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Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins.

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Abstract

The interest in biopreservation of food has prompted the quest for new natural antimicrobial compounds from different origins. Bacteriocins have been widely recognized as natural food biopreservatives but lastest advances on bateriocin biology have opened new fields to explore. On the contrary, the use of bacteriophages and endolysins has only been considered in the last five years and recent developments have produced promising perspectives. This review provides an overview of the current and foreseen applications of bacteriocins, bacteriophages and phage-encoded endolysins along the food chain and highlights research topics to be addressed in the future.

Keywords: Food safey, natural antimicrobials, biopreservation, food chain

Introduction

Food borne diseases are among the most serious and costly public health concerns worldwide, being a major cause of morbidity. In spite of modern technologies, good manufacturing practices, quality control and hygiene and safety concepts such as risk assessment and HACCP, the reported numbers of food-borne illnesses and intoxications still increased over the past decade. The most common food-borne infections in the European Union (EU) are caused by bacteria, namely *Campylobacter*, *Salmonella* and *Listeria*, and viruses. They are reported to affect over 380,000 EU citizens each year (EFSA, 2009).

Food market globalization, the introduction of novel foods, new manufacturing processes and the growing demand for minimally processed, fresh-cut and ready-to-eat products may require a longer and more complex food chain, increasing the risk of microbiological contamination. Thus, novel and complementary food preservation technologies that comply with these demands from “farm to fork” are continuously seeked. Among alternative food preservation technologies, particular attention has been paid to biopreservation to extent the shelf-life and to enhance the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products. Biopreservation rationally exploits the antimicrobial potential of naturally occurring (micro-) organisms in food and/or their metabolites with a long history of safe use. Bacteriocins, bacteriophages and bacteriophage-encoded enzymes fall in this concept. This review will summarize basic knowledge and current applications of these natural antimicrobials along the food chain. Based on this state-of-the-art, future trends and areas of research that deserve more attention will be discussed.

Bacteriocins: structure and mode of action

Bacteriocins are bacterial ribosomally synthesized peptides or proteins with antimicrobial activity. They were primarily described in *E. coli* (colicins). Most of the colicins are relatively large proteins (up to 80 kDa) that kill very closely related bacteria upon binding to the inner membrane or other cytosolic targets (Cascales et al., 2007). Nowadays, the term bacteriocin is mostly used to describe the small, heat-stable cationic peptides synthesised by Gram positive bacteria, namely lactic acid bacteria (LAB), which display a wider spectrum of inhibition (Cotter, Hill, & Ross, 2005). Since LAB have been traditionally associated to food and are regarded as safe, food biopreservation has mostly focused on LAB bacteriocins.

Bacteriocins comprise a very heterogeneous group regarding their primary structure, composition and physico-chemical properties. A "universal" classification of bacteriocins is still a matter of debate. A scheme has been recently proposed by Heng & Tagg (2006) which evolves from previous classification schemes and takes into account the nature of colicins. Thereby, bacteriocins are grouped in four main classes (Table 1).

Class I or lantibiotics include post-translationally modified peptides characterized by the distinctive thioether-based intramolecular rings of lanthionine and β-methyl-lanthionine (Xie & van der Donk, 2004). Class II encompasses heat stable non-modified peptides and is by far the largest class among Gram positive bacteriocins. In general, they are short cationic peptides with high isoelectric points. Of particular relevance for food biopreservation is the potent-antilisteria activity display by the pediocin-like bacteriocins (Class IIa). Class III comprises large heat labile proteins with modest prospects as food biopreservatives. With the exception of colicin V and microcins, Gram negative bacteriocins fall in this class. Finally, circular peptides characterized by a peptide bond between the C- and N-terminus are clustered in class

IV. Examples of bacteriocins whose activity resides on the concerted action of two independent peptides are found in both classes I and II. Most LAB bacteriocins which have been applied in food biopreservation belong to Class Ia, II and IV (Table 1).

As ribosomally synthesised peptides, bacteriocins are encoded by a plasmid- or chromosome-borne structural gene which is often clustered with genes coding for immunity protein(s) and dedicated transport. In particular examples, genes specifying modification enzymes and regulatory genes may also be present.

The mode of action of LAB bacteriocins has been extensively studied although most pioneering work was basically carried out with nisin, the first described Gram positive bacteriocin. Based on their cationic and their hydrophobic nature, most of these peptides act as membrane permeabilizers. Pore formation leads to the total or partial dissipation of the proton motive force, ultimately causing cell death. Bacteriocin pore formation seems to be target-mediated. Nisin and other lantibiotics use the cell wall precursor lipid II as a docking molecule (Breukink, Wiedemann, van Kraaij, Kuipers, Sahl, & de Kruijff, 1999). Thereby, two modes of action, i.e. inhibition of cell wall biosynthesis and pore formation, are combined within one molecule for potent antimicrobial activity (Wiedemann et al., 2001). This strategy is also used by other lantibiotics and non-pore forming bacteriocins such as the non-lantibiotic Lcn972 (Martínez, Böttiger, Schneider, Rodríguez, Sahl, & Wiedemann, 2008a). Recently, several class II bacteriocins were shown to use the membrane-associated component of the mannose-phosphotransferase system as specific receptor in target cells (Diep, Skaugen, Salehian, Holo, & Nes, 2007).

Many LAB bacteriocins are active against many food-borne and spoilage Gram positive microorganisms including antibiotic resistant bacteria. Gram negative bacteria are intrinsically resistant due to the protective role of the external membrane. However,

some can be active in combination with other membrane destabilizing agents (e.g. EDTA).

