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1 **Bacteriocins: Antibiotics in the Age of the Microbiome**

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15 Key words: Bacteriocin, Microbiome, Antibiotic, Probiotic, Antimicrobial Resistance

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23 Running title: Bacteriocins as therapeutic antimicrobials in the context of the

24 microbiome.

Abstract

Antibiotics have revolutionised the treatment of infectious disease and improved the lives of billions of people worldwide over many decades. With the rise in antimicrobial resistance (AMR) and corresponding lack of antibiotic development, we find ourselves in dire need of alternative treatments. Bacteriocins are a class of bacterially produced, ribosomally synthesised, antimicrobial peptides that may be narrow or broad in their spectrums of activity. Animal models have demonstrated the safety and efficacy of bacteriocins in treating a broad range of infections, however, one of the principal drawbacks has been their relatively narrow spectra as compared with small molecule antibiotics. In an era where we are beginning to appreciate the role of the microbiota in human and animal health, the fact that bacteriocins cause much less collateral damage to the host microbiome makes them a highly desirable therapeutic. This review makes a case for the implementation of bacteriocins as therapeutic antimicrobials, either alone or in combination with existing antibiotics to alleviate the AMR crisis and to lessen the impact of antibiotics on the host microbiome.

41 **Abbreviations used:** AAD, Antibiotic Associated Diarrhoea; AMR,

42 Antimicrobial Resistance; AOM, Acute Otitis Media; CDAD, *Clostridium difficile*

43 Associated Disorder; CDC, Centres for Disease Control and Prevention; EcN,

44 *Escherichia coli* Nissle; GI, Gastrointestinal tract; MIC, Minimum Inhibitory

45 Concentration ;VRE, Vancomycin Resistant *Enterococci*.

Introduction

Antimicrobial resistance (AMR) has been recognised as one of the major threats to public health in the 21st century. In a report commissioned by the UK government in 2014, it was estimated that AMR could be responsible for 10 million deaths worldwide by 2050, with a global financial cost of $100 trillion (1). Meanwhile the Centers for Disease Control and Prevention (CDC) estimates the annual cost of AMR in the US to range from $20 billion in direct healthcare costs to $35 billion in additional costs to society due to lost productivity (2). Apart from the human and financial costs associated with AMR, there are also ethical considerations that need to be addressed surrounding how we as a society respond and deal with the AMR crisis (3). There are multiple reasons for the present AMR crisis, but significant factors include the incorrect/indiscriminate administration and use of antibiotics and a dry antibiotic development pipeline (4, 5). The CDC also recently estimated that in the US approximately 50% of antibiotics are incorrectly prescribed. Moreover, the use of antibiotics in agriculture has continued, despite undeniable evidence that this practice adds to the antimicrobial resistance crisis. Resistance to a key "last-resort" antibiotic, colisitin, has been observed in the US, Europe and Asia (6-8). We have also seen the rapid spread of resistance to another "last resort" class of antibiotics, the carbapenems (9). With the emergence of these new resistant strains and the emergence of pan-resistant bacteria, it is safe to say we have truly arrived in the much predicted post-antibiotic era (10).

It is important that we acknowledge that broad-spectrum antibiotic therapy has revolutionised the treatment of infectious diseases within the last century, but we must also admit to unintended consequences of antibiotic use, such as potentially negative

71   effects on the host microbiome and their potential toxicity (5, 11). Although the field

72   of microbiome research is in its infancy relative to that of antibiotic therapy, evidence

73   strongly suggests that the composition of the microbiome can be an indicator of health

74   and is likely to be involved in many aspects of human health and disease (12). Strides

75   in DNA sequencing technology and bioinformatics have increased our understanding

76   of the role of the microbiome in a variety of disease states. Indeed the administration

77   of antibiotics in early life and the subsequent disruption of the microbiota may

78   contribute to risk of obesity in later life (13, 14). Furthermore, when subjected to

79   broad-spectrum antibiotic therapy, non-target commensal microbes may evolve and/or

80   acquire resistance mechanisms to evade the effects of the antibiotic, thereby

81   contributing to the antibiotic resistance crisis.

