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**Bacteriocin diversity, function, discovery and application as
antimicrobials**

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13 **Abstract**

14 Bacteriocins are potent antimicrobial peptides that are produced by bacteria. Since their
15 discovery almost a century ago, diverse peptides have been discovered and described, and some
16 are currently used as commercial food preservatives. Many bacteriocins exhibit extensively
17 post-translationally modified structures encoded on complex gene clusters whereas others have
18 simple linear structures. The molecular structures, mechanisms of action and resistance have
19 been determined for a number of bacteriocins, but most remain incompletely characterised.
20 These gene-encoded peptides are amenable to bioengineering strategies and heterologous
21 expression that would enable metagenomic mining of novel antimicrobials. The ongoing global
22 antimicrobial resistance crisis demands that novel therapeutics be developed to combat
23 infectious pathogens. New compounds that are target-specific and compatible with the resident
24 microbiota would be valuable alternatives to current antimicrobials. As bacteriocins can be
25 broad or narrow spectrum in nature they are promising tools for this purpose. However, few
26 bacteriocins have gone beyond preclinical trials and none are currently used therapeutically in
27 humans. In this Review, we explore the broad diversity in bacteriocin structure and function,
28 describe identification and optimisation methods, and discuss the reasons behind the lack of
29 translation beyond the lab of these potentially valuable antimicrobials.

30

[H1] Introduction

Antimicrobial peptides are produced ubiquitously across the kingdoms of life as an ancient form of host defence¹. Bacteriocins are a subset of bacterially produced, ribosomally synthesised antimicrobial peptides that have been studied for almost a century². In that time bacteriocins have been the subjects of thousands of publications, describing diverse peptide families many of which have reported the ability of the peptides and/or their producers to control the outgrowth of food spoilage organisms and preserve foods³. Many bacteriocins are the products of safe and food grade fermenting species such as lactic acid bacteria and some, exemplified by nisin, have transitioned in the past century from unknown antimicrobial byproducts² to characterised⁴, regulated and approved natural food additives⁵ in widely consumed commercial products. Bacteriocins have found success as food preservatives in the present but are not limited to it for the future, as they have also been investigated for various applications such as growth promotion in production animals⁶, plant growth promotion⁷, and as clinical therapeutics for the treatment of fungal⁸, protozoal⁹, viral¹⁰ and, foremostly, bacterial infections¹¹.

The increasing rates of antimicrobial resistance threatens the use of current antibiotics and it is predicted that antibiotic-resistant microorganisms will cause millions of deaths worldwide every year by 2050, severely affecting what are now considered routine medical treatments¹².

The World Health Organisation has generated a priority pathogen list highlighting an urgent need for novel treatments against antibiotic-resistant pathogens, with special mention of *Mycobacterium tuberculosis* and carbapenem-resistant Gram-negative bacteria for which only a few treatment options are available, followed by the Gram-positive vancomycin-resistant *Enterococcus faecium* within and other high-priority pathogens¹³. Bacteriocins represent a potential solution as current antibiotic alternatives or adjuncts^{14–17}. They offer several

56 pharmacological advantages such as activity against pathogens in the nanomolar and picomolar
57 ranges, narrow spectra, general non-toxicity and distinct mechanisms of action¹⁸. The diversity
58 in bacteriocin structures and mechanisms represent a range of antimicrobials that are
59 ribosomally produced and therefore amenable to genetic engineering strategies that enable the
60 production of bioengineered variants with superior efficacy against target microorganisms¹⁹.

61 As natural agents of microbial competition, bacteriocins offer an advantage to bacteria
62 over sensitive neighbouring species, enabling invasion, colonisation and protection of a niche
63 in complex environments such as the gut microbiome²⁰. By exploiting these natural
64 mechanisms they can be used as a tool for humans to deliver antimicrobial activity as pure
65 peptides, composite nano particles or codified within live biotherapeutics for *in situ* expression
66 in microbiomes. Advances in microbiome metagenomics and other ‘omics’ technologies over
67 the past two decades have revealed catalogues of genome- and metabolome-mined
68 bacteriocins, and new families are continually being discovered, but currently discovery is out
69 pacing characterisation and application. Narrow-target spectrum bacteriocins are also
70 increasingly desirable as we advance our understanding of host–microbiota interactions
71 (response and regulation of host processes to microbiota composition and products²¹) and are
72 realising that the impact of antibiotics on microbiome diversity should be considered²². Despite
73 lack of investment, bacteriocins are currently undergoing clinical studies to treat infections
74 caused by antimicrobial-resistant pathogens the outlook is bright for diverse bacteriocin
75 applications in the next century.