Current bacteriocin food applications

The traditional role of LAB on food and feed fermentations is the main load-bearing pillar on which the use of bacteriocins in biopreservation relies. LAB and their bacteriocins have been consumed unintentionally for ages, laying down a long history of safe use. Their spectrum of inhibition, bactericidal mode of action, relative tolerance to technologically relevant conditions (pH, NaCl, heat treatments) and the lack of toxicity towards eukaryotic cells further support their role as biopreservatives in food. Since the first use of nisin in the 50´s to inhibit the outgrowth of *Clostridium tyrobutyricum* responsible for late cheese blowing, there have been numerous reports in the literature on the application of many LAB bacteriocins, mostly in food processing. Excellent comprehensive reviews on bacteriocin-based biopreservation technologies are available (Gálvez, Abriouel, López, & Ben, 2007; De Arauz, Jozala, Mazzola, & Vessoni-Penna, 2009; Settanni & Corsetti, 2008). Thus, only a few examples will be cited to give an overview of bacteriocin applications along the food chain (Fig. 1).

Examples of bacteriocin application in the production of primary food commodities are found in veterinary, agriculture and aquaculture. Nisin and lacticin 3147 have been incorporated into commercial prophylactic measures against mastitis. Bacteriocins have also been suggested as an alternative to antibiotic feeding and the use of bacteriocin producers able to colonize the gastrointestinal tract has successfully reduced the carriage of zoonotic pathogens (Calo-Mata, Arlindo, Boehme, de Miguel, Pascoal, & Barros-Velazquez, 2008; Diez-Gonzalez, 2007; Line et al., 2008). In aquaculture, most pathogens are Gram negatives and the colicin-like bacteriocins are

139 those with the best prospects for biocontrol. Bacterial plant pathogens also synthesised
140 bacteriocins able to prevent plant infections (Holtsmark, Eijsink, & Brurberg, 2008).

141 The largest field of investigation has been the application of bacteriocins to
142 inhibit pathogenic and spoilage bacteria during food processing (Fig. 1). The
143 bacteriocins which have been thoroughly examined are the lantibiotics nisin and lacticin
144 3147, several class IIa or pediocin-like bacteriocins and, among the circular peptides,
145 enterocin AS-48 has proven to be very effective against a wide range of spoilage and
146 foodborne pathogens in several foodstuffs including dairy, meat and vegetable products.
147 Bacteriocins may be applied basically in three different formats: i) *in situ* production by
148 starter or protective cultures, ii) as an ingredient (fermentate of a bacteriocinogenic
149 strain), or iii) as an additive in a semi- or purified preparation. *In situ* production is
150 readily cost-effective provided that the bacteriocin producers are technologically
151 suitable. Nisin-producing dairy starters have been designed to specifically inhibit
152 *Staphylococcus aureus* in acid-coagulated cheeses and *C. tyrobutyricum* in semi-hard
153 cheeses (Rilla et al., 2003; 2004). Protective cultures, which do not contribute to the
154 sensory attributes of food, have been mainly applied to enhance the hygienic quality of
155 raw meat and fish products (Devlieghere, Vermeiren & Debevere, 2004). The use of
156 bacteriocins as ingredients or additives requires new strategies for large scale
157 production in suitable low-cost food-grade media. For example, lacticin 3147 and the
158 enterocin AS-48 have been produced in whey-based media suitable as a dairy ingredient
159 (Ananou, Muñoz, Gálvez, Martínez-Bueno, Maqueda & Valdivia, 2008; Morgan,
160 Galvin, Kelly, Ross & Hill, 1999). The use of whey as a substrate is an attractive option
161 because it also contributes to recycle a by-side product of the dairy industry.

162 Besides food biopreservation, bacteriocins have been shown to accelerate cheese
163 ripening by promoting the release of intracellular enzymes to the cheese matrix and a

subsequent increase in the concentration of volatile and other compounds responsible of the sensory attributes of the matured cheese (Martínez-Cuesta, Requena, & Peláez, 2006). Bacteriocins producers were also shown to hold back the adventitious microbiota and guarantee homogenous fermented products (Ryan, Ross & Hill, 2001). Food grade markers based on the bacteriocin immunity proteins offer the possibility to replace antibiotic selective markers for genetic engineering of food-related bacteria (Brede, Lothe, Salehian, Faye & Nes, 2007).

Bacteriophages and their antibacterial life cycle.

Bacteriophages or phages are the most abundant microorganisms on Earth (10^{31} particles) and widely spread including foods of various origins (Brüssow and Kutter, 2005). Bacteriophages are viruses that specifically infect and multiply in bacteria. Thus, they are harmless to humans, animals, and plants. The phages are classified into 13 families based on their shape, size, type of nucleic acid and presence/absence of envelope or lipids in their structure. Most of them belong to the *Caudovirales* order (5360 of 5568 reported to date) with an icosahedral head and a tail and double-stranded DNA (Fig. 2a). According to the morphological features of the tail, they are classified into three families: *Myoviridae* (contractile tail), *Siphoviridae* (long non contractile tail), and *Podoviridae* (extremely short tail). The rest of the phages are cubic, filamentous, or pleomorphic phages with dsDNA, single-stranded DNA, double-stranded RNA, or single-stranded RNA (Ackermann, 2007).

