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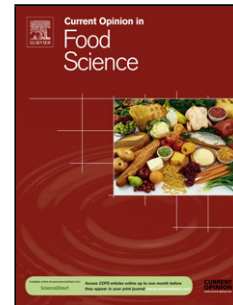
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1     Developing bacteriocins of lactic acid bacteria into  
2                     next generation biopreservatives

3

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7

8     Key Words: Bacteriocin, lactic acid bacteria, nisin, preservative, antimicrobial peptide, food  
9     safety, bioengineering.

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20     Running Title: Developing LAB bacteriocins

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34 **Abstract**

35 Bacteriocins are ribosomally synthesized peptides produced by bacteria which can kill other  
36 bacteria. Those produced by lactic acid bacteria (LAB) are of great interest as they are often  
37 employed in food processing and food fermentations as natural biopreservatives. In this  
38 review, we discuss the implementation of bioengineering to enhance the antimicrobial  
39 activity, antibacterial spectrum and physico-chemical properties of LAB bacteriocins.  
40 Additionally, we discuss the potential applications of bacteriocin derivatives for use as  
41 promising food preservatives alone or in combination with other naturally derived  
42 antimicrobials as a form of hurdle technology and the regulatory status of strains engineered  
43 through food-grade approaches.

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## 58 **Highlights**

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- 60 • Bioengineering can generate novel bacteriocin variants for specific purposes.
- 61
- 62 • Genome mining has identified new bacteriocin biosynthetic gene clusters.
- 63
- 64 • Bioengineered bacteriocins show great promise as synergists in hurdle technology.
- 65
- 66 • Bacteriocin producing strains which have been tailored through food-grade methods  
67 can be directly added to food.
- 68
- 69

## 70 **Introduction**

71

72 The growth in world population and the globalization of food commerce has led to large scale  
73 food production practices requiring ever longer transport networks and extended storage  
74 times until final distribution to consumers. In addition, growing consumer demand for food  
75 products that are minimally processed and free from chemical additives presents a  
76 complicated and difficult challenge for food processors. Such demand has opened up new  
77 opportunities for the use of natural antimicrobials derived from plant, animal or microbial  
78 sources to control the growth of undesirable micro-organisms in food [1,2]. Bacteriocins  
79 (ribosomally-produced, small, heat-stable peptides that are active against other bacteria)  
80 provide one potential solution. While bacteriocins can be produced by a range of Gram-  
81 positive and Gram-negative bacteria [3], those produced by lactic acid bacteria (LAB) are of  
82 particular interest to the food industry for several reasons. Firstly, members of the LAB group  
83 have a history of safe use as starter cultures in food fermentations and many possess  
84 “Generally Regarded as Safe (GRAS)” status according to the US Food and Drug

85 Administration [4]. Secondly, besides being non-toxic to eukaryotic cells, LAB bacteriocins  
86 are extremely potent against many food spoilage microbes and pathogenic bacteria,  
87 demonstrating killing activity in the nanomolar range. Thirdly, they do not interfere with the  
88 sensory quality of foods. Finally, the ribosomal origin of bacteriocins has enabled the  
89 manipulation of the associated structural gene in a more direct fashion than is possible for  
90 other classes of antimicrobials to obtain variants with potentially beneficial properties.  
91 Indeed, several groups have reported on the enhancement of bacteriocins' performance in  
92 food environments, including the engineering of derivatives with enhanced activity and  
93 inhibition spectra, increased resistance to proteolytic enzymes as well as the combination of  
94 such derivatives with other natural antimicrobials in the form of hurdle technology. This  
95 review will focus on recent developments with regard to these achievements and present the  
96 latest innovations which aim to harness the full potential of these highly potent  
97 antimicrobials.