76 In this Review, we summarise recent advances in bacteriocin research, including the
77 identification of novel peptides and mechanisms of action. We discuss bioengineering as a
78 means to optimise bacteriocin activity and expression in addition to novel methods of
79 bacteriocin discovery in the ‘omics’ era. Finally we outline the potential applications of

bacteriocins producers, including live-biotherapeutics for microbiome modulation and highlight those peptides that are currently under investigation for clinical application.

[H1] Bacteriocin diversity

[H2] Structural diversity.

Bacteriocins are diverse and widespread across the phyla that produce them, with enormous heterogeneity in gene cluster organisation, biosynthetic machinery and peptide structures (Fig. 1). Despite such diversity, bacteriocins are usually classified into two major groups, those with post-translational modifications (class I) and unmodified peptides (class II) (Box 1). In recent years, there have been increased reports of novel ribosomally produced and post-translationally modified peptides (RiPPs) and characterisation of their biosynthetic machinery (extensively reviewed in Ref. ²³). However, not all RiPPs are bacteriocins and nor are all bacteriocins RiPPs as some do not undergo substantial post-translational modification. Bacteriocins are generally considered large molecule antimicrobials relative to small molecule antibiotics but can range from bicyclic peptides like the recently described darobactin (965 Da)¹⁶ that is smaller than the clinically used colistin (~1200 Da)²⁴, to larger circular peptides such as pumilarin (7083 Da)²⁵ (Fig 1).

Class I bacteriocins undergo extensive modification with enzymes that may dehydrate residues and form inter-amino acid bonds leading to their intricate and stable structures. Such modifications can result in peptides that are extensively cyclised and dehydrated, such as polyheterocyclic klebsazolicin²⁶ and corynaridin, the latter of which contains eight dehydrated residues²⁷. Lantibiotics (lanthionine-containing antibiotics) are well established class I bacteriocins, but novel structurally related peptide families continue to be identified; such as the type V lanthipeptides (lanthidins), which are glycosylated and N-terminally demethylated, as evident in the founding member cacaoidin²⁸. Class II bacteriocin structures include simple

linear alpha-helical peptides like plantaricin EF²⁹, peptides with conserved complex secondary structures like the four helix peptide BacSp222³⁰, or linear peptides with specific conserved disulphides such as pediocin-like maltaricin CPN and actifensin^{31,32}. Class II bacteriocins do not feature the significant modifications, such as dehydration and formation of unusual amino acids or linkages of class I, and previously included circular bacteriocins that are subject to N-terminal to C-terminal covalent linkage resulting in their simple circular shape³³, and the microcins such as MccH47 that are postrationally conjugated to siderophore (iron binding) moiety³⁴. For consistency within the two major classification scheme (Box 1.) we suggest circular and siderophore-modified peptides are henceforth referred to as class I bacteriocins, as they undergo modification despite relatively simple mature structures.

For most bacteriocins the structural peptide-encoding gene also encodes a leader sequence, which comprises N-terminal residues removed from the mature peptide, and can fulfil various functions in peptide export, cleavage and modification²³. These roles are linked to the specific type of bacteriocin they accompany, shaping peptide production and post-translational alterations. For example, in the case of lantibiotics, conserved motifs like the FNLD motif are observed within leader sequences, whereas circular bacteriocins exhibit considerable diversity in their leader sequences, ranging from minimal lengths as seen in lactocyclin Q (two residues³⁵) to more extensive sequences like in amylocyclicin (47 residues³⁶). The flexibility of circular bacteriocin leader sequences was highlighted by modification of circularin A, which demonstrated functional expression with a leader of only one residue (Met), suggesting a limited role for the leader in active circularin A biosynthesis³⁷. Additionally, specific motifs within the nisin leader sequence have been shown to guide peptide export, modification³⁸ and cleavage³⁹, whereas in lanthipeptides such as mersacidin, the leader undergoes a two-step cleavage process involving initial cleavage upon exiting the cell by the transporter-peptidase MrsT⁴⁰ and subsequent cleavage outside the cell by specific extracellular