Depending on their life style, phages are divided into virulent and temperate phages (Fig. 2b). Virulent phages strictly follow a lytic cycle whereby they multiply within the bacterial cell to finally lyse the cell to release the phage progeny. By contrast, temperate phages may enter the lysogenic cycle by inserting their DNA into the

bacterial chromosome (prophage) where it replicates as part of the host genome until it may be induced to enter the lytic cycle. Of note, lysogenic bacteria become immune against superinfection with the same or a closely related phage.

Several phases are distinguished in the lytic cycle (Fig. 2b). First, host recognition and adsorption takes place, partly mediated by tail-associated proteins that distinctively recognize specific bacterial receptors. Upon irreversible adsorption, the phage injects the nucleic acid that is transcribed by the host cell RNA polymerase. The phage genome is replicated in multiple copies and the newly synthesised proteins sequester the entire host cell machinery and force it to exclusively produce the structural phage proteins, which assemble into the new virions, and lysis proteins which, ultimately, will lyse the host bacterium.

This last lytic step is precisely where the phage antimicrobial activity resides. In fact, phages have been extensively used in the former Soviet Union to treat human infections. Their results undoubtedly indicate that phages are suitable for clinical treatment or prophylaxis of infectious diseases caused by both Gram positive and Gram negative bacteria (Sulakvelidze & Kutter, 2005; Hanlon, 2007).

Current bacteriophage-based food applications

The concept of combating pathogens in food using phages is recent but several applications along the food chain have already been approached (Fig. 1) and several companies have already begun investing in phage technology (García, Martínez, Obeso & Rodríguez, 2008). Bacteriophages are suitable i) to prevent or reduce colonization and diseases in livestock (phage therapy), ii) to decontaminate carcasses and other raw products, such as fresh fruit and vegetables, and to disinfect equipment and contact

surfaces (phage biosanitation), and iii) to extend the shelf life of perishable manufactured foods as natural preservatives (phage biocontrol).

Phages have been applied to reduce pathogen carriage in livestock farming and also after slaughter or milking. Several studies have been undertaken to treat chickens with phages against *Salmonella* (Fiorentin, Vieira & Barioni, 2005) and *Campylobacter* (Atterbury et al., 2005) and to treat ruminants with phages targeted against pathogenic *E. coli* (Raya et al., 2006). Significant reduction of bacterial load was observed after phage treatment, particularly when applied just before slaughtering. Phages were also active on fresh-cut produce (Leverentz et al., 2003). Another phage-based approach has been to fight bacterial plant diseases as exemplified by the commercially available *AgriPhage* (Intralytix) to combat tomato and pepper spot. In the same line, phage-based biosanitation has been proposed to reduce biofilm formation (Azeredo & Sutherland, 2008) or to eradicate or reduce *S. aureus* nasal or skin colonisation in food handlers (Mann, 2008).

Experimental evidence of the antimicrobial activity of phages during food processing and storage is still scarce but results are encouraging. The host specificity of phages, sometimes restricted to a few strains, pose a burden to their wide use as food biopreservatives. However, it is precisely this feature what makes them very attractive candidates as biopreservatives in fermented products to avoid interference with proper starter performance or the development of the secondary microbiota. The incorporation of phages into milk contaminated with *Salmonella* in cheddar production reduced viable cells after storage (Modi, Hirvi, Hill & Griffiths, 2001). Similarly, *S. aureus* growth in milk and during curd manufacture was inhibited by phages (García et al., 2007; 2009) and inhibition proceeded during ripening, and storage of acid coagulated and semi-hard cheeses (our unpublished results). Complete eradication of *Listeria monocytogenes*

depending on dosage and treatment was achieved on surface ripened cheese by surface application of the virulent phage P100 (Carlton, Noordman, Biswas, de Meester & Loessner, 2005). Other examples of phage-based biopreservation approaches are inhibition of *Enterobacter sakazakii* in reconstituted infant formula milk (Kim, Klumpp & Loessner, 2007) and *Salmonella typhimurium* on chicken frankfurters (Whichard, Sriranganathan & Pierson, 2003).

Recently, two phage cocktails against *L. monocytogenes*, *Listex* (EBI Food Safety, www.ebifoodsafety.com) and *LMP 102* (Intralytics, www.intralytics.com) were approved by the Food and Drug Administration (FDA) in ready-to-eat meat. In 2007, OmniLytics Inc. (www.omnilytics.com) received FDA approval for an anti-*E. coli* and an anti-*Salmonella* phage-based product to treat live animals prior to slaughtering.

Another contribution of phages to food safety is their use in the detection of foodborne pathogens. Phages have long been used for bacterial typing and several phage-based methods have already been developed to detect bacteria in food (Hagens & Loessner, 2007). These methods basically exploit the phage specificity and the efficacy of host recognition.

Endolysins: structure and mode of action

Bacteriophages have developed two basic ways to release the new virions from the infected bacterial cells. In filamentous bacteriophages the progeny is continuously extruded from bacteria cells without killing, whereas non-filamentous bacteriophages destroy the cell wall of the host bacterium by phage-encoded lytic enzymes. Small RNA and DNA phages encode specific proteins that interfere with host enzymes responsible for peptidoglycan biosynthesis. In large DNA phages, endolysins (also termed lysins) are produced during the late phase of gene expression in the lytic cycle and are

responsible of the enzymatic cleavage of peptidoglycan (Young, Wang & Roof, 2000; Loessner, 2005). Endolysins are also capable of degrading the peptidoglycan of Gram positive bacteria when applied externally to the bacterial cell, thereby acting as antibacterial agents.

