Bacteriocins: Antibiotics in the Age of the Microbiome

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

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

Abbreviations used: AAD, Antibiotic Associated Diarrhoea; AMR, Antimicrobial Resistance; AOM, Acute Otitis Media; CDAD, *Clostridium difficile* Associated Disorder; CDC, Centres for Disease Control and Prevention; EcN, *Escherichia coli* Nissle; GI, Gastrointestinal tract; MIC, Minimum Inhibitory 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

effects on the host microbiome and their potential toxicity (5, 11). Although the field of microbiome research is in its infancy relative to that of antibiotic therapy, evidence strongly suggests that the composition of the microbiome can be an indicator of health and is likely to be involved in many aspects of human health and disease (12). Strides in DNA sequencing technology and bioinformatics have increased our understanding of the role of the microbiome in a variety of disease states. Indeed the administration of antibiotics in early life and the subsequent disruption of the microbiota may contribute to risk of obesity in later life (13, 14). Furthermore, when subjected to broad-spectrum antibiotic therapy, non-target commensal microbes may evolve and/or acquire resistance mechanisms to evade the effects of the antibiotic, thereby contributing to the antibiotic resistance crisis.

Bacteriocins represent a class of powerful antimicrobial peptides that may provide at least part of a solution to the AMR crisis. We aim to demonstrate their efficacy in the treatment of infectious disease and their reduced impact on the host microbiome by comparison to broad-spectrum antibiotic therapy.

Bacteriocins: potent antimicrobial peptides

Many excellent reviews have been written about bacteriocins (11, 15, 16) , but in brief they are a diverse group of peptides that may be classified into three distinct groups; class I (modified), class II (unmodified or cyclic) and Class III (>10kDa peptides). Apart from their potent antimicrobial activity (with minimum inhibitory concentrations [MIC's] often in the nanomolar range) they have also been shown to have antiviral (17), anticancer (18) and immunomodulatory properties (19). Bacteriocins typically have a narrow spectrum of activity, but broad-spectrum

peptides are also present in this class of antimicrobials (e.g. nisin and lacticin 3147 inhibit a wide range of Gram-positive bacteria). As a result these peptides may be suitable for treating infections of unknown aetiology, using broad-spectrum bacteriocins, or may allow more precise targeting of known infectious agents using highly active narrow spectrum bacteriocins. Bacteriocins are gene encoded, which makes them amenable to genetic alterations to improve functional characteristics. Furthermore, their toxicity is low and they may be administered as either purified peptide or produced *in situ* by bacteriocin producing probiotic bacteria (11). Bacteriocins are also known to interact with a variety of receptors, which are different to those targeted by antibiotics, making cross-resistance less likely (20). Although a more targeted approach may still ultimately lead to resistance development in the infectious agent, it does reduce the likelihood of resistance development in commensal populations outside of the target range of the bacteriocin. Resistance mechanisms involving the class II receptors, the mannose phosphotransferase system (Man-PTS), have been identified (21) along with a variety of resistance mechanisms to the class I lantibiotics (22).

The microbiota perspective

The term “superorganism” or “holobiont” has commonly been applied to describe the relationship that exists between humans and its commensal microbes and viruses (23). Understanding the role of the microbiota in health and protecting its diversity during the treatment of infectious disease is a key element of why bacteriocins may be suitable as alternatives to antibiotics.

The two-peptide sactibiotic bacteriocin, Thuricin CD, is a narrow spectrum bacteriocin. Thuricin CD is highly active against one of the main causative agent of antibiotic associated diarrhoea (AAD), *Clostridium difficile*, which is responsible for 20-30% of AAD cases (24). Briefly, AAD is caused by a disruption of the microbiota (often referred to as dysbiosis) following broad spectrum antibiotic treatment, and notably has a recurrence rate of 15-60% (25). Thuricin CD was shown to exhibit comparable activity to both vancomycin and metronidazole (two antibiotics used for the treatment of AAD which has progressed to *C. difficile* associated disease, CDAD). Importantly, it showed almost no effect on microbial diversity when compared to both metronidazole and vancomycin in a distal colon model (26). The modified R-Type bacteriocin, Av-CD291.2, has also been shown to prophylactically prevent colonization of *C. difficile* in a mouse model without perturbing the microbiota (27). There are other broad spectrum bacteriocins which are attractive therapeutic agents by virtue of their activity against *C. difficile*, but while the broad spectrum lantibiotic lacticin 3147 is effective at killing *C. difficile*, it has a significant impact on the resident microbiome populations such as *Bifidobacterium*, *Lactobacillus* and *Enterococcus* species (28). It has also been shown that a commercially available product containing the lantibiotic nisin, Nisaplin®, can eliminate a *C. difficile* infection when added at a concentration of 20X MIC in a simulated human colon model. However a significant decrease in the total microbiota count was observed, with Gram-positives being adversely affected (29).