#### 98 **Classification of LAB Bacteriocins**

99 Bacteriocins produced by LAB represent a heterogeneous group of peptides encoded by a  
100 diverse genetic repertoire. Some are post-translationally modified and this aspect, together  
101 with their mode of action, has traditionally been used as a basis for their classification [5].  
102 The simplest scheme comprises two classes; i.e. Class I bacteriocins also known as RiPPs  
103 (Ribosomally Produced and Post-translationally modified Peptides) encompasses all the  
104 peptides that undergo enzymatic modification during their biosynthesis (including  
105 lanthionines, glycosylation and/or heterocycles). Class II do not contain unusual  
106 modifications. However, recent extensive genome mining analysis of LAB suggests that the  
107 repertoire of antimicrobials that are encoded in publicly available sequence databases could  
108 be even more extensive than previously thought, with some putative classes thus far not  
109 reported in LAB (e.g. lasso peptides and sactipeptides) [6]. Furthermore, an *in silico*

110 screening approach of genome-sequenced isolates from the human gastrointestinal tract (GIT)  
111 identified more than 70 clusters of note from almost 60 unique members including  
112 Firmicutes, Bacteroidetes, Actinobacteria, and others [7]. The most commonly identified  
113 class of bacteriocin was the >10 kDa class, formerly known as bacteriolysins, followed by  
114 lantibiotics and sactipeptides [7]. Consequently, a revised scheme (that is also valid for  
115 bacteriocins from non-LAB micro-organisms) proposes three classes in which Class I is  
116 divided into six subclasses representing different modifications, Class II comprises four  
117 subclasses of unmodified peptides of <10kDa and Class III are large-molecular-weight  
118 (>10kDa) proteins and are subdivided into the bacteriolysins and the non-lytic bacteriocins  
119 [6].

#### 120 **Bioengineering to modulate the physicochemical properties of LAB bacteriocins**

121 Despite the amount of research that has been carried out on the discovery, characterization  
122 and mode of action of LAB bacteriocins over the last few decades, to date, just a handful  
123 have been commercialized to any extent. These include nisin, a lantibiotic peptide produced  
124 by *Lactococcus lactis* [8], pediocin PA-1 produced by *Pediococcus acidilactici* [9] and  
125 carnocyclin A produced by *Carnobacterium maltaromaticum* UAL307 [10]. However,  
126 studies with other LAB bacteriocins including enterocin AS-48 [11] or lacticin 3147 [12]  
127 demonstrate their enormous potential as biopreservatives in food. Nisin is used in most major  
128 food-producer countries as a concentrated fermentate powder (e.g. Nisaplin) in a wide variety  
129 of dairy and non-dairy products to control the growth of Gram positive bacteria [13]. A  
130 fermentate powder produced from the pediocin-producing strain *Pediococcus acidilactici*  
131 (ALTA 2351, Kerry Biosciences, Ireland) can be used to protect meat products from *L.*  
132 *monocytogenes* contamination [14]. Carnocyclin A is marketed as Micocin in the US and  
133 Canada and has been developed to inhibit *Listeria monocytogenes* in ready-to-eat meat (RTE)  
134 products [10]. Accordingly, these bacteriocins have been the subject of several

135 bioengineering strategies (For comprehensive reviews see [15-17]) that have sought to  
136 improve bacteriocin efficacy in the food environment. Peptide function can be influenced by  
137 a number of factors including fat content, proteolytic degradation, polar or non-polar food  
138 components, pH (which influences the solubility of the bacteriocin) and sodium chloride  
139 concentrations [18]. For example, the limited activity spectrum of nisin with respect to pH  
140 and its intrinsic insolubility has emphasized the need for alternative versions that exhibit  
141 superior stability and are suitable for food fermentation and preservation practices. The  
142 natural variant nisin Z, which differs from nisin A by one amino acid (asparagine rather than  
143 histidine at position 27) provides an example of a derivative with improved functional  
144 characteristics since, while it has similar antimicrobial activity to nisin A, nisin Z displays a  
145 higher rate of diffusion [19] and is less soluble at low pH [20]. Recently, bioengineered nisin  
146 derivatives were identified with an enhanced ability to diffuse through complex polymers that  
147 enabled the peptides to surpass nisin A in restricting growth of *Listeria monocytogenes* in  
148 commercially produced chocolate milk containing carrageenan as a stabilizer [21]. Despite  
149 the fact that derivatives of the unmodified class II bacteriocins can be generated with relative  
150 ease [22], there are comparatively few examples of instances in which LAB producers of the  
151 class II bacteriocins have been engineered to positive effect. However, in the case of pediocin  
152 PA-1/AcH, greater resistance to oxidation was achieved by the replacement of a methionine  
153 moiety with a hydrophobic one, which had only minor effects on antimicrobial activity [23].  
154 Such modifications are an important step in the development of pediocin PA-1 into an  
155 advantageous food additive. Some studies have aimed to incorporate protease resistance into  
156 LAB-derived peptides. For example, particular modification of trypsin recognition sites in the  
157 class IIb bacteriocin salivaricin P produced by *Lactobacillus salivarius* had only minor  
158 effects on activity [24].