Most of the endolysins lack secretory signals and their access to the peptidoglycan from inside the cell is dependent on small hydrophobic proteins, termed holins, which enable the endolysin molecules to cross the cytoplasmic membrane and gain access to the cell wall (Wang, Smith & Young, 2000) (Fig. 3a). A few others contain signal peptides recognized by the host general secretion pathway (Sao-Jose, Parreira, Vieira & Santos, 2000).

Depending on the enzymatic specificity, endolysins are 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. Noteworthy, muramidases and amidases that hydrolyze the most conserved bonds in the peptidoglycan seem to be the most widely spread (Fischetti, 2008). Peptidoglycan damage ultimately leads to hypotonic lysis of the host. Some endolysins contain sequences at the C-terminus similar to those typical of cationic antimicrobial peptides that disrupt the bacterial membranes (Düring, Porsch, Mahn, Brinkmann & Gieffers, 1999).

Gram positive endolysins display a modular structure composed of at least two distinct functional domains (Fig. 3b). The N-terminal domain contains the catalytic activity, mostly with one muralytic activity but bifunctional lysins have also been described. At the C- terminus, a cell wall binding domain (CBD) confers some degree

of specificity to the enzyme. Besides, CDBs keep the endolysin bound to its substrate once the host is lysed. In this way, endolysins are not freely released to the environment avoiding the lysis of putative new phage host cells. CDBs are not often found among endolysins from Gram negative phages (Briers et al., 2007).

Most endolysins display a narrow spectrum of lytic activity often restricted to the host bacterial species of the phage from which it is derived although some are genus specific. An exception is an enterococcal phage lysin that not only lyses enterococci but also *Streptococcus pyogenes*, group B streptococci, and *S. aureus*, making it one of the broadest acting lysins identified so far (Yoong, Schuch, Nelson & Fischetti, 2004).

Endolysins in food applications

Most of work that supports the role of endolysins as powerful antimicrobials has been focused on prophylaxis and treatment of bacterial infections in animal models. In regard to food biopreservation, research is still at its infancy. However, the number of endolysins active against numerous zoonotic and food-borne pathogens which are being isolated and characterized is increasing exponentially and future applications are foreseen. Worth mentioning is the fact that to date no resistance to endolysins has been reported even by repeated exposure or by stimulating mutant development. Although it may be still premature to be fully confident, lack of resistance is a clear advantage over other antimicrobial agents.

To date only very few reports have addressed the antimicrobial potential of endolysins *in situ* along the food chain. At the primary production step, protection against the phytopathogen *Erwinia amylovora* was demonstrated in transgenic potatoes synthesising the T4 lysozyme (Düring, Porsch, Fladung & Lörz, 1993) or by surface application of the recombinant phiEa1h endolysin on pears (Kim, Salm & Geider,

2004). Transgenic cows expressing endolysins have also been suggested to reduce mastitis and *S. aureus* milk contamination (Donovan, Lardeo & Foster-Frey, 2006). As a prophylactic measurement, aerosolized PlyC endolysin contributed to eradicate or reduce *Streptococcus equi* load on a variety of materials even in the presence of non ionic detergents, hard water, or organic materials (Hoopes, Stark, Kim, Sussman, Donovan & Nelson, 2009). Likewise, a staphylococcal endolysin has been shown to remove *S. aureus* biofilms (Sass & Bierbaum, 2007).

In food processing, pathogen biocontrol by endolysins has been basically approached in dairy manufacturing. Pioonering work has been carried out with the staphylococcal phage endolysin LysH5 (Obeso, Martínez, Rodríguez & García, 2008). The purified endolysin killed *S. aureus* in pasteurized milk, although higher amounts than those anticipated *in vitro* were needed. Recombinant lactic acid bacteria were able to secrete active *Listeria* endolysin but their antagonistic activity in milk or alternative food matrices has not been assessed (Turner, Waldherr, Loessner & Giffard, 2007).

A very relevant role that endolysins play in food safety is based on the high specificity of their CBDs. These recognition domains have been used to develop rapid and sensitive identification and detection systems (Fujinami, Hirai, Sakai, Yoshino & Yasuda, 2007). Magnetic beads coated with recombinant CBDs enabled immobilization and recovery of more than 90% of *L. monocytogenes* cells from food samples (Kretzer et al., 2007).

Topics for the future

Despite of the vast knowledge generated on bacteriocin and bacteriophage biology and the increasing attention paid to endolysins, there are still several basic and

applied issues that deserve further attention to fully exploit their antimicrobial potential in food safety (Table 2).

Special needs in basic research may be grouped in three main fields: i) resistant mechanisms, ii) new and/or enhanced antimicrobials, and iii) safety concerns which may emerge by the use of these biopreservatives. Development of resistance is a major concern when designing new biopreservation approaches. Adaptation to bacteriocins is easily achieved under laboratory conditions. Besides, little is known about bacteriocin immunity and the chance of genetic transfer. Noteworthy, despite of the extensive use of nisin as a food biopreservative, resistance has not posed a problem yet. Nevertheless, the consequences of adaptation to bacteriocins must be considered when designing combined treatments as cross-resistant phenomena may occur (Martínez, Obeso, Rodríguez & García, 2008b). High-throughput technologies (omics) will help to clarify how cells respond to bacteriocin treatment. Resistance could also threaten bacteriophage-based approaches. However, phage resistance may reduce the fitness or virulence of the bacteria and the use of phages mixtures decrease the probability of resistance. Moreover, phages mutate at frequencies significantly higher than that of bacteria and selection of new phages might easily overcome bacterial resistance. Lysogeny also makes bacteria resistant to superinfection, thereby temperate phages should be avoided.

