

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

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26     **Abstract**

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

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38     **Keywords:** Food safety, natural antimicrobials, biopreservation, food chain

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40           **Introduction**

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

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

63

64 **Bacteriocins: structure and mode of action**

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

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

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

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

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

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

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

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

116

117 **Current bacteriocin food applications**

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

131 Examples of bacteriocin application in the production of primary food  
132 commodities are found in veterinary, agriculture and aquaculture. Nisin and lacticin  
133 3147 have been incorporated into commercial prophylactic measures against mastitis.  
134 Bacteriocins have also been suggested as an alternative to antibiotic feeding and the use  
135 of bacteriocin producers able to colonize the gastrointestinal tract has successfully  
136 reduced the carriage of zoonotic pathogens (Calo-Mata, Arlindo, Boehme, de Miguel,  
137 Pascoal, & Barros-Velazquez, 2008; Diez-Gonzalez, 2007; Line et al., 2008). In  
138 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

164 subsequent increase in the concentration of volatile and other compounds responsible of  
165 the sensory attributes of the matured cheese (Martínez-Cuesta, Requena, & Peláez,  
166 2006). Bacteriocins producers were also shown to hold back the adventitious microbiota  
167 and guarantee homogenous fermented products (Ryan, Ross & Hill, 2001). Food grade  
168 markers based on the bacteriocin immunity proteins offer the possibility to replace  
169 antibiotic selective markers for genetic engineering of food-related bacteria (Brede,  
170 Lothe, Salehian, Faye & Nes, 2007).

171

## 172 **Bacteriophages and their antibacterial life cycle.**

173 Bacteriophages or phages are the most abundant microorganisms on Earth ( $10^{31}$ <sup>31</sup>  
174 particles) and widely spread including foods of various origins (Brüssow and Kutter,  
175 2005). Bacteriophages are viruses that specifically infect and multiply in bacteria. Thus,  
176 they are harmless to humans, animals, and plants. The phages are classified into 13  
177 families based on their shape, size, type of nucleic acid and presence/absence of  
178 envelope or lipids in their structure. Most of them belong to the *Caudovirales* order  
179 (5360 of 5568 reported to date) with an icosahedral head and a tail and double-stranded  
180 DNA (Fig. 2a). According to the morphological features of the tail, they are classified  
181 into three families: *Myoviridae* (contractile tail), *Siphoviridae* (long non contractile tail),  
182 and *Podoviridae* (extremely short tail). The rest of the phages are cubic, filamentous, or  
183 pleomorphic phages with dsDNA, single-stranded DNA, double-stranded RNA, or  
184 single-stranded RNA (Ackermann, 2007).

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

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

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

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

205

## 206 **Current bacteriophage-based food applications**

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

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

215 Phages have been applied to reduce pathogen carriage in livestock farming and  
216 also after slaughter or milking. Several studies have been undertaken to treat chickens  
217 with phages against *Salmonella* (Fiorentin, Vieira & Barioni, 2005) and *Campylobacter*  
218 (Atterbury et al., 2005) and to treat ruminants with phages targeted against pathogenic  
219 *E. coli* (Raya et al., 2006). Significant reduction of bacterial load was observed after  
220 phage treatment, particularly when applied just before slaughtering. Phages were also  
221 active on fresh-cut produce (Leverentz et al., 2003). Another phage-based approach has  
222 been to fight bacterial plant diseases as exemplified by the commercially available  
223 *AgriPhage* (Intralytix) to combat tomato and pepper spot. In the same line, phage-based  
224 biosanitation has been proposed to reduce biofilm formation (Azeredo & Sutherland,  
225 2008) or to eradicate or reduce *S. aureus* nasal or skin colonisation in food handlers  
226 (Mann, 2008).