159



160 **Bioengineering to modulate the antimicrobial activity and spectrum of LAB**  
161 **bacteriocins**

162 The range of inhibitory activity by LAB bacteriocins can be either narrow, inhibiting only  
163 those strains that are closely related to the producer organism, or wide, inhibiting a broad  
164 range of Gram-positive micro-organisms [25]. Several investigations have sought to identify  
165 nisin derivatives that are enhanced with respect to the purpose for which nisin is most  
166 renowned, the inhibition of Gram-positive bacteria [15]. One remarkable derivative, nisin A  
167 M21V (Nisin V) (Fig. 1) exhibits enhanced potency against a wide range of targets, most  
168 notably *L. monocytogenes*. Furthermore, this enhanced activity was apparent in food model  
169 experiments with purified peptide [26]. *L. monocytogenes* is of major concern to the food  
170 industry. Apart from the risk to human health, food product recalls due to *Listeria*  
171 contamination present an enormous financial burden, estimated to be in the billions of dollars  
172 per year in the United States [27]. Recently, nisin V in the form of a fermentate, combined  
173 more effectively than nisin A with the essential oils carvacrol, thymol and trans-  
174 cinnamaldehyde to inhibit *L. monocytogenes* in a validated food model system [28].  
175 Worryingly, *L. monocytogenes* has the ability to form biofilms providing it with the means to  
176 survive on contact surfaces, with higher levels of resistance to disinfectants and potential for  
177 growth under the rigorous conditions used for food processing. This could lead to  
178 contamination of food products. A recent study demonstrated the effectiveness of nisin  
179 M21A, in combination with natural food-grade additives, in targeting biofilms of *L.*  
180 *monocytogenes* F6854 [29], a strain that has been associated with contaminated turkey  
181 frankfurters. Similarly, the advantage of shortening or extending the hinge region of nisin has  
182 generated variants with improved bioactivity against one or more indicator targets, including  
183 *L. monocytogenes*, *E. faecalis* and *B. sporothermodurans* [30]. Bioengineering to enhance the  
184 efficacy of Class IIa bacteriocins has also been considered. Mutated peptides in which

185 residues were substituted within the N-terminal half of pediocin PA-1 exhibited increased  
186 activity against *Micrococcus luteus* and *Staphylococcus aureus* [31], while variants within the  
187 C-terminus displayed increased potency towards *L. monocytogenes* [32]. Directed  
188 mutagenesis studies have been employed to manipulate enterocin AS-48, a broad-spectrum  
189 circular bacteriocin produced by a strain of *E. faecalis* [33]. The authors determined that  
190 enterocin AS-48 was active in a dimeric form, and established the essential residues involved  
191 in these interactions [34]. Such knowledge may facilitate the design and expression of novel  
192 variants with improved antimicrobial activity. Notably, the successful chemical synthesis of  
193 circular bacteriocins including enterocin AS-48 has recently been achieved [35], providing a  
194 more suitable means to generate novel variants, the production of which can often be  
195 compromised in the native producer.

196 The poor activity of LAB bacteriocins toward Gram negative bacteria is due to the outer  
197 membrane (OM) of the Gram negative cell wall [36]. The OM functions as an efficient  
198 permeability barrier and is able to exclude macromolecules such as bacteriocins or enzymes.  
199 Importantly, bioengineered nisin variants S29A and S29G have been shown to display  
200 improved activity against Gram negative bacteria [37]. Moreover, while nisin A has been  
201 shown to be effective against Gram negatives when used in combination with chelating  
202 agents such as EDTA [38], perhaps a more attractive option for food applications is the  
203 combination of nisin with natural phytochemical compounds such as essential oils, which act  
204 by permeabilization/disruption of the OM. [39]. Recent investigations have also sought to  
205 extend the antimicrobial spectrum of pediocin-like bacteriocins to include Gram-negative  
206 bacteria. The enterocin CRL35, a pediocin-like bacteriocin, has a potent antilisterial activity  
207 but is inactive against Gram-negative targets. In contrast, microcin V (previously known as  
208 colicin V) is specifically active against Gram-negative bacteria [40]. A hybrid bacteriocin  
209 named Ent35–MccV resulting from the gene fusion of the enterocin CRL35 and microcin V

210 genes (*munA* and *cvaC*, respectively) displayed inhibitory activity against *E. coli*, *L.*  
211 *monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria [41].  
212 Synthetic biology approaches are another promising means to provide insights into structure-  
213 stability relationships and generate novel derivatives with improved function. For example,  
214 analogues of Lacticin 481 containing non-proteinogenic amino acids were found to have  
215 enhanced antibacterial activity [42].