82

83   Bacteriocins represent a class of powerful antimicrobial peptides that may provide at

84   least part of a solution to the AMR crisis. We aim to demonstrate their efficacy in the

85   treatment of infectious disease and their reduced impact on the host microbiome by

86   comparison to broad-spectrum antibiotic therapy.

87

88   **Bacteriocins: potent antimicrobial peptides**

89   Many excellent reviews have been written about bacteriocins (11, 15, 16) , but in

90   brief they are a diverse group of peptides that may be classified into three distinct

91   groups; class I (modified), class II (unmodified or cyclic) and Class III (>10kDa

92   peptides). Apart from their potent antimicrobial activity (with minimum inhibitory

93   concentrations [MIC's] often in the nanomolar range) they have also been shown to

94   have antiviral (17), anticancer (18) and immunomodulatory properties (19).

95   Bacteriocins typically have a narrow spectrum of activity, but broad-spectrum

96 peptides are also present in this class of antimicrobials (e.g. nisin and lacticin 3147

97 inhibit a wide range of Gram-positive bacteria). As a result these peptides may be

98 suitable for treating infections of unknown aetiology, using broad-spectrum

99 bacteriocins, or may allow more precise targeting of known infectious agents using

100 highly active narrow spectrum bacteriocins. Bacteriocins are gene encoded, which

101 makes them amenable to genetic alterations to improve functional characteristics.

102 Furthermore, their toxicity is low and they may be administered as either purified

103 peptide or produced *in situ* by bacteriocin producing probiotic bacteria (11).

104 Bacteriocins are also known to interact with a variety of receptors, which are different

105 to those targeted by antibiotics, making cross-resistance less likely (20). Although a

106 more targeted approach may still ultimately lead to resistance development in the

107 infectious agent, it does reduce the likelihood of resistance development in

108 commensal populations outside of the target range of the bacteriocin. Resistance

109 mechanisms involving the class II receptors, the mannose phosphotransferase system

110 (Man-PTS), have been identified (21) along with a variety of resistance mechanisms

111 to the class I lantibiotics (22).

112

113 **The microbiota perspective**

114 The term "superorganism" or "holobiont" has commonly been applied to describe the

115 relationship that exists between humans and its commensal microbes and viruses (23).

116 Understanding the role of the microbiota in health and protecting its diversity during

117 the treatment of infectious disease is a key element of why bacteriocins may be

118 suitable as alternatives to antibiotics.

119

120　The two-peptide sactibiotic bacteriocin, Thuricin CD, is a narrow spectrum

121　bacteriocin. Thuricin CD is highly active against one of the main causative agent of

122　antibiotic associated diarrhoea (AAD), *Clostridium difficile*, which is responsible for

123　20-30% of AAD cases (24). Briefly, AAD is caused by a disruption of the microbiota

124　(often referred to as dysbiosis) following broad spectrum antibiotic treatment, and

125　notably has a recurrence rate of 15-60% (25). Thuricin CD was shown to exhibit

126　comparable activity to both vancomycin and metronidazole (two antibiotics used for

127　the treatment of AAD which has progressed to *C. difficile* associated disease, CDAD).

128　Importantly, it showed almost no effect on microbial diversity when compared to both

129　metronidazole and vancomycin in a distal colon model (26). The modified R-Type

130　bacteriocin, Av-CD291.2, has also been shown to prophylactically prevent

131　colonization of *C. difficile* in a mouse model without perturbing the microbiota (27).

132　There are other broad spectrum bacteriocins which are attractive therapeutic agents by

133　virtue of their activity against *C. difficile*, but while the broad spectrum lantibiotic

134　lacticin 3147 is effective at killing *C. difficile*, it has a significant impact on the

135　resident microbiome populations such as *Bifidobacterium*, *Lactobacillus* and

136　*Enterococcus* species (28). It has also been shown that a commercially available

137　product containing the lantibiotic nisin, Nisaplin[®], can eliminate a *C. difficile*

138　infection when added at a concentration of 20X MIC in a simulated human colon

139　model. However a significant decrease in the total microbiota count was observed,

140　with Gram-positives being adversely affected (29).