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

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

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

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

254

## 255 **Endolysins: structure and mode of action**

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

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

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

273       Depending on the enzymatic specificity, endolysins are divided into five main  
274 classes: i) N-acetylmuramidases (lysozymes), ii) endo- $\beta$ -N-acetylglucosaminidases, and  
275 iii) lytic transglycosylases, all cleaving at the sugar backbone moiety of peptidoglycan,  
276 iv) endopeptidases, which cleave the peptide moiety, and v) N-acetylmuramoyl-L-  
277 alanine amidases, which cut the amide bond between both moieties. Noteworthy,  
278 muramidases and amidases that hydrolyze the most conserved bonds in the  
279 peptidoglycan seem to be the most widely spread (Fischetti, 2008). Peptidoglycan  
280 damage ultimately leads to hypotonic lysis of the host. Some endolysins contain  
281 sequences at the C-terminus similar to those typical of cationic antimicrobial peptides  
282 that disrupt the bacterial membranes (Düring, Porsch, Mahn, Brinkmann & Gieffers,  
283 1999).

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

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

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

297

298 **Endolysins in food applications**

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

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

313 2004). Transgenic cows expressing endolysins have also been suggested to reduce  
314 mastitis and *S. aureus* milk contamination (Donovan, Lardeo & Foster-Frey, 2006). As  
315 a prophylactic measurement, aerosolized PlyC endolysin contributed to eradicate or  
316 reduce *Streptococcus equi* load on a variety of materials even in the presence of non  
317 ionic detergents, hard water, or organic materials (Hoopes, Stark, Kim, Sussman,  
318 Donovan & Nelson, 2009). Likewise, a staphylococcal endolysin has been shown to  
319 remove *S. aureus* biofilms (Sass & Bierbaum, 2007).

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

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

333

### 334 **Topics for the future**

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

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

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

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

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

366 Considering the use of bacteriocins, bacteriophages and endolysins as food  
367 additives, it is important to address the effect of oral administration. The inclusion of  
368 bacteriocinogenic strains in probiotic preparations demands a better knowledge of the  
369 ecological role that bacteriocins may play in complex ecosystems as the gastrointestinal  
370 tract (e.g. outcompeting pathogens) (Corr, Li, Riedel, O'Toole, Hill & Gahan, 2007).  
371 The new molecular tools to study the intestinal microbiome will definitively be very  
372 useful. More detailed cytotoxic and immunogenicity studies are also needed  
373 (Jasniewski, Cailliez-Grimal, Chevalot, Milliere & Revol-Junelles, 2009). So far, no  
374 adverse effects of oral administration of phages have been described (Bruttin & Brüsow,  
375 2005). On the contrary, no data are available about endolysins, although no signs of  
376 anaphylaxis were observed after both systemic and mucosal administration (Fischetti,  
377 2008). Other safety issues related to the use of phages must be carefully analysed prior  
378 to selecting biopreservation candidates. This includes a deep knowledge of the role of  
379 phages in DNA exchange and virulence. Phages may carry harmful genes and some  
380 may even promote intergeneric transfer (Cheng & Novick, 2009).

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

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

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

406 Within the food chain, there are several unexploited fields in which these natural  
407 antimicrobials may be relevant. For example, the use of bacteriocinogenic starter for  
408 silage fermentation has hardly been addressed. Moreover, these natural antimicrobials  
409 are suitable for organic food production, thereby promoting an environmentally  
410 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

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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 <sup>a</sup>
IIIb-Non-lytic	Cytosolic targets	Colicins <sup>b</sup> E2-E9
IV-Circular peptides	Heat stable, tail-head peptide bond	AS-48, Gassericin A, Acidocin B

<sup>a</sup> Lcn972 binds to the cell wall precursor lipid II and blocks cell wall biosynthesis, 15 kDa.

<sup>b</sup> Colicins are synthesised by *E. coli*.

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

Topic	Specific issues	
<b>Basic Research</b>	<b>Bacteriocins</b>	<b>Phage/endolysins</b>
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; toxicity
<b>Applied Research</b>	<b>Bacteriocins/phage/endolysins</b>	
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**

- Prevention/treatment of infections
- Bacteriocinogenic probiotics
- Alternative to antibiotic-based growth promoters
- Reduction of zoonotic bacteria

## **Food processing**

- In situ* production (starter/protective cultures) or additive/food ingredient
- Processing aids in cheese ripening and control of fermentation