proteases like AprE⁴¹. Although many bacteriocins with leader sequences possess a dedicated leader sequence essential for their biosynthesis and activity, with exceptions such as circularin A described above, leaderless bacteriocins lack this and instead are directly translated as active peptides³³ and the mechanisms of their production and export are poorly understood. Two pleckstrin homology (PH) domain-containing proteins DdE and DdF have been linked to the export of enterocin DD14 peptides in *Enterococcus faecalis*⁴², but peptide export mechanisms have yet to be characterised.

[H2] Biosynthetic gene clusters.

Bacteriocin-encoding gene clusters encode essential factors involved in their production and protection, typically incorporating genes encoding a core peptide, modifying enzymes for modified peptides⁴³, export machinery, self-immunity proteins that protect the producer⁴⁴, and many clusters also contain genes encoding regulatory proteins⁴⁵. In the case of the unmodified bacteriocin bactofencin, a ~4kbp gene cluster encodes the peptide and an ABC transporter with a protease domain that exports and removes the leader sequence. These two genes alone (*bfnA* and *DLSL_0052*) have been demonstrated to produce active peptide when expressed heterologously in *E. coli*⁴⁶. By contrast, the native producer of bactofencin A, *Ligilactobacillus salivarius* DPC6502, produces an immunity protein that provides self-protection, and an additional protein containing a thioredoxin family domain that is hypothesised to assist in disulphide bond formation⁴⁶. Other small gene clusters result in more complicated peptide structures such as a cluster of 6.2 kbp that contains genes for darbobactin A production; a core-peptide (*darA*), an ABC transporter (*darBCD*) and a single radical S-adenosylmethionine (rSAM) enzyme (encoded by *darE*) that catalyses formation of a Trp-Lys C-C bond and a C-O-C Trp-Trp ether bond via an unknown mechanism¹⁶. Radical SAM enzymes also catalyse the formation of alpha carbon to thioether (sactionine) bonds in sactibiotics (sactionine-

containing antibiotics), such as the ruminococcins, where the gene cluster in *Ruminococcus*
gnavus E1 consists of 17 open reading frames over 15 kbp that can result in five distinct
peptides⁴⁷. In the case of the lanthidin cacaoidin, the gene cluster spans 30 kbp and contains 27
genes that encode the necessary enzymes for multiple modifications, including rhamnose
synthesis and glycosyl transferases, dehydration and C-terminal S-[(Z)-2-aminovinyl-3-
methyl]-D-cysteine (AviMeCys) formation²⁸. Many bacteriocin gene clusters are not
constrained to specific phyla, such as glycocins and lasso peptides, which are encoded within
the genomes of both Gram-positive and Gram-negative bacteria^{48,49}. Bacteriocin gene clusters
may be present on plasmids capable of conjugation or transposable elements, thus facilitating
transfer of the bacteriocin production phenotype in a competitive environment⁵⁰. Recently, it
was found that up to 3% of prophage encode biosynthetic gene clusters, the majority of which
are for bacteriocin production and can demonstrably transfer production phenotype to nascent
strains⁵¹.

[H2] Functional diversity.

Though this Review mainly focuses on the antibacterial nature of bacteriocins, they are known
to have a diverse set of other biological functions. A wide range of peptides possess antiviral
activities⁵² such as subtilosin from *Bacillus amyloliquefaciens* that displays antiviral activity
against herpes simplex viruses⁵³. Peptides have been demonstrated to have cytotoxic effects
towards cancer cell lines such as laterosporulin⁵⁴, or possess *in vivo* anti-tumor activity as
was shown for microcin E492 against human colorectal cancer cells using a xenografted
zebrafish model⁵⁵. Bacteriocins can modulate immune systems such as sublancin that can
enhance phagocytic activity of macrophages and increase T-cell frequency in mesenteric lymph
nodes in mouse models of methicillin-resistant *Staphylococcus aureus* (MRSA) infection^{56,57}.
Thuricin 17, which is produced by *Bacillus thuringiensis*, has been shown to promote the

growth of canola plants (*Brassica napus* L.) at high temperatures through an unknown mechanism, displaying a potential role for bacteriocins in supporting plant growth in changing climates⁷. Finally, human streptococcal pathogens coregulate complex bacteriocin production and competence pathways linking fratricide and natural transformation implicating a role for bacteriocins in gene acquisition⁵⁸.