141

142　Notably, in recent years the emergence of Vancomycin Resistant *Enterococci* (VRE)

143　has become a great concern and therefore raises the issues surrounding the efficacy of

144　treating CDAD with vancomycin if it presents a risk to the general population and the

145 spread of antibiotic resistance. In this light, the treatment of CDAD with bacteriocins

146 could be a valuable alternative to vancomycin. When VRE development has taken

147 place, it has been shown that mice colonised with VRE can be decolonized through

148 the use of an *Enterococcus* probiotic containing a conjugation defective plasmid

149 which produces a bacteriocin named Bac-21 (30).

150

151 A defensin-like bacteriocin, bactofencin A, displays *in vitro* activity against *L.*

152 *monocytogenes* and *S. aureus* (31, 32). Although one might expect this medium to

153 broad spectrum antimicrobial peptide to cause drastic changes in the host

154 microbiome, this was in fact not the case and it was observed that the bactofencin

155 peptide only subtly modulated an *ex vivo* host microbiome (distal colon model) when

156 introduced as a bacteriocin producing probiotic or purified peptide. While the purified

157 peptide resulted in higher levels of beneficial microbes such as *Bifidobacterium*, it

158 was also associated with lower levels of *Clostridium*, which has been linked to

159 obesity and gut pathogenesis. Interestingly, although bactofencin does not show

160 inhibitory activity *in vitro* against strains from the genera *Clostridium*, *Fusobacterium*

161 and *Bacteroides*, the reduction of these populations in the bactofencin treated faecal

162 samples indicates that the consequence of bactofencin altering the overall microbiota

163 structure impacts, directly or indirectly, on these normally insensitive populations

164 when in the gut environment (33).

165

166 It has also been shown, using bacteriocin producing probiotic strains and their

167 isogenic mutants, that the production of bacteriocins can aid the colonisation of a

168 murine host (34). Sequencing data revealed that although bacteriocin production by

169 the probiotics did not affect bacterial diversity at the phylum level, broad spectrum

170 bacteriocins (enterocins and garvicin ML) had a more significant impact on the
171 genus/family diversity of the host microbiome than narrow spectrum bacteriocins
172 (sakacin A, plantaricins and pediocin PA-1).

173

Bacteriocins in animal models

175 Bacteriocins have been shown to be effective in the treatment of variety of infectious
176 bacteria using two delivery methods, either as purified peptides (Table 1) or when
177 delivered *in situ* by probiotics.

178

179 It has been hypothesized that there are three mechanisms by which bacteriocins
180 mediate their producers probiotic properties (42); (i) **Competitive Inhibition:**
181 bacteriocins may support colonization of the host through competitive inhibition of
182 the autologous microbiota, (ii) **Pathogen Inhibition:** bacteriocins may interact
183 directly on a pathogenic target, or (iii) **Immunomodulation:** bacteriocins may act as
184 signalling peptides, recruiting other bacteria or recruiting immune cells to the site of
185 infection to aid elimination of the pathogen **(Figure 1).**

186

187 *Preventing infection*

188

189 The concept of oral replacement therapy is another interesting example of
190 prophylactic probiotic therapy, which has been investigated using the mutacin 1140
191 producing *streptococcus mutans* BCS3-L1. This bacteriocin producing strain is
192 suitable for replacement therapy as it has reduced cariogenic potential because it does
193 not produce lactic acid, mediated through the removal of its entire lactic acid
194 dehydrogenase operon (43). Another interesting probiotic that has shown promise in

195 the limitation of dental caries, plaque accumulation and acidification is *Streptococcus*

196 *salivarius* M18. This strain has 3 plasmid and 1 chromosomally encoded bacteriocins,

197 which is perhaps why it can colonise the oral cavity so effectively. It also produces

198 two enzymes, urease and dextranase, which reduce saliva acidity and counteract

199 plaque formation (44). In a clinical trial, both the safety and efficacy of this strain's

200 probiotic potential was demonstrated, and it was shown to significantly reduce plaque

201 formation in subjects who received the probiotic, over those who received the placebo

202 (45). Furthermore, the treatment of children who have a high risk of dental caries

203 development, with an oral formulation of the *S. salivarius* M18 probiotic (Carioblis[®])

204 was shown to reduce the likelihood of new dental caries development (46).