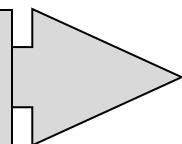
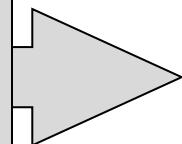
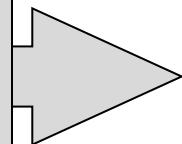
## **Food storage**

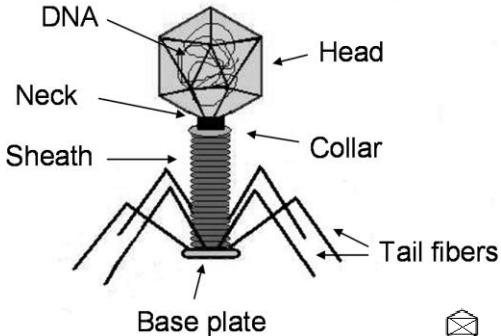
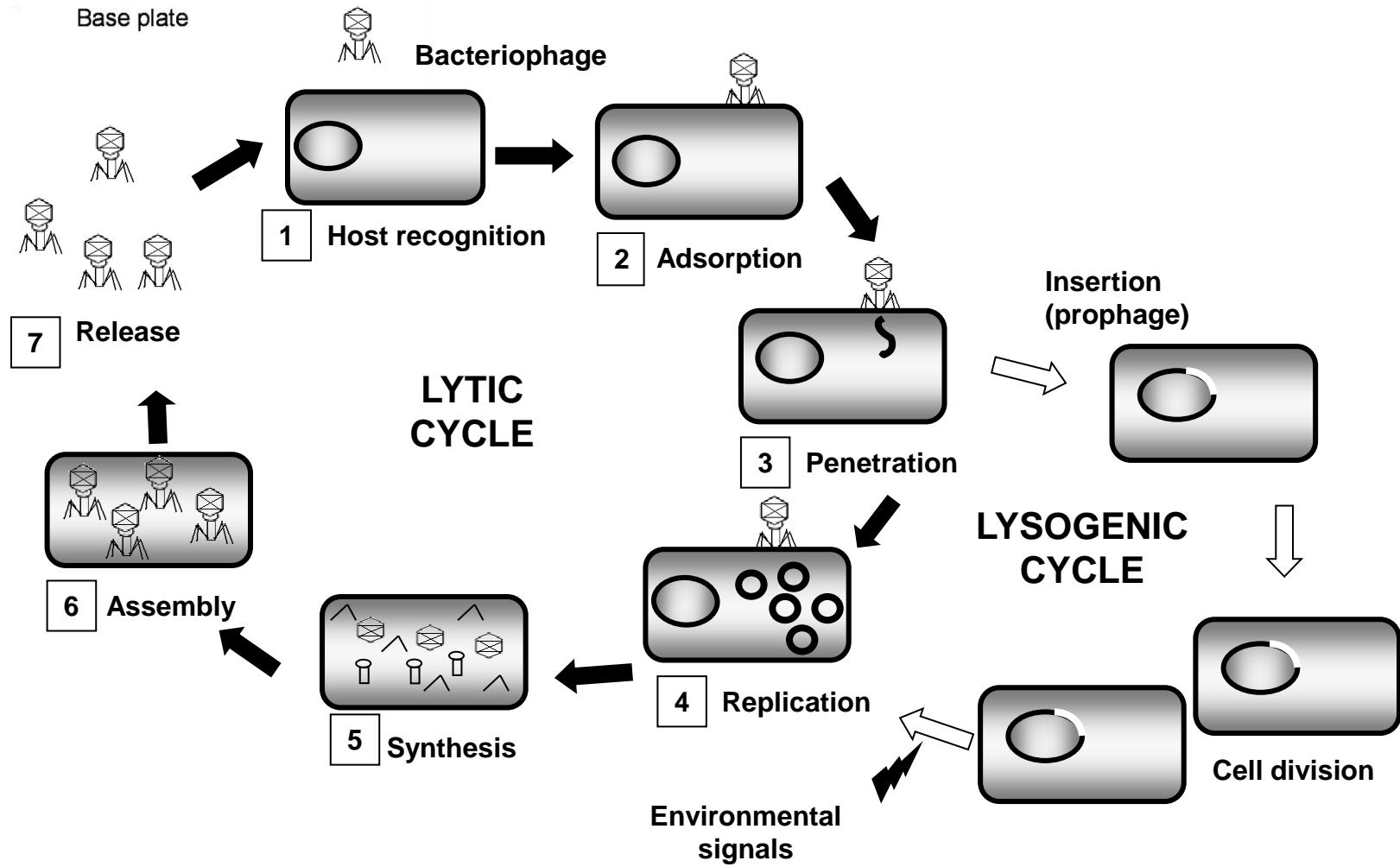
- Extended shelf-life
- Hurdle technology
- Active packaging
- Lower heat treatments
- Emerging technologies