[H1] Narrow to broad spectrum peptides

Bacteriocins, like antimicrobial peptides across all kingdoms of life, are typically cationic hydrophobic peptides, many of which are active at negatively charged bacterial cell surfaces^{59–61}. The activities of bacteriocins, once they reach the cell surface, are diverse, including specific interactions with cell wall or membrane components, general membrane interaction and pore formation, or receptor-mediated cell penetration and interference with intracellular processes (for extensive mechanistic reviews see Refs. 62, 63, and 64.)(Fig. 2).

[H2] Extracellular targets.

Nisin is well established to have a dual mechanism of action, binding the cell wall precursor molecule lipid-II, thereby inhibiting peptidoglycan biosynthesis and subsequent pore formation leading to cell death⁶⁵. Initially, it was believed to form a pore complex of eight nisin and four lipid II molecules, though more recent evidence has shown the pore complexes continue to grow causing massive cell membrane damage⁶⁶ (Fig. 2). Numerous post-translationally modified bacteriocins share a conserved nisin-like N-terminal lipid-II binding domain (rings A and B), but do not possess the same C-terminal domain, and do not form pores as a result⁶⁷. This has been recently been demonstrated for the nisin-like peptides romboicin A⁶⁸ and cesin⁶⁹. Many bacteriocins do not target lipid II and instead use a different receptor or lipid component as a binding site^{70,71}.

Pediocin-like bacteriocins (class II) interact with the mannose phosphotransferase system (Man-PTS) forcing the channel into an open state, which was recently confirmed by cryo-electron microscopy⁷². The Man-PTS transporter is a receptor for several class II bacteriocins such as lactococcins A and B, and garvicins A, B and C, as was recently confirmed by deletion mutants, protein modelling, and cryo-EM^{73,74}, and is the target for microcin E492 after ‘Trojan horse’ translocation across the outer membrane⁷⁵. The circular bacteriocin garvicin ML relies on the maltose ABC transporter to target *Lactococcus lactis* whereas AS-48 is believed to interact based solely on electrostatic and hydrophobic interactions to insert directly into the lipid bilayer, leading to destabilisation^{76,77}. Direct membrane interaction is also the mechanism for the four helix-bundle bacteriocins epidermicin NI01 and aureocin A53, which have been found to have distinct multi-modal ‘flowering’ pore-forming activity⁶¹.

Bacteriocin activities are typically restricted within the Gram-status of the producer; that is, peptides derived from Gram-positive bacteria are typically far less active against Gram-negative bacteria without assistance of an outer-membrane permeabilising agent^{78,79}. Contradictory to this, a semipurified supernatant containing bacteriocins from *Lacticaseibacillus paracasei* CNCM I-5369 was found to be active against a panel of Gram-negative bacteria which was confirmed by heterologous expression⁸⁰. Bioengineering and rational design have been utilised in the absence of an outer-membrane permeabilising compound (that would typically be required for Gram-positive derived bacteriocins to inhibit Gram-negative bacteria) to produce variants of nisin with up to 12-fold greater activity including bactericidal activity against *E. coli* and *Acinetobacter baumannii*⁸¹. A hybrid peptide consisting of enterocin CRL35 fused with microcin V (Ent35–MccV) was shown to be active against the targets of both individual peptides, including *Listeria monocytogenes* and *E. coli*⁸².

[H2] Intracellular targets.

Some bacteriocins interfere with essential intracellular processes, in particular those that target Gram-negative bacteria, which lack exposed peptidoglycan and inner cell membrane components. The TOMM klebsazolicin inhibits protein synthesis by blocking the ribosome exit tunnel, but its extracellular uptake mechanism is unknown²⁶. The crystal structures of the lasso peptides microcin J25 and capistruin have revealed their specific sites of transcription inhibition within the RNA polymerase secondary channel following uptake mediated by a host of transport proteins⁸³. Sactibiotics are a class known for their narrow spectra of inhibition. Recently, it was suggested that the *Ruminococcus gnavus*-produced and *Clostridioides*-targeting sactibiotic ruminicoccin C could act through nucleic acid synthesis inhibition in a manner similar to metronidazole, but this has yet to be confirmed⁴⁷.