205

206 It has been demonstrated that dosing mice orally with the bacteriocin producer *Lb.*

207 *salivarius* UCC118 three days prior to infection with *L. monocytogenes* resulted in a

208 significant reduction in subsequent infection by *L. monocytogenes* (47). Nisin Z and

209 pediocin AcH have also been shown to reduce and prevent the colonisation of a

210 mouse model with vancomycin resistant *Enterococci* (VRE), where the

211 bacteriocinogenic probiotic was administered 8 days prior to infection (48). It has also

212 been demonstrated using a porcine model that *Salmonella enterica* serovar

213 *typhimurium* shedding is reduced and disease symptoms of infection are alleviated

214 when a mix of five probiotic strains was administered 6 days before infection (49).

215 One of the probiotics, *L. salivarius*, produces salivaricin P, which can kill the other 4

216 strains in the probiotic mix. Interestingly, this bacteriocinogenic strain dominated in

217 the ileum (the primary attachment site of the infecting *Salmonella*) whereas it was

218 only detected as a minor component in the faeces of the same animals. This suggests

219 that bacteriocin production may play a role where colonisation can occur along the

220   gastrointestinal (GI) tract (50). The concept of using prophylactic probiotics to

221   competitively colonise a pathogens niche could be an effective strategy in agriculture

222   to reduce antibiotic usage. If, as expected, regulations limiting the use of antibiotics in

223   agriculture come into force, probiotics may be invaluable alternative.

224

225   Acute otitis media (AOM) is a type of inflammatory disease of the middle ear,

226   characterised typically by rapid inflammation, potential tympanic membrane

227   perforation, along with fullness and erythema. It has been reported that the levels of

228   normal α-haemolytic *Streptococcus* colonising the nasopharynx of otitis prone

229   children is much lower than in healthy individuals and that recolonization can

230   significantly reduce the episodes of AOM (51, 52). It has been demonstrated treating

231   otitis prone children with a history of AOM, with a nasal spray containing safe

232   *Streptococcus salivarius* 24SMB (a strain which produces a bacteriocin-like

233   substance), reduces the incidences of AOM over the placebo treated group (53).

234

235   ***Treating infection***

236

237   *Helicobacter pylori* infection and colonisation results in a variety of disease states and

238   may even lead to the development of gastric carcinoma. More recently, the prevalence

239   of antibiotic resistant *H. pylori* has been increasing, creating a need for a new

240   therapeutic agent (54). It has been shown in mice that eradication of *H. pylori* was

241   achieved using a bacteriocinogenic probiotic treatment of *P. acidilactici* BA28 (55).

242   Using a mixture of cranberry juice and the bacteriocin producing probiotic culture

243   *Lactobacillus johnsonii* str. La1 supernatant, the carriage of *H. pylori* was also

244   reduced in children after three weeks of treatment (56).

245

246   One barrier to the use of probiotics as a therapeutic is their ability to survive and

247   colonise the area of infection. It has been shown that *Pediococcus acidilactici* UL5

248   and *Lactococcus lactis* ATCC 11454 can produce the bacteriocins pediocin PA-1 and

249   nisin, respectively, *in situ* under simulated upper gastric conditions (57). Interestingly,

250   the *in vitro* activity of a bacteriocin does not always correspond to the *in vivo* activity,

251   where the bacteriocin is sometimes more or less active in an animal model, as is the

252   case with mersacidin which is more active *in vivo* than *in vitro* (58).