Notably, in recent years the emergence of Vancomycin Resistant *Enterococci* (VRE) has become a great concern and therefore raises the issues surrounding the efficacy of treating CDAD with vancomycin if it presents a risk to the general population and the

spread of antibiotic resistance. In this light, the treatment of CDAD with bacteriocins could be a valuable alternative to vancomycin. When VRE development has taken place, it has been shown that mice colonised with VRE can be decolonized through the use of an *Enterococcus* probiotic containing a conjugation defective plasmid which produces a bacteriocin named Bac-21 (30).

A defensin-like bacteriocin, bactofencin A, displays *in vitro* activity against *L. monocytogenes* and *S. aureus* (31, 32). Although one might expect this medium to broad spectrum antimicrobial peptide to cause drastic changes in the host microbiome, this was in fact not the case and it was observed that the bactofencin peptide only subtly modulated an *ex vivo* host microbiome (distal colon model) when introduced as a bacteriocin producing probiotic or purified peptide. While the purified peptide resulted in higher levels of beneficial microbes such as *Bifidobacterium*, it was also associated with lower levels of *Clostridium*, which has been linked to obesity and gut pathogenesis. Interestingly, although bactofencin does not show inhibitory activity *in vitro* against strains from the genera *Clostridium*, *Fusobacterium* and *Bacteroides*, the reduction of these populations in the bactofencin treated faecal samples indicates that the consequence of bactofencin altering the overall microbiota structure impacts, directly or indirectly, on these normally insensitive populations when in the gut environment (33).

It has also been shown, using bacteriocin producing probiotic strains and their isogenic mutants, that the production of bacteriocins can aid the colonisation of a murine host (34). Sequencing data revealed that although bacteriocin production by the probiotics did not affect bacterial diversity at the phylum level, broad spectrum

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

Bacteriocins in animal models

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

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

Preventing infection

The concept of oral replacement therapy is another interesting example of prophylactic probiotic therapy, which has been investigated using the mutacin 1140 producing *streptococcus mutans* BCS3-L1. This bacteriocin producing strain is suitable for replacement therapy as it has reduced cariogenic potential because it does not produce lactic acid, mediated through the removal of its entire lactic acid 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

gastrointestinal (GI) tract (50). The concept of using prophylactic probiotics to competitively colonise a pathogens niche could be an effective strategy in agriculture to reduce antibiotic usage. If, as expected, regulations limiting the use of antibiotics in agriculture come into force, probiotics may be invaluable alternative.

Acute otitis media (AOM) is a type of inflammatory disease of the middle ear, characterised typically by rapid inflammation, potential tympanic membrane perforation, along with fullness and erythema**.** It has been reported that the levels of normal α-haemolytic *Streptococcus* colonising the nasopharynx of otitis prone children is much lower than in healthy individuals and that recolonization can significantly reduce the episodes of AOM (51, 52). It has been demonstrated treating otitis prone children with a history of AOM, with a nasal spray containing safe *Streptococcus salivarius* 24SMB (a strain which produces a bacteriocin-like substance), reduces the incidences of AOM over the placebo treated group (53).

Treating infection

Helicobacter pylori infection and colonisation results in a variety of disease states and may even lead to the development of gastric carcinoma. More recently, the prevalence of antibiotic resistant *H. pylori* has been increasing, creating a need for a new therapeutic agent (54). It has been shown in mice that eradication of *H. pylori* was achieved using a bacteriocinogenic probiotic treatment of *P. acidilactici* BA28 (55). Using a mixture of cranberry juice and the bacteriocin producing probiotic culture *Lactobacillus johnsonii* str. La1 supernatant, the carriage of *H. pylori* was also reduced in children after three weeks of treatment (56).

One barrier to the use of probiotics as a therapeutic is their ability to survive and colonise the area of infection. It has been shown that *Pediococcus acidilactici* UL5 and *Lactococcus lactis* ATCC 11454 can produce the bacteriocins pediocin PA-1 and nisin, respectively, *in situ* under simulated upper gastric conditions (57). Interestingly, the *in vitro* activity of a bacteriocin does not always correspond to the *in vivo* activity, where the bacteriocin is sometimes more or less active in an animal model, as is the case with mersacidin which is more active *in vivo* than *in vitro* (58).