[H2] Unknown targets.

Despite ongoing characterisation of the mechanisms of action of bacteriocins, many peptides of interest remain uncharacterised. Similarly, the recently described bacteroidetocins, a family of anti-Bacteroidales pediocin-like bacteriocins, have an unknown mechanism of action as *Bacteroides* species lack the mannose-PTS system⁸⁴. Although most research to date has focused on mechanisms of bacteriocins as free agents in liquid culture, a two-component Gram-negative bacteria-derived bacteriocin CdzCD was recently identified on the basis of cell-to-cell contact-dependent killing for which the outer-membrane protein PerA was required for sensitivity, and has been suggested as the target receptor for CdzCD binding⁸⁵. With the vast recent improvements in protein structural modelling and prediction, we may begin to see more in-depth bacteriocin–target complex structures and gain insights into yet unknown mechanisms.

[H2] Immunity and resistance.

The risk of target strains developing resistance against bacteriocins because of continuous use is a key concern, given the current situation with AMR and the antimicrobial arms race. Bacteriocin producers are self-protected by the action of immunity proteins, that may bind or export the peptides⁴⁴, or block the action of peptides on protein complexes⁷². Resistance to bacteriocin activity can occur through general mechanisms, such as cell wall thickening causing resistance to the lipid II binding lasso peptide, siamycin I⁵⁹, or by truncation of a protein target like the transporter of the mannose-PTS⁸⁶ but high-level resistance occurs through acquisition of specific resistance genes. Bacteriocins can be inactivated due to the activity of specific enzymes such as the nisin resistance protein which degrades nisin⁸⁷. Worryingly, some pathogenic species express generalised transporter proteins such as the *Streptococcus agalactiae* bacitracin type efflux protein (SaNsrFP) that protects against numerous cell-wall targeting compounds including lantibiotics⁸⁸. As bacteriocins have not been extensively used in the clinical setting, the extent or risk for resistance in clinical use is unknown and to our knowledge for many classes of bacteriocins, no resistance mechanisms have been characterised yet.

[H1] Production and bioengineering

One of the most appealing attributes of bacteriocins is their gene-encoded nature, that enables the alteration of peptides using simple techniques and the generation of new-to-nature structures with unknown characteristics (Fig. 3). Wild-type bacteriocin producers often generate low bacteriocin yields because production is a high-energy process for the cell and is thus tightly controlled. This can result in excessive production costs that render bacteriocins non-viable commercially⁸⁹. Production optimisation has therefore been examined by altering growth parameters in addition to exploiting their gene-encoded nature by manipulating the native producers⁹⁰, and/or peptide expression in more efficient or peptide-insensitive heterologous hosts^{91,92}.

[H2] Heterologous expression optimisation.

Bacteriocins of all classes have been subject to heterologous expression to express novel silent genes⁹³, understand their mechanism of biosynthesis^{94,95}, or to improve their production for commercial viability⁹². Whereas post-translationally modified (class I) bacteriocins require essential modification enzymes to produce active mature peptides, class II bacteriocins are particularly suited to heterologous expression as many are ‘simple’ linear peptides. A range of Gram-positive bacteria, Gram-negative bacteria, eukaryotic hosts and diverse plasmids have been exploited for this such as *Escherichia coli* for expression of pediocin PA-1 (syn. pediocin AcH), bactofencin A and lactocin AB^{46,96}, *L. lactis* for expression of plantaricin JK and enterocin NKR-5-3B^{97,98}, and the yeasts *Pichia pastoris* X-33 and *Kluyveromyces lactis* for the expression of enterocins^{99,100}.