Current molecular biology techniques and the genetic amenability of bacteriocins, phages and endolysins offer attractive options to develop new antimicrobials. Bacteriocins and endolysins are suitable for DNA shuffling and protein engineering to generate highly potent variants with expanded activity spectrum (Field, Connor, Cotter, Hill & Ross, 2008; Manoharadas, Witte & Bläsi, 2009). Bacteriophages may be also genetically modified to fullfill specific requirements such as an expanded

host range. Moreover, phages encode other proteins or peptides that inhibit the bacterial growth during infection as well as virion-associated peptidoglycan hydrolases responsible for "lysis from without" and enzymes involved on degradation of surface polysaccharides. All of them might be regarded as future antimicrobials.

Considering the use of bacteriocins, bacteriophages and endolysins as food additives, it is important to address the effect of oral administration. The inclusion of bacteriocinogenic strains in probiotic preparations demands a better knowledge of the ecological role that bacteriocins may play in complex ecosystems as the gastrointestinal tract (e.g. outcompeting pathogens) (Corr, Li, Riedel, O'Toole, Hill & Gahan, 2007). The new molecular tools to study the intestinal microbiome will definitively be very useful. More detailed cytotoxic and immunogenicity studies are also needed (Jasniewski, Cailliez-Grimal, Chevalot, Milliere & Revol-Junelles, 2009). So far, no adverse effects of oral administration of phages have been described (Bruttin & Brüsow, 2005). On the contrary, no data are available about endolysins, although no signs of anaphylaxis were observed after both systemic and mucosal administration (Fischetti, 2008). Other safety issues related to the use of phages must be carefully analysed prior to selecting biopreservation candidates. This includes a deep knowledge of the role of phages in DNA exchange and virulence. Phages may carry harmful genes and some may even promote intergeneric transfer (Cheng & Novick, 2009).

From a practical point of view, a major effort is needed to enhance the effectiveness of these biopreservatives in the food matrix as their antimicrobial activity may be extremely disminished *in situ*. Food composition, microbial load, and technological treatments have already been shown to interfere largely with bacteriocin activity (reviewed by Gálvez et al., 2007). It also applies to phages as infection proceeds upon contact with the host, clearly hindered in solid or semi-solid environments such as

food. Many other variables such as adsorption rate, burst size, latent period, initial phage dose, bacterial concentration, etc. are also involved. All these critical parameters may be modelled (Cairns, Timms, Jansen, Connerton & Payne, 2009). However, a deeper insight into the dynamics of phage infection in different food matrices is still missing.

Currently, efforts to improve preservation technologies are mainly focused on hurdle technology. Bacteriocins have been succesfully combined with other hurdles. They have been incorporated into packaging films and combined with modified atmosphere packaging (MAP). Bacteriocins also help to apply less harsh conditions of traditional preservation methods (i.e. less chemical preservatives or lower heat treatments) with the subsequent energy and costs saving. They also act synergistically with emerging preservation technologies such as high hydrostatic pressure and pulsed electric fields showing synergic effects (Gálvez et al., 2007). On the contrary, the use of phages and endolysins in hurdle technology has hardly been explored but results are promising. Phages and endolysins have been successfully combined with nisin and high hydrostatic pressure enhanced endolysin activity by making the peptidoglycan of Gram negatives accessible (Briers et al., 2008). Bacteriophages and endolysins have been proposed as disinfectants in food environments, including food handlers, but delivery strategies have to be implemented.

Within the food chain, there are several unexploited fields in which these natural antimicrobials may be relevant. For example, the use of bacteriocinogenic starter for silage fermentation has hardly been addressed. Moreover, these natural antimicrobials are suitable for organic food production, thereby promoting an environmentally responsible food industry.

411 It is expected that a better and deeper understanding of the molecular basis of the
412 antimicrobial activity of bacteriocins, bacteriophages and endolysins will definitively
413 result in safer food in the near future. Following a knowledge-based approach, new
414 biopreservation strategies as well as unique biotechnological applications of these
415 natural antimicrobials are envisaged.
416
417 **Acknowledgements**
418 The DairySafe group at IPLA-CSIC is financially supported by grants from the
419 Spanish Government BIO2007-65061 and AGL2009-13144-C02-01 and the regional
420 research program FICYT IB08-052.
421

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627

628 **Figure captions**

629

630 **Figure 1.** Proposed bacteriocin, bacteriophages and endolysin applications of their
631 antimicrobial activitiy along three main stages of the food chain (based on published
632 reports).

633 **Figure 2.** Structure of a typical tailed bacteriophage (a) and the steps during the
634 bacteriophage lytic and lysogenic life cycles (b). Temperate phages may follow the
635 lysogenic cycle by integration into the host genome. After infection, or prophage
636 activation, the host is lysed to release the new progeny.

637 **Figure 3.** Schematic representation of the modular structure (a) and mode of action (b)
638 of phage-encoded-endolysins. Most endolysins are characterized by one or two catalytic
639 domains and one cell wall binding domain involved in substrate recognition. Access of
640 the endolysin to the peptidoglycan (PG) layer is often aided by insertion of the holin
641 into the cytoplasmic membrane (CM).

642

643

644

Table 1. Bacteriocin classification according to Heng and Tagg (2006).