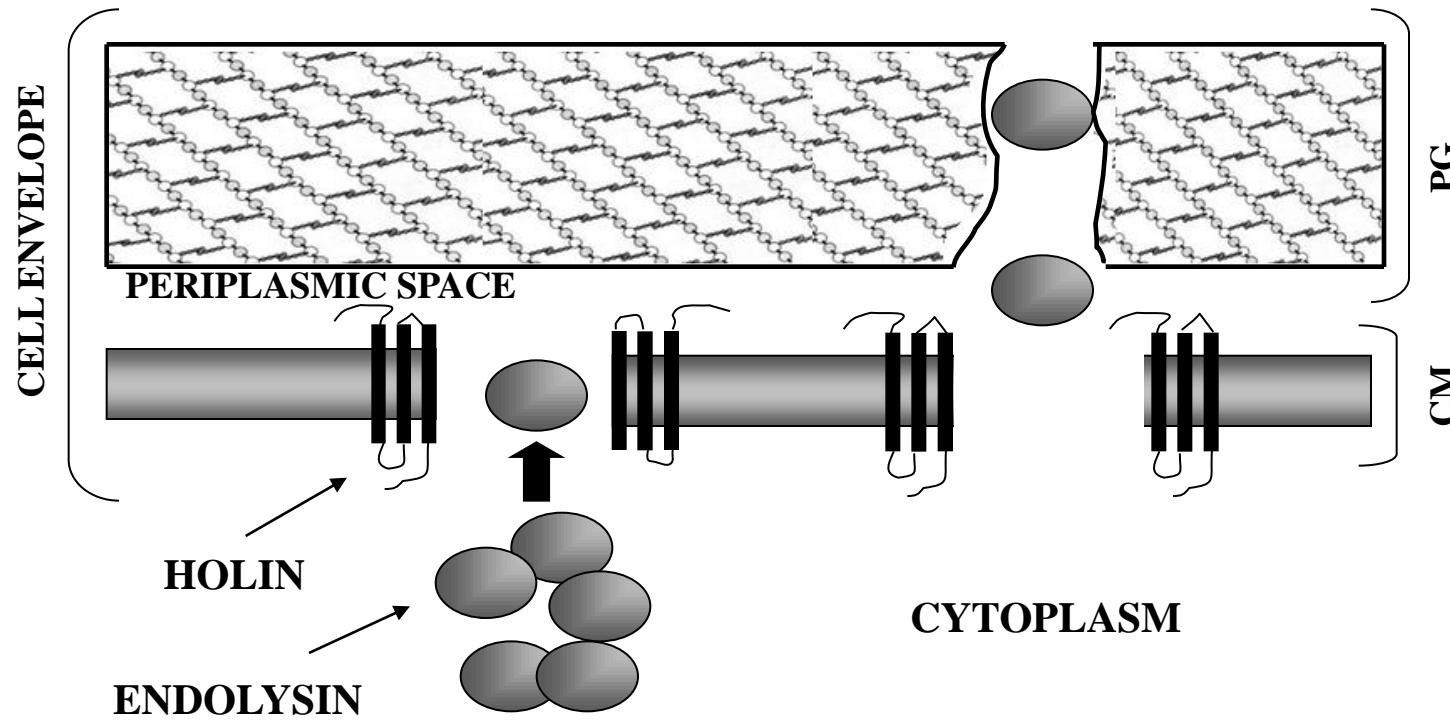
**BACTERIOCINS**

**BACTERIOPHAGES**

**ENDOLYSINS**



**a****b**

**a****b**