Beyond heterologous expression, simple optimisation of culture conditions can increase bacteriocin yield, as researchers demonstrated by improving garvicin KS production from *Lactococcus garviae* as high as 60 fold when cultured in pasteurised milk and tryptone, compared to GM17 media⁹⁰. Combining the improved media with increased gene dosage of the native producer’s core peptide further increased production to the high concentration of 1.2g of peptide per liter⁹⁰. Increasing gene copy number has also improved production of the sacitibiotic thurincin H, where an additional three copies of the structural gene, *thnA*, resulted in double the yield from broth culture¹⁰¹. Synthesis of plantaricin E/F in *Lactobacillus plantarum* strains is induced by both an auto-inducing peptide PlnA1 and by acetate via a distinct interaction with the PlnB1 response regulator¹⁰². Exploiting this mechanistic understanding enabled an increase in the yield of plantaricin in culture by addition of sodium acetate at a concentration of 1g.L⁻¹ (Ref. ¹⁰²). Limited studies report extensive optimisation or comparative purification yields, as peptide purification is typically a means of characterising

peptides at small scale *in vitro* rather than for potential commercial application. However, to scale up to *in vivo* experiments with pure peptide and for bacteriocin production to become more commercially viable optimisation must be explored in greater detail.

[H2] Expanding the functional diversity of bacteriocins

Nisin is perhaps the most genetically manipulated bacteriocin with thousands of unique variants having been generated using random and site-direct PCR mutagenesis techniques, in addition to an entire expression system developed based on the tight regulation of nisin production¹⁰³. Mutagenesis methods have produced nisin variants with increased activity against biofilms and variants with greater activity against Gram-negative bacteria such as *Cronobacter sakazakii*, *Salmonella typhimurium* and *E. coli*¹⁰⁴. Rombocin K, a bioengineered variant of nisin-like rombocin A, was found to have a two-fold lower minimum inhibitory concentration (MIC) than the original peptide when tested against *L. monocytogenes*⁶⁸. The inhibition spectrum of nisin has also been refined in the derivatives M17Q, T2L and HTK, which possess increased anti-*Staphylococcus* activity and reduced anti-*Lactococcus* activity^{19,105}. Similarly, saturation mutagenesis of mutacin 1140 generated 418 single amino acid variants of which Phe1Ile ultimately emerged as the most promising *in vivo* candidate for *Clostridioides difficile* infection and associated diarrhoea, which in turn underwent a second round of mutagenesis optimisation resulting in a more potent peptide^{106,107}. Such methods may improve a promising bacteriocin for application, or be used to overcome a limitation, such as the oxidation-resistant M31L variant of pediocin PA-1, where oxidation-associated activity reduction challenges the wider application of wild-type peptide¹⁰⁸. Bioengineering can be used as a means to overcome proteolytic degradation, such as nisin S29P that is immune to the action of the nisin resistance serine protease¹⁰⁹, and cesin R15G which has improved resistance to trypsin⁶⁹.

Recently developed methods have utilised incorporation of non-canonical amino acids into the peptide backbone which can then be conjugated to other compounds via ‘click chemistry’. Using the non-canonical and more-hydrophobic amino acid ethionine in the place of methionine, heterodimeric and fluorescently labelled nisin variants have been generated each of which retained antimicrobial activity¹¹⁰. A similar method lipidated nisin variants which had improved bioactivity and specificity against pathogenic strains¹¹¹. Conjugation of bacteriocin peptides to molecules such as fatty acids can be exploited to produce bacteriocins that form colloidal stable assemblies, a feature which could be utilised to overcome problems with solubility of hydrophobic peptides¹¹². Novel methods such as this can be used to further expand, edit and hone already diverse peptide functionality and future downstream applications.

[H1] Discovery in the meta-omics era

In recent years, bacteriocin discovery has been catapulted into the meta-omics era with the availability of vast genomic datasets and dedicated bioinformatic software enabling *in silico* screening of bacterial genomes and large-scale metagenomic mining for the presence of novel bacteriocin gene clusters (Fig. 3). Despite convincing evidence that bacteriocin-encoding genes are widely distributed across many genera, confirming bacteriocin production from individual strains isolated during screening studies has proven difficult. Prior to the increased accessibility of next-generation sequencing technologies, the majority of novel bacteriocins were identified *in vitro* through laborious screening of hundreds or thousands of bacterial isolates¹¹³. These culture-dependent screening methods face logistical microbiological limitations such as the choice of media on which samples are plated, the choice of indicator species and the growth conditions (pH, atmosphere composition, temperature), which can all lower the likelihood of bacteriocin detection. Failure to detect bacteriocin production does not mean lack of

bacteriocin presence, but could be due to the inadvertent choice of an insensitive indicator species or a limited or lack of induction and production of an active bacteriocin. Genomic approaches that are agnostic of active expression and rely on bioinformatic predictions of the presence of biosynthetic gene clusters can overcome these issues but are not without their own limitations. For example, *in silico* approaches alone do not confirm biological activity nor do they enable the purification and characterisation of a bacteriocin. It is likely that a combination of *in silico* and culture-dependent methods will prove most effective in terms of identifying and isolating novel functional bacteriocins.