Class	General features	Produced by lactic acid bacteria
I-Lantibiotics	Modified, heat stable, <15 kDa	
Ia-Linear	Pore forming, cationic	Nisin, Lacticin 481, Plantaricin C
Ib-Globular	Enzyme inhibitors, no cationic	None
IIc-Multi-component	Two peptides	Lct3147, Plantaricin W
II-Unmodified peptides	Heat stable, <15 kDa	
IIa-Pediocin-like	anti-listeria, YGNGV consensus	Pediocin PA1/AcH, Enterocin A, Sakacin A
IIb-Miscellaneous	Non-pediocin-like	Enterocin B, L50, Carnobacteriocin A
IIc- Multi-component	Two peptides	Lactococcin G, Plantaricin S, Lactacin F
III-Large proteins	Heat labile, >30 kDa	
IIIa-Bacteriolytic	Cell wall degradation	Enterolysin A, Lcn972[a]
IIIb-Non-lytic	Cytosolic targets	Colicins[b] E2-E9
IV-Circular peptides	Heat stable, tail-head peptide bond	AS-48, Gassericin A, Acidocin B

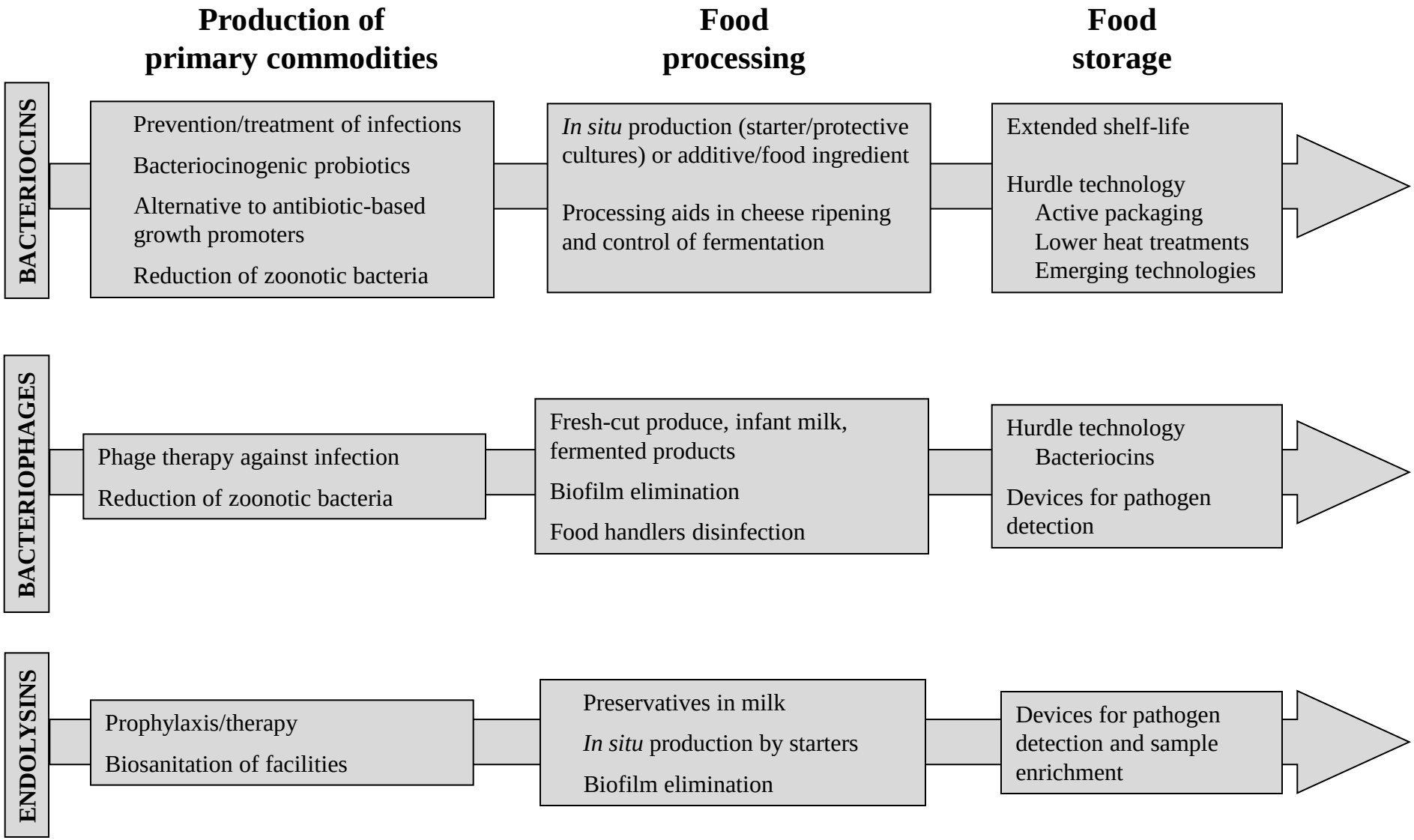
[a] Lcn972 binds to the cell wall precursor lipid II and blocks cell wall biosynthesis, 15 kDa.

[b] Colicins are synthesised by *E. coli*.

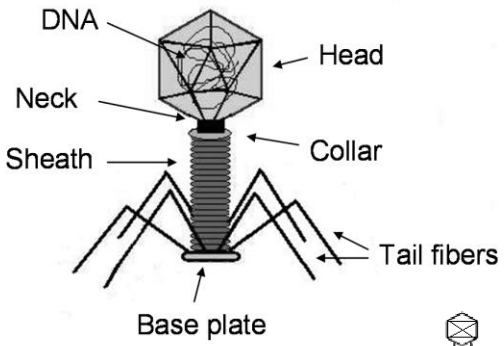
Table 2. Research topics on bacteriocins, bacteriophages and endolysins to be addressed in the future.