253

254   **Bacteriocins against Gram-negatives**

255   Comparatively speaking, Gram-negative bacteria are relatively insensitive to

256   bacteriocins compared to their Gram-positive counterparts, largely owing to the outer

257   membrane which acts as a physical barrier. Until recently the treatment of Gram-

258   negative infections with bacteriocins has not been favoured due to the efficacy of

259   conventional antibiotics in the treatment of these infections. The rise of antibiotic

260   resistant Gram-negative bacteria to the last line of antibiotics (6) means the treatment

261   of Gram-negative infections using bacteriocins can no longer be ignored .

262

263   Widespread use requires a solution to the relative insensitivity of Gram-negative

264   microorganisms. One possibility is to use bacteriocins in combination with other

265   antimicrobial agents, including conventional antibiotics. Although conventional

266   antibiotics will have an impact on the host microbiota (as previously discussed),

267   certain bacteriocin/antibiotic combinations can be synergistic (59-62) and therefore

268   lead to a reduced dose of both antimicrobial agents needed to treat an infection,

269   thereby lowering the potential effect on the host microbiome, the cytotoxic effects on

270   the host and may potentially reduce the development of resistance.

271

272   Success of antibiotics is also hindered by Gram-negative bacteria residing within

273   biofilms, where they are highly resistant to antibiotic treatments. Bacteriocin/

274   antibiotic combinations have shown great promise in overcoming biofilm mediated

275   resistance for important Gram-negative pathogens such as *Pseudomonas aeruginosa*

276   (63) and *Escherichia coli* (64).

277

278   Although this review mainly focuses on Gram-positive bacteriocins, it is important to

279   also to identify Gram-negative bacteriocins which may have potential therapeutic

280   significance. Microcins are ribosomally-synthesized peptides commonly produced by

281   Gram-negative bacteria which are active against Gram-negative strains, and are an

282   interesting alternative to Gram-positive bacteriocins. They have been shown to

283   display potent antimicrobial activity *in vitro* (65, 66) and more recently also *in vivo*

284   (67). It has been demonstrated that the microcin producer *E. coli* Nissle 1917 (EcN)

285   can prevent colonisation of competing *Enterobacteriaceae* in the gut, whilst still

286   having a minimal impact on the diversity of the gut microbiota. However, EcN

287   microcins exhibit their mechanism of action by targeting specific siderophore

288   receptors on other *Enterobacteriaceae* which are only displayed during iron

289   starvation, making their spectrum of activity quite narrow. Additionally to its

290   prophylactic applications, EcN has also been demonstrated to reduce inflammation

291   and weight loss associated with *Salmonella* infections. Another microcin produced by

292   *E. coli* G3/10, microcin S, has been shown to inhibit other *E. coli* strains and

293 furthermore can prevent the adherence of Enteropathogenic *E. coli* (EPEC) to
294 intestinal epithelial cells (68).

295

296 **Overcoming the limitations/outlook**

297 In previous decades significant emphasis was placed on functional characteristics of
298 bacteriocins, such as spectrum of activity, pH and temperature stability, which were
299 essential for the use of bacteriocins in food applications. For their use as therapeutics
300 additional characteristics such as proteolytic resistance, stability and solubility of
301 bacteriocins will also be important.

302

303 With advancements in the field of bioengineering many intrinsic limitations have
304 been overcome, and it has been shown using the prototypic lantibiotic, nisin, that
305 bioengineering strategies can improve functional qualities such as antimicrobial
306 activity (69-72), solubility (73, 74) diffusion properties (75) and effectiveness against
307 Gram-negative bacteria (71). Indeed, similar bioengineering strategies could be
308 applied to other bacteriocins once suitable expression systems have been developed.
309 Although the sensitivity of bacteriocins to proteolytic cleavage was previously
310 regarded as a desirable trait when using these peptides as food preservatives, it does
311 represent a major concern with regard to their administration and widespread use,
312 both orally and intravenously. Bioengineering strategies could be once again used to
313 manipulate peptide residues so they are no longer recognisable by host proteases and
314 therefore are not proteolytically cleaved, thereby improving peptide functional
315 qualities (76). Notably, the therapeutic application of the prototypic bacteriocin, nisin,
316 has been in part hampered by its sensitivity to host proteases (77. Other approaches
317 include prospecting for bacteriocins that display innate resistance to proteases, as was