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## 220 **Heterologous expression to increase yield and multiple bacteriocin production**

221

222 One of the issues that remains to be tackled is that of bacteriocin production, which can be  
223 low or inconsistent in lactic acid bacteria and which can be affected by poor growth of  
224 producing strains in particular food environments. Although the heterologous production of  
225 bacteriocins by LAB is reliant on several factors, bioengineering techniques can facilitate  
226 increased levels of bacteriocin production. Some efforts to increase bacteriocin yield have  
227 involved using synthetic genes encoding bacteriocins cloned and expressed in yeasts. For  
228 instance, the use of codon optimization was recently employed to overcome the bottleneck of  
229 low yield of Enterocin A, a class IIa bacteriocin produced by *Enterococcus faecium* CTC492  
230 [43]. Likewise, a recent study involving synthetic biology approaches describes the  
231 development of a genetic system that facilitates significant overproduction of nisin [44]. Such  
232 innovative systems could potentially reduce the cost of bacteriocin production and also

233 provides a means by which sufficient quantities of bacteriocin can be produced *in situ*. In  
234 addition, novel bacteriocin clusters identified through genome mining and considered highly  
235 advantageous to the food industry could be cloned and expressed in suitable hosts. In fact,  
236 bacteriocin producing bioprotective cultures that target particular pathogens are commercially  
237 available under various trade names [45]. Remarkably, naturally occurring multi-bacteriocin  
238 producing LAB have been reported [46]. Consequently, the use of bacteriocin combinations  
239 or bacteriocin ‘loading’ may represent a useful approach whereby an assortment of  
240 bacteriocins produced *in situ* provide an effective cocktail that can act synergistically to  
241 inhibit desired target pathogens. Indeed, some optimistic reports are emerging on the use of  
242 multi-bacteriocin mixtures for the effective control of foodborne pathogens such as *Listeria*  
243 [47,48].

244

#### 245 **Bioengineering and the Regulation of Genetically Modified Micro-organisms**

246 Although the application of bioengineering has been instrumental in the fundamental  
247 analyses of bacteriocin biology, mode of action studies, and the ability to design more potent  
248 peptides with enhanced properties and target selectivity, the application of such peptides as  
249 food preservatives may face a significant regulatory obstacle in some jurisdictions. The  
250 genetic manipulation of bacteriocins or the producer strains needs to pass through strict safety  
251 regulations and guidelines laid down by regulatory agencies such as the FDA (or the  
252 European Food Safety Authority [EFSA] in Europe) for approval to be used in human  
253 consumption. Indeed, some of the strategies employed to bioengineer many of the  
254 bacteriocins described above involve methods that could result in the producer being labelled  
255 as a genetically modified micro-organism (GMM). Alternatively, self-cloning of non-  
256 pathogenic micro-organisms is not considered to lead to a GMM so long as containment of

257 the organism is guaranteed (directive 90/219/EC). Notably, the temporary introduction of  
258 plasmid vectors or the use of recombinant vectors with an extended history of safe use in the  
259 particular micro-organisms, or introduction of DNA from another micro-organism belonging  
260 to the same species fall within the definition of self-cloning. Thus, minimal changes to  
261 bacteriocin structural genes (such as the alteration of single codons) made using food grade  
262 strategies [26,49] fall outside the remit of the EFSA Contained Use legislation and therefore  
263 are not regulated as GMMs.