Topic	**Specific issues**	
Basic Research	**Bacteriocins**	**Phage/endolysins**
Resistant mechanisms	Transfer of immunity; cross resistance; bacteriocin receptors	Lysogeny, molecular basis of host specificity and frequency of mutations
New antimicrobials	Protein engineering	Unknown phage proteins with antimicrobial activity/domain shuffling
Safety	Effects in complex ecosystems (GT, fermented foods); toxicity	Role of phages in DNA exchange and host virulence; toxicityy
Applied Research	**Bacteriocins/phage/endolysins**	
Food processing	Influence of the food matrix and food processing (modelling) Use in hurdle technology Biofilm removal and Biosanitation	
Unexploited fields	Silage, organic production	

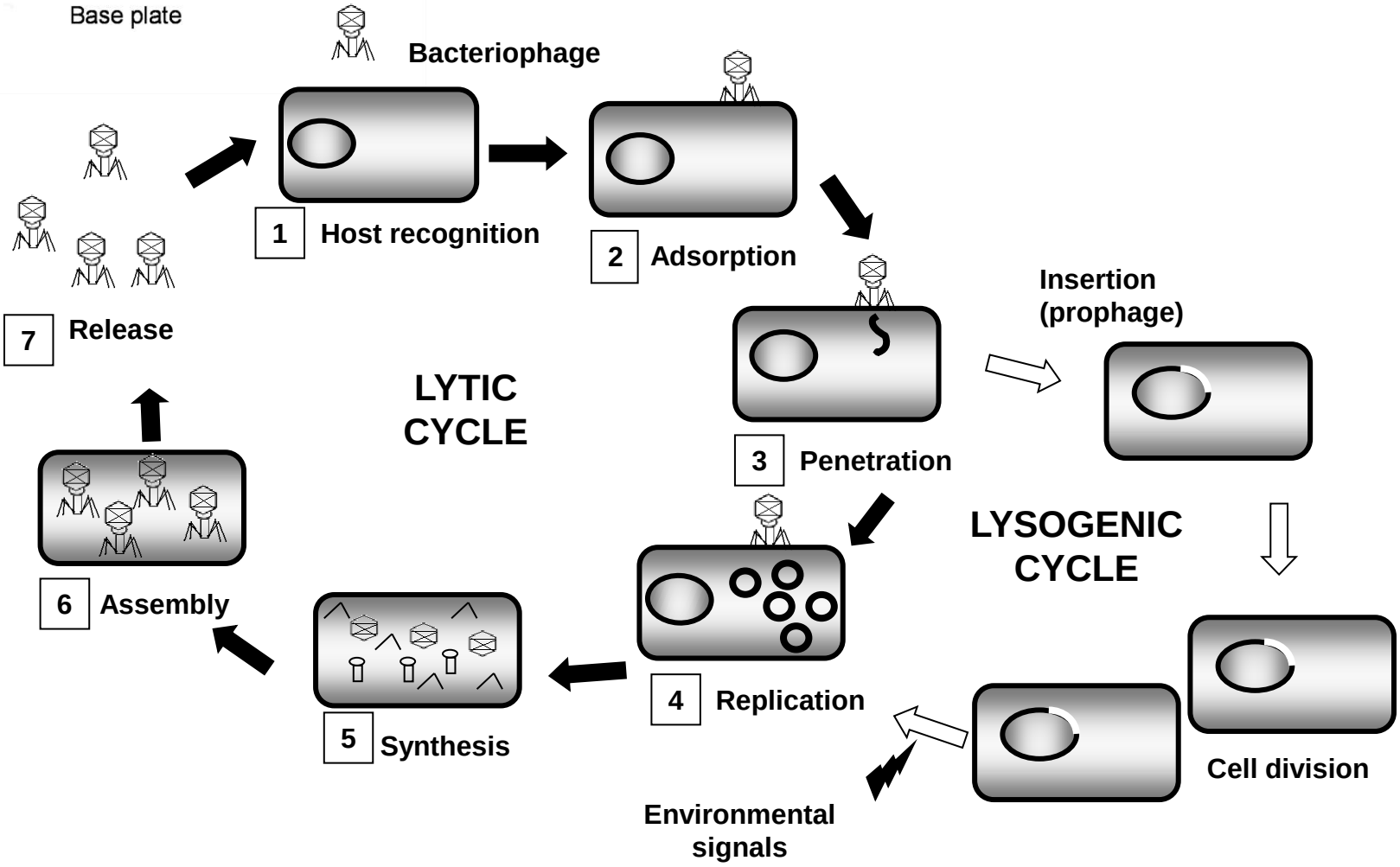
GT, Gastrointestinal tract


Production of primary commodities
Food processing
Food storage
BACTERIOCINS
Prevention/treatment of infections
Bacteriocinogenic probiotics
Alternative to antibiotic-based growth promoters
Reduction of zoonotic bacteria
In situ production (starter/protective cultures) or additive/food ingredient
Processing aids in cheese ripening and control of fermentation
Extended shelf-life
Hurdle technology
Active packaging
Lower heat treatments
Emerging technologies
BACTERIOPHAGES
Phage therapy against infection
Reduction of zoonotic bacteria
Fresh-cut produce, infant milk, fermented products
Biofilm elimination
Food handlers disinfection
Hurdle technology
Bacteriocins
Devices for pathogen detection
ENDOLYSINS
Prophylaxis/therapy
Biosanitation of facilities
Preservatives in milk
In situ production by starters
Biofilm elimination
Devices for pathogen detection and sample enrichment