[H2] Bacteriocin gene cluster prediction.

As sequencing technology has improved and sequence databases have grown, a range of tools has been developed for mining bacteriocin genes encoded in bacterial DNA. Tools have been developed that span from ‘point-and-click’ interfaces suitable for non-bioinformaticians, to command-line controlled packages to detect biosynthetic gene clusters in genomic and metagenomic datasets at a range of assembly levels. BAGEL and antiSMASH are well-tested and frequently updated tools that are designed for the identification of bacteriocin (and secondary metabolite) biosynthetic gene clusters in assembled bacterial genomes^{114,115}. Both tools are useful for the characterisation of an unknown antimicrobial producing-isolate but are limited by predictions based on enzyme homology and predicted core peptide characteristics which may miss truly novel compounds. In addition, they can be low- to medium-throughput based on the skill level of the user. Despite this they have enabled the discovery of bacteriocins in several hundred to thousands of genomes, such as the identification of 101 lanthipeptide gene clusters from 223 *Bacillus cereus* strains¹¹⁶, the characterisation of the biotechnological potential of 213 diverse lactic acid bacteria¹¹⁷, and the genome-guided expansion of a bacteriocin family from an initial 11 members to widespread detection of homologues in

hundreds of strains²⁵. More recently a simple tool for mining specific RiPP classes based on BAGEL has been developed to interrogate metagenomic datasets for bacteriocin biosynthetic gene clusters¹¹⁸.

With continuous growth in computing power and bioinformatic tool development, artificial intelligence has also been introduced into bacteriocin research, with machine learning being utilised to predict biosynthetic gene clusters and predict chemical structures following post-translational modification. RODEO (rapid ORF description and evaluation online) was the first tool available to utilise machine learning and was able to expand the number of known lasso peptides tenfold upon its initial publication, and subsequently increased the number of known glycocins from six to ten distinct characterised members using only a single enzyme as a starting point^{49,119}.

[H2] *From in silico to in vitro.*

Bioinformatics can have an important role in identifying potential bacteriocins, but this should be supported by functional characterisation either by isolation of a producing strain, chemical synthesis, or by heterologous expression from genomic or synthetic DNA in a suitable host. When candidate bacteriocin-producing strains are identified *in silico* but production cannot be demonstrated, or when putative producers are not available, *E. coli* has been used as a heterologous bacteriocin expression ‘workhorse’. Expression in *E. coli* strains has enabled the characterisation of structural peptides and biosynthetic machinery for glycocins and lantibiotics^{40,120,121}. Bioinformatically mined class II bacteriocins have also been expressed in *E. coli*, including pediocin PA-1 and lactobacilli-derived pediocin-like peptides, enabling the identification of genes essential for bacteriocin production^{46,93}. Gram-positive hosts have also been extensively utilised for the expression of bacteriocins, including the reliable nisin-inducible expression system in *L. lactis* which has recently been used for the functional

characterisation of the circular bacteriocin circularin A mined from a clostridial genome⁹⁵. Genome mining paired with heterologous expression is a powerful combination to explore novel compounds but can be low-throughput and failure-prone depending on the choice of host and vector. Researchers recently developed a powerful platform combining high-throughput bioinformatics and an integrated robotic system to express 96 RiPP gene clusters from diverse bacterial phyla involving 383 biosynthetic genes, with a success rate of 86% (Ref. ¹²²). High-throughput methods such as this could be used to explore the Integrated Microbial Genome Atlas of biosynthetic Gene Clusters (IMG-ABC) database that consists of 20,000 gene clusters, of which a small number have been experimentally validated¹²³.

[H2] Integrating 'omics' technologies.