Bacteriocins against Gram-negatives

Comparatively speaking, Gram-negative bacteria are relatively insensitive to bacteriocins compared to their Gram-positive counterparts, largely owing to the outer membrane which acts as a physical barrier. Until recently the treatment of Gram-negative infections with bacteriocins has not been favoured due to the efficacy of conventional antibiotics in the treatment of these infections. The rise of antibiotic resistant Gram-negative bacteria to the last line of antibiotics (6) means the treatment of Gram-negative infections using bacteriocins can no longer be ignored .

Widespread use requires a solution to the relative insensitivity of Gram-negative microorganisms. One possibility is to use bacteriocins in combination with other antimicrobial agents, including conventional antibiotics. Although conventional antibiotics will have an impact on the host microbiota (as previously discussed), certain bacteriocin/antibiotic combinations can be synergistic (59-62) and therefore lead to a reduced dose of both antimicrobial agents needed to treat an infection,

thereby lowering the potential effect on the host microbiome, the cytotoxic effects on the host and may potentially reduce the development of resistance.

Success of antibiotics is also hindered by Gram-negative bacteria residing within biofilms, where they are highly resistant to antibiotic treatments. Bacteriocin/ antibiotic combinations have shown great promise in overcoming biofilm mediated resistance for important Gram-negative pathogens such as *Pseudomonas aeruginosa* (63) and *Escherichia coli* (64).

Although this review mainly focuses on Gram-positive bacteriocins, it is important to also to identify Gram-negative bacteriocins which may have potential therapeutic significance. Microcins are ribosomally-synthesized peptides commonly produced by Gram-negative bacteria which are active against Gram-negative strains, and are an interesting alternative to Gram-positive bacteriocins. They have been shown to display potent antimicrobial activity *in vitro* (65, 66) and more recently also *in vivo* (67). It has been demonstrated that the microcin producer *E. coli* Nissle 1917 (EcN) can prevent colonisation of competing *Enterobacteriaceae* in the gut, whilst still having a minimal impact on the diversity of the gut microbiota. However, EcN microcins exhibit their mechanism of action by targeting specific siderophore receptors on other *Enterobacteriaceae* which are only displayed during iron starvation, making their spectrum of activity quite narrow. Additionally to its prophylactic applications, EcN has also been demonstrated to reduce inflammation and weight loss associated with *Salmonella* infections. Another microcin produced by *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

achieved with pseudomycoicidin (78), which is naturally resistant to trypsin due to the presence of a thioether ring structure. The field of bioinformatics and the use of such programs as BAGEL 3.0 (79) and antiSMASH (80) could be a fundamental aspect of this prospecting, as these bacteriocin amino acid prediction tools from genome sequences may also allow researchers to identify protease resistant peptides before investing large amounts of time and effort in characterising such bacteriocins. Finally, understanding bacteriocin pharmacodynamics and pharmacokinetics are also essential to their safe implementation as therapeutics, which has been under investigated in comparison to other aspects of bacteriocin research. If bacteriocins are indeed to become an alternative to conventional antibiotics, a greater emphasis must be placed on research surrounding these host-drug interactions, such as was achieved with MU1140 (81). Addressing these limitations of bacteriocin research to date could provide a turning point for the flagging interest of the pharmaceutical industry and make bacteriocins an attractive therapeutic alternative to current antibiotics (10).

Although there is considerable evidence that narrow spectrum bacteriocins have a minimal effect on the host microbiome by comparison to current broad-spectrum antibiotics, it should also be recognised that more work in this regard is needed to strengthen the argument for the use of bacteriocins as antibiotics, along with overcoming the previously outlined limitations. Ultimately, we believe given the safe history of use of bacteriocins in food and the large body of literature surrounding this field, that they are useful candidates for antimicrobial therapeutics as the AMR crisis 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 or financial relationships that could be construed as a potential conflict of interest.

Funding information

KE, CH and RR are supported by the Irish Government under the National Development Plan, through the Food Institutional Research Measure, administered by the Department of Agriculture, Fisheries and Food, Ireland (DAFM 13/F/462). CH and RR are also supported by the SFI Investigator awards (10/IN.1/B3027) and the APC Microbiome institute under Grant Number SFI/12/RC/2273.