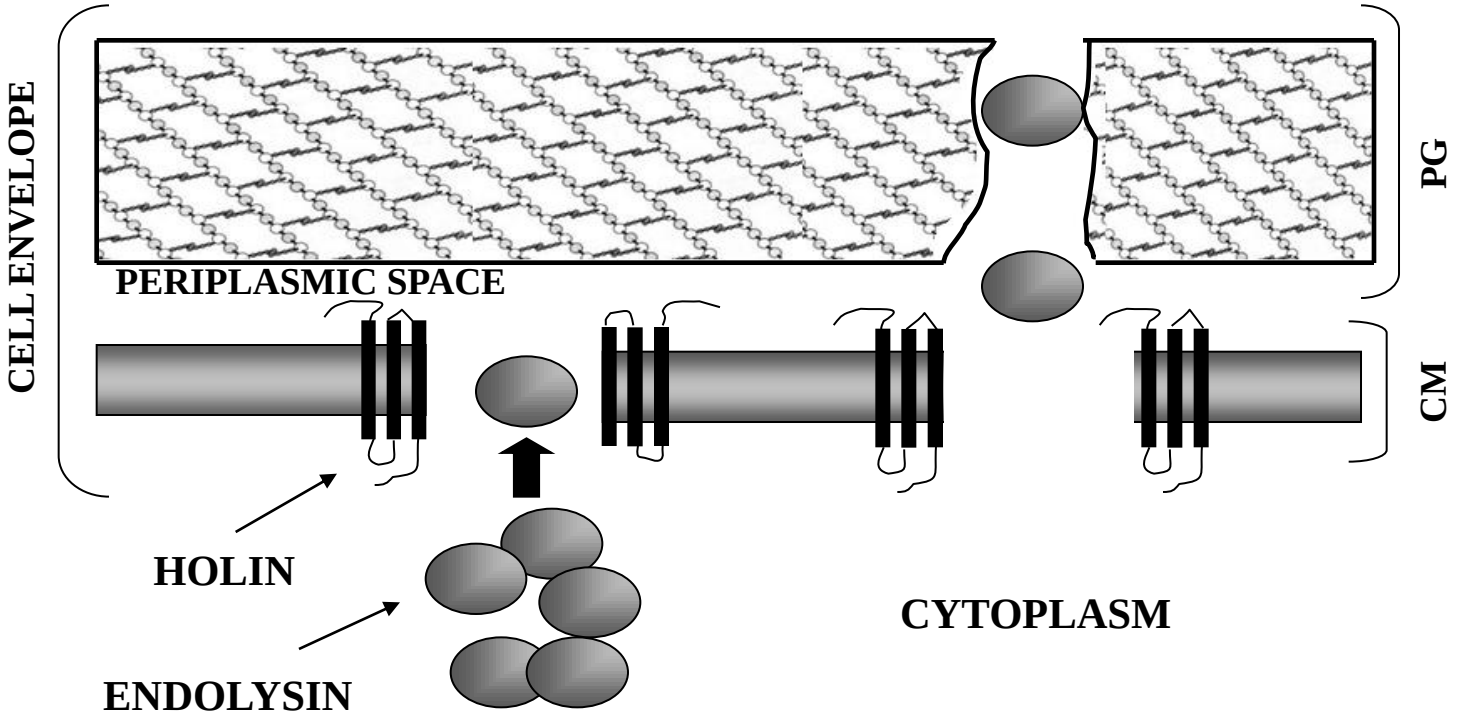
a


DNA
Head
Neck
Collar
Sheath
Tail fibers
Base plate

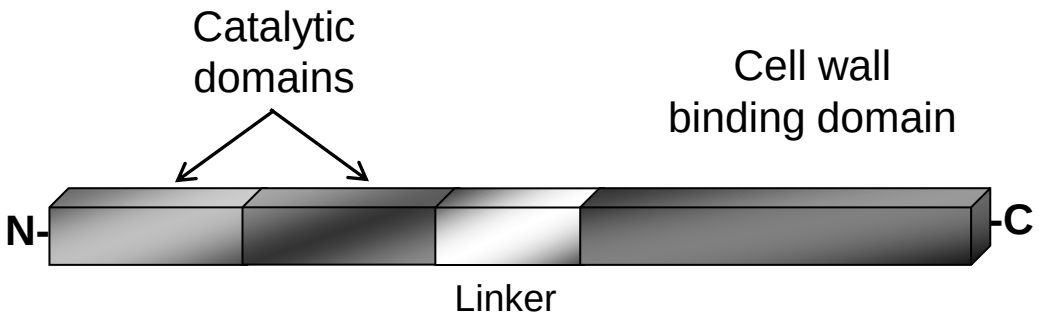
b


Bacteriophage
1 Host recognition
2 Adsorption
3 Penetration
Insertion (prophage)
LYTIC CYCLE
LYSOGENIC CYCLE
4 Replication
5 Synthesis
6 Assembly
7 Release
Cell division
Environmental signals

a


CELL ENVELOPE
PERIPLASMIC SPACE
PG
CM
HOLIN
ENDOLYSIN
CYTOPLASM

b


Catalytic domains
Cell wall binding domain
N-
-C
Linker