76].

To circumvent the potential interactions of EOs with food components or the negative effect on the sensory properties of food due to their intensive aroma, alternative approaches for using EOs in food preservation have been proposed such as their incorporation in active packaging systems. Regarding this, they can be incorporated into polymers of edible and biodegradable coating that allows the slow release to the food surface. For instance, a great reduction of *Campylobacter jejuni* population in chicken breast wrapped observed in apple-based films in which cinnamaldehyde had been incorporated [77]. In addition, the preparation of nanoemulsions enhances the stability and the antimicrobial activity of EOs as well as minimizes the negative effect on food organoleptic properties [78].

**BACTERIOPHAGE-ENCODED LYTIC ENZYMES**

Bacteriophages are viruses that infect exclusively bacteria. Double-stranded bacteriophages encode enzymes (endolysins) intended to degrade the bacterial cell wall peptidoglycan during the last step of their lytic cycle. Generally, endolysins access the periplasmic space through holes in the cytoplasmic membrane that have been previously formed by other proteins named holins. The breakages made in the peptidoglycan destabilize its structure and the final outcome of both proteins is the explosion of host cell and the release of new formed virions (Figure 3A). An important property of endolysins is their ability to digest the peptidoglycan of Gram-positive bacteria when are added exogenously becoming them in antibacterials [79].

In addition to endolysins, some bacteriophages encode other proteins with peptidoglycan hydrolytic activity, virion-associated peptidoglycan hydrolases. These proteins are generally associated to the viral particle, contacting the bacterial surface during phage infection (Figure 3B). Their action provokes the local degradation of peptidoglycan which facilitates the injection of bacteriophage genetic material [80]. The antimicrobial action of these proteins is well known as there are responsible for the “lysis from without” that occurs by the adsorption of a high number of phages to the cell.

At the last years, there is a growing interest in bacteriophage-lytic proteins because of the need of new weapons against antibiotic multiresistant bacteria. In addition, these proteins have a number of properties that made them attractive for several applications including biopreservation of foodstuffs [19]. Most of these properties are directly related with their protein structure, i. e., Gram positive lytic enzymes have a modular structure composed of at least two functional domains (Figure 4A). The N-terminal domain exerts the catalytic activity, which can be divided into five main classes: i) N-acetylmuramidases (lysozymes), ii) endo-b-N-acetylglucosaminidases, and iii) lytic transglycosylases, all cleaving at the sugar backbone moiety of peptidoglycan, iv) endopeptidases, which cleave the peptide moiety, and v) N-acetylMuramoyl-L-alanine amidases, which cut the amide bond between both moieties. The C-terminal domain contains the cell wall binding domain (CBD) involved in the specific recognition of substrate.
The spectrum of activity of most endolysins is restricted to the host bacterial genus which it is infected by the phage that encodes them. The high specificity of endolysins implies a significant advantage for their application since it allows the removing of undesirable bacteria without modifying other components of nearby microbiota [81]. Regarding the modular structure, the presence of different domains facilitates the engineering of these proteins to obtain new chimeric proteins with improved activity and specificity [82, 83, 84].

Recently, endolysins have been engineered to make them active against Gram negative bacteria. Fusion of an outer membrane permeabilizing (OMP)-peptide and an endolysin results in a new protein (Artilysin®) with ability to go through the outer membrane and to target and degrade the peptidoglycan [85] (Figure 4B). Finally, due to the peptidoglycan target backbone of endolysins and virion-associated peptidoglycan hydrolases, it seems unlikely that bacteria can develop resistance to them. In fact, no bacteria resistant to endolysins have been described so far even after repeated exposure to these proteins [86]. The presence of several catalytic domains in these proteins might also contribute to hinder resistance development [87]. Regarding virion-associated peptidoglycan hydrolases, there have similar characteristics to endolysins but their lack of recognizable CBD [88].

To date, the application of bacteriophage-lytic proteins as biopreservatives in food is a limited exploited field. Most of the work published about antimicrobial uses of endolysins has been focused on therapy of infectious diseases [89, 90]. However, there are also data that

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support their effectiveness for improvement of food safety. Indeed, bacteriophage-lytic proteins can be used along the complete food chain, including primary production (agriculture, veterinary), processing (preservation of food, cleaning and disinfection of industry surfaces) and storage [91]. In addition, the high specificity and affinity of CBDS for the bacterial cell wall has taken advantage in developing very sensitive detection systems for *L. monocytogenes* and *Bacillus cereus* [92, 93, 94].