Author Contribution statement

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

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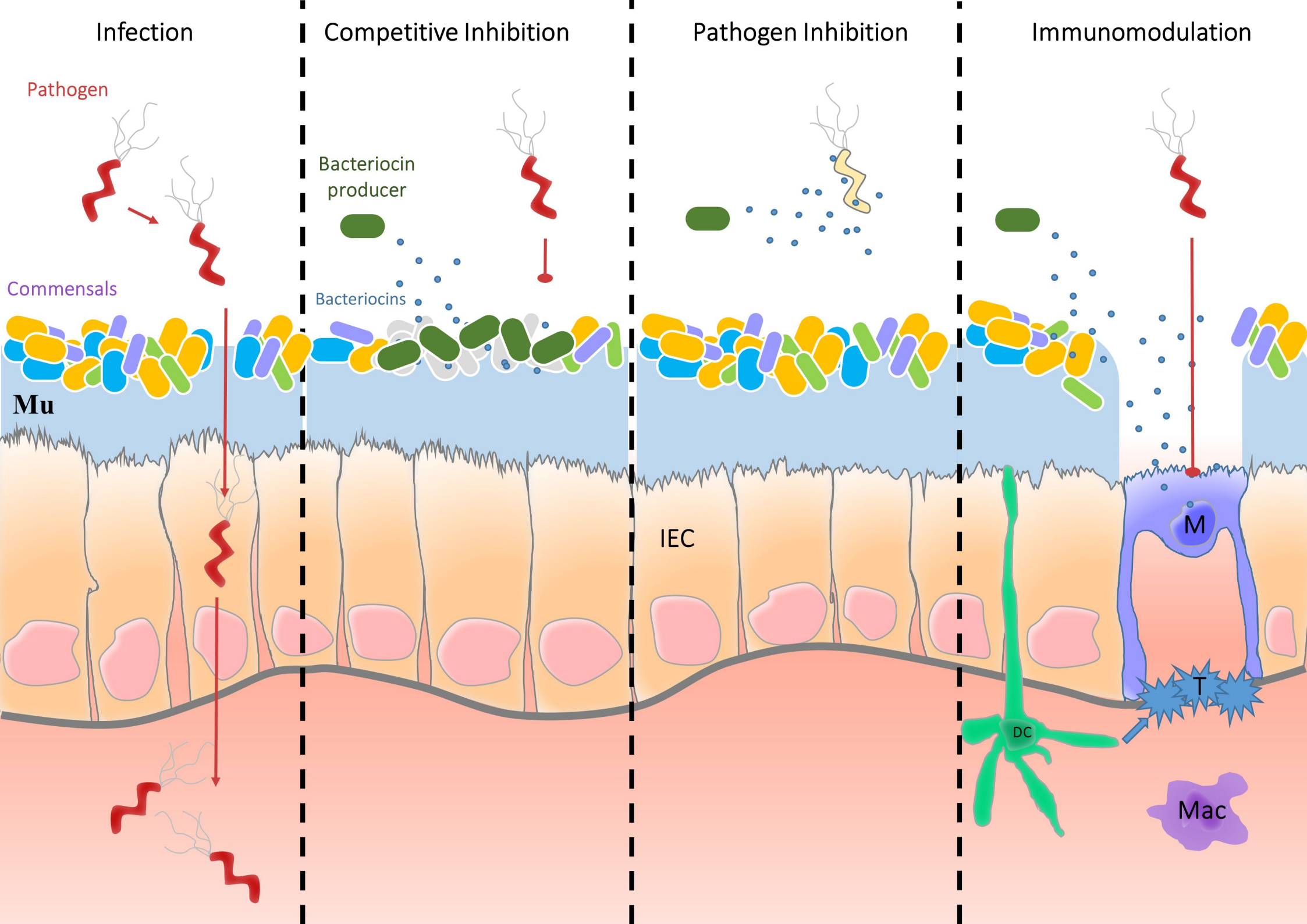
Figure 1: Bacteriocinogenic probiotics can be utilized either prophylactically or therapeutically to treat an infection. M, M cell; Mac, macrophage; Mu, mucous; T, T cell; IEC, intestinal epithelial cell; DC, dendritic cell.


Infection
Competitive Inhibition
Pathogen Inhibition
Immunomodulation
Pathogen
Commensals
Bacteriocin producer
Bacteriocins
Mu
IEC
M
T
DC
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.