264

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266

## 267 **Conclusions**

268 A broad range of technologies have emerged in recent years that provide a battery of valuable  
269 tools to expand the potential of bacteriocinogenic strains for food applications. The  
270 knowledge gained will improve our understanding on the global effects of bacteriocins in  
271 food ecosystems and permit more rational approaches for their application in foods. Several  
272 bioengineered bacteriocins capable of inhibiting food-associated Gram positive and Gram-  
273 negative bacteria of concern have been recently described. A number have already been  
274 tested with satisfactory results in terms of the control of pathogenic bacteria in model food  
275 systems. Moreover, their use in combination with other naturally derived antimicrobials in  
276 the form of hurdle technology may open new possibilities for the control of a broad range of  
277 undesirable organisms. Although genetic manipulation by recombinant and bioengineering-  
278 based approaches hold great promise, only bacteriocins which have been tailored through  
279 food-grade methodologies can be directly added to food. Furthermore, as the number of  
280 microbial genome sequences has increased exponentially, an even larger collection of

281 putative bacteriocin biosynthetic gene clusters has been revealed. These clusters can be used  
282 to identify producer strains, or the information gained from their analysis can be used  
283 indirectly to guide the bioengineering of new and existing peptide structures with enhanced  
284 functionality for use in the food industry.

285

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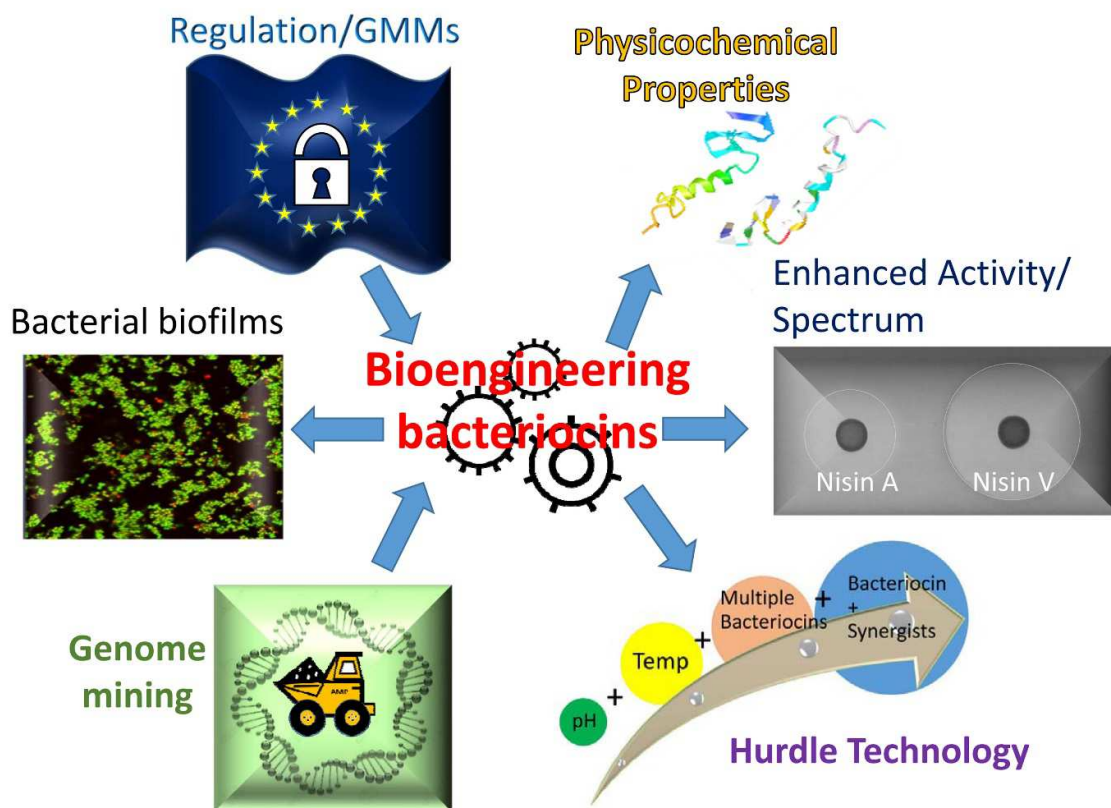
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446 **Figure 1.** Bioengineering bacteriocins: bacteriocins with improved physicochemical  
 447 properties (pH, solubility) have been generated as well as novel variants with improved  
 448 activity, target spectrum and anti-biofilm efficacy. Bioengineered variants can act  
 449 synergistically to inhibit desired target pathogens in the form of hurdle technology. Genome  
 450 mining has identified an even larger collection of new bacteriocin biosynthetic gene clusters  
 451 which can be used to guide the bioengineering of new and existing peptide structures.  
 452 Bioengineered strains which have been tailored through food-grade approaches are not  
 453 considered GMMs and can be directly added to food.

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