318 achieved with pseudomycoicidin (78), which is naturally resistant to trypsin due to the

319 presence of a thioether ring structure. The field of bioinformatics and the use of such

320 programs as BAGEL 3.0 (79) and antiSMASH (80) could be a fundamental aspect of

321 this prospecting, as these bacteriocin amino acid prediction tools from genome

322 sequences may also allow researchers to identify protease resistant peptides before

323 investing large amounts of time and effort in characterising such bacteriocins. Finally,

324 understanding bacteriocin pharmacodynamics and pharmacokinetics are also essential

325 to their safe implementation as therapeutics, which has been under investigated in

326 comparison to other aspects of bacteriocin research. If bacteriocins are indeed to

327 become an alternative to conventional antibiotics, a greater emphasis must be placed

328 on research surrounding these host-drug interactions, such as was achieved with

329 MU1140 (81). Addressing these limitations of bacteriocin research to date could

330 provide a turning point for the flagging interest of the pharmaceutical industry and

331 make bacteriocins an attractive therapeutic alternative to current antibiotics (10).

332

333 Although there is considerable evidence that narrow spectrum bacteriocins have a

334 minimal effect on the host microbiome by comparison to current broad-spectrum

335 antibiotics, it should also be recognised that more work in this regard is needed to

336 strengthen the argument for the use of bacteriocins as antibiotics, along with

337 overcoming the previously outlined limitations. Ultimately, we believe given the safe

338 history of use of bacteriocins in food and the large body of literature surrounding this

339 field, that they are useful candidates for antimicrobial therapeutics as the AMR crisis

340 continues to worsen.

Summary

- Antimicrobial resistance (AMR) is a major threat to public health requiring immediate attention.

- Bacteriocins are potent antimicrobial peptides, active in the nanomolar range and have a reduced impact on the host microbiota.

- Bacteriocins may be used to treat a broad range of infections and can be delivered as purified peptides or as bacteriocinogenic probiotics.

- Combining antibiotics and bacteriocins is a strategy to reduce the negative impacts on the host microbiota and also alleviate the AMR crisis.

- Overcoming the current limitations of bacteriocin-based therapeutics should be a key goal of bacteriocin research in the future.

Declarations of interest

The authors declare that the research was conducted in the absence of any commercial

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361   **Author Contribution statement**