![Figure 4. Structure and activity of A) Endolysins and B) Artilysin.](image)

Direct addition of bacteriophage-lytic proteins to foodstuffs is the most straightforward use of these proteins to inhibit the growth of undesirable bacteria in foods. Thus, endolysin LysH5 was added directly to milk deliberately contaminated with *S. aureus*. After 4 h of incubation at 37°C a total removing of the pathogenic bacteria was achieved [95]. Similarly, chimeric proteins derived from the virion-associated peptidoglycan hydrolase HydH5 have activity against *S. aureus* in milk. Remarkably, CHAP-SH3b is quite stable and effective in raw milk [96]. Other phage lytic proteins with antimicrobial activity in milk are λSA2-E-LysK-SH3b and λSA2-E-LysK-SH3b, two chimeric proteins derived from the fusion of the streptococcal λSA2 endolysin and the CBD from either lysostaphin or LysK endolysin, respectively. The addition of these proteins resulted in a reduction in the bacterial load to up 3 units log in 3 h [97]. Similar results were obtained with the chimeric fusion Ply187-AN-KSH3b derived from f Ply187 and LysK endolysins [98].

Removing of *S. aureus* in dairying was also approached by taking advantage of synergistic interactions between several antimicrobials. Thus, endolysin LysH5 was used in combination with nisin [99] and with other peptidoglycan hydrolases [84] with the aim to reduce the effective dose needed to remove target bacteria.

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Bearing in mind the need to protect some products during fermentation processes, endolysins can be delivered through starter cultures. Consequently, the endolysin LysH5 fused to the signal peptide of the bacteriocin lactococin 972 was expressed and secreted by Lactococcus lactis. The low lytic activity detected in the extracellular fraction of cultures might hinder the inhibition of S. aureus growth [100]. A similar strategy was successfully used to reduce S. aureus during cheese manufacture. [101] engineered Lactobacillus casei BL23 to express and deliver the endolysin Lysdb from L. delbrueckii. Cheese making using this strain, as part of the starter culture, resulted in a significantly reduction in S. aureus contamination (5 log units), which support the use of protective cultures expressing lytic proteins. The endolysin encoded by bacteriophage ΦCTP1 is also active in milk against C. tyrobutyricum and Clostridium sporogenes [102].

Finally, although a lot of work was performed in L. monocytogenes endolysins [103], there are not reports about their use on foods. As exception, the endolysin LysZ5 from phage FWLLm3, was able to kill L. monocytogenes (4 log units reduction) in soya milk even at 4°C incubation [104].

CONCLUSION

A substantial progress has been made by the scientific community to isolate and characterized antimicrobial compounds that are naturally present in food. This effort has resulted in a wide variety of structures encompassing low-molecular weight molecules as essential oils to peptides and enzymes, all of them from microbial, animal or plant origin and with an inhibitory spectrum that comprises food pathogens and food spoilers. However, only lysozyme and nisin along with a few LAB fermentates have been approved by the legal authorities in some countries. This fact may be explained by several factors. On one hand, the complexity of the food matrix involving its physicochemical properties (pH, ionic strength, proteases, viscosity) and structure (liquid, solid) has been shown to compromise the efficacy of these natural antimicrobials as compared to their activity in vitro. Moreover, processing conditions may be incompatible with the use of certain natural antimicrobials as they become inactivated (e.g., phage endolysins) or they have to be used at such high concentrations to ensure activity that the organoleptic properties of the final products are modified (e.g., essential oils). Therefore, further research should aim at developing innovative strategies to implement these antimicrobials in food processing, for example, as enhancers of new emerging preservation technologies such as high pressure treatments or pulse electric fields. Another field that deserves further efforts is the development of high-yield production and purification protocols for antimicrobials intended to be used as food biopreservatives. This will ensure their availability and affordability. Reaching these key goals will definitively pave the way towards a more universal implementation of natural antimicrobials with a long history of safe use in the food industry.
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