362   KE drafted the manuscript. RR and CH revised and approved the final manuscript.

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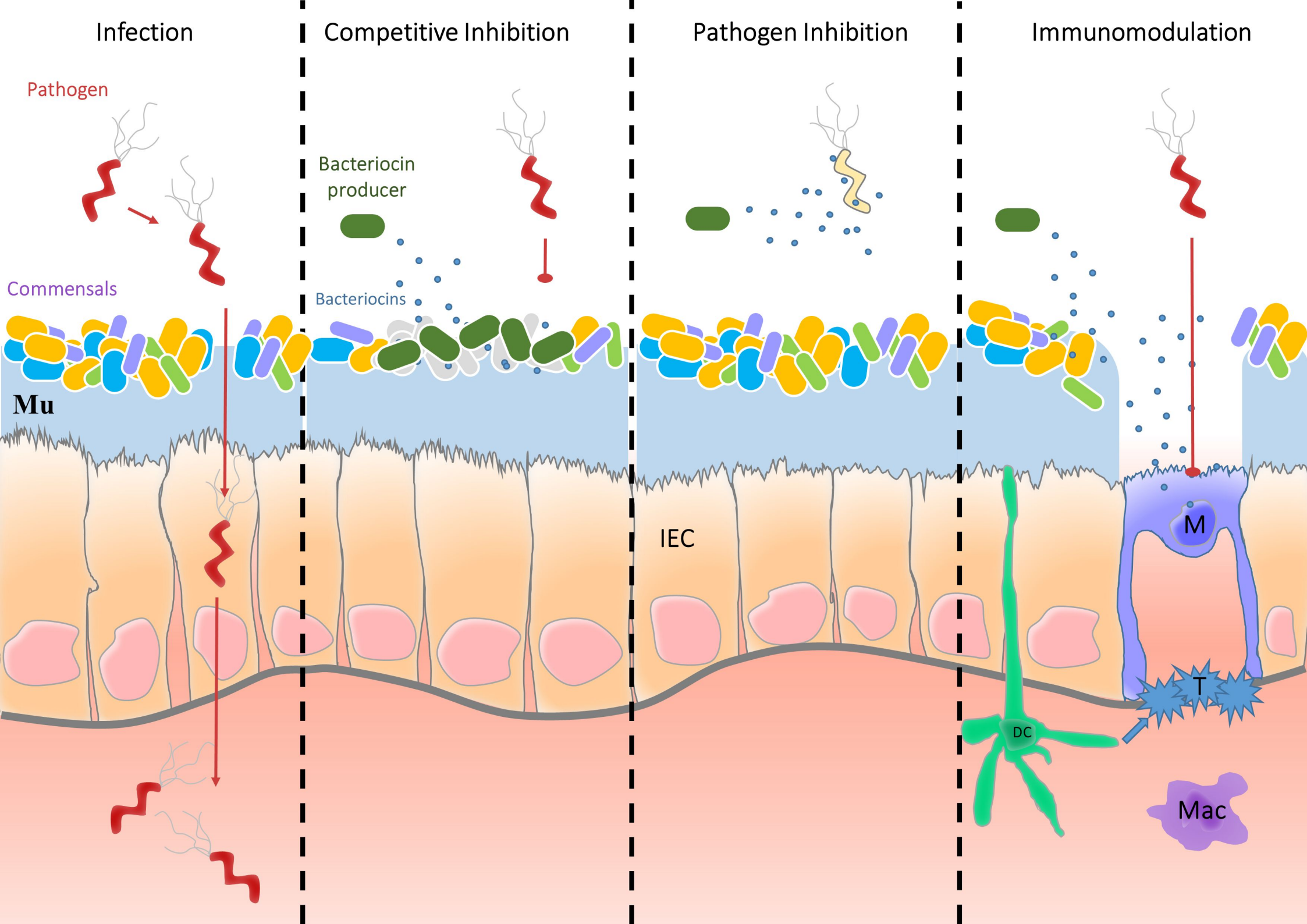
685   sciences. 2010;99(5):2521-8.

686   **Figure 1: Bacteriocinogenic probiotics can be utilized either prophylactically or**

687   **therapeutically to treat an infection.** M, M cell; Mac, macrophage; Mu, mucous; T,

688   T cell; IEC, intestinal epithelial cell; DC, dendritic cell.

Infection Competitive Inhibition Pathogen Inhibition Immunomodulation

Pathogen

Commensals

Bacteriocin producer

Bacteriocins

Mu

IEC

DC

M

T

Mac

Peptide	Strain inhibited	Model	Purity	Reference
Nisin F	*S. aureus*	Immunosuppressed Wistar rats	Semi-pure	(35)
	S. aureus	Brushite cement in BALB/c mice	Semi-pure	(36)
Lacticin NK34	*S. aureus/* *S. simulans*	ICR mice	Semi-pure	(37)
Nisin V	*L. monocytogenes*	BALB/c mice	Pure	(38)
Divercin V41	*L. monocytogenes*	BALB/c mice	Pure	(39)
Mutacin B-Ny266	*S. aureus*	Unknown	Pure	(40)
Mersacidin	Methicillin-resistant *Staphylococcus aureus* (MRSA)	BALB/cA mice	pure	(41)

Table 1. Bacterial infections in animal models successfully treated using purified bacteriocins.