Whole metabolomic methods are of growing interest in bacteriocin and RiPPs research (for an extensive recent review see Ref. ¹²⁴). Peptidogenomics, which is mass spectrometry-guided genome mining for peptides, have successfully identified a novel lanthipeptide and production gene cluster from the cell extract and genome of a single strain¹²⁵. MetaMiner has been developed to search for novel compounds from billions of mass spectra and compare with diverse metagenomic datasets such as human microbiome samples¹²⁶. 'Omic-mining' can successfully identify novel peptides and biosynthetic gene clusters in an untargeted manner, but without 'wet-laboratory' demonstration of compound-specific antimicrobial activity all such compounds should not be classed as bacteriocins. As the number of known biosynthetic gene clusters continues to grow, it is inevitable that biological characterisation will lag behind and will have to be upscaled to explore applications of novel peptides.

[H1] Translation from food to clinic

[H2] Food preservatives.

Fermentation as a means of food preservation, and bacteriocins unknowingly as by-products of thereof, have been utilised by humanity for millennia. Much of early bacteriocin research was performed in the context of food fermenting organisms, such as the description of nisin in the context of delayed dairy fermentation due to producer inhibition and continues to be studied to this day^{2,127}. Vast amounts of historical and ongoing research continues to describe the efficacy of bacteriocin producers (as fermentation starters or adjuncts) and their products to inhibit spoilage organisms in different products. However, despite decades of bacteriocin use in the food industry (nisin was designated as generally recognized as safe by the US FDA in 1988 (Ref. ¹²⁸)) and the array of bacteriocins that have been identified to date, only a small number are currently commercially available as food preservatives (mainly nisin A, nisin Z and pediocin PA-1). Most bacteriocin preserving agents are available as partially purified fermentates, some of which also contain the producing organism. Nisin is available as a powder with degrees of purity under the product names Nisaplin® (Danisco, Dupont), Chrisin® (Chr. Hansen) and vegetal or white NisinA/Z® and NisinA/Z® P (Handary). Pediocin PA-1 is utilised as an anti-listerial additive in food production in a number of powdered fermentate forms such as ALTA 2351/2341 (Kerry Group) and Fargo 23® (formerly in production by now defunct Quest International) approved by EFSA's Qualified Presumption of Safety (QPS) status¹²⁹. A formulation containing *Carnobacterium maltaromaticum* CB1 and derived bacteriocins labelled Micocin® (Griffith Foods) has been approved for use in North America and Canada for the preservation of meats¹³⁰.

[H2] *Live strains and microbiome modulators.*

[H3] *Microbiome modulation.*

Microbiome research continues to grow exponentially with considerable efforts directed towards associating particular microorganisms or microbial compositions with human

diseases or conditions^{131–133}. Identifying such correlations will naturally lead to attempts to develop tools for modulating the microbiome to enhance specific health-associated taxa or to reduce disease-associated taxa. Nutrition and lifestyle are obvious microbiome modulating strategies but these are essentially ‘blunt tools’, generating largely non-specific and potentially widespread changes to gut microbiota composition and functionality. Antibiotics are also blunt tools and despite being necessary for treatment and prevention of infectious agents their lack of precision causes collateral damage to beneficial gut microorganisms, risking adverse health events such as *Clostridioides difficile* overgrowth or the expansion of antibiotic-resistant strains^{22,134,135}. This effect is not restricted to the gut, with antibiotic treatment also restructuring microbiomes of the skin, vagina and oral cavity^{136–138}. Bacteriocins may prove to be an exciting alternative as potent antimicrobials which may have narrow target spectra to target specific genera and species, and can therefore be utilised to more precisely inhibit pathogens or pathobionts, leaving benign commensals unharmed. Several class II bacteriocins have been found not to alter the overall gut microbiota composition but to reduce specific taxa such as *Enterococcaceae* and *Clostridium* spp. when consumed by mice¹³⁹. Thuricin CD, produced by *Bacillus thuringiensis*, has also been shown to specifically reduce clostridia in a faecal fermentation model whereas vancomycin, a commonly used treatment in clinical settings, has a broad impact on the bacterial community¹⁴⁰. Even broad-spectrum bacteriocins can induce only subtle changes in microbiome compositions when compared with traditional antibiotics, as was demonstrated with microcin J25 relative to rifampicin in a continuous culture porcine model¹⁴¹. Targeted microbiome modulation should also consider potential ecosystem effects as removal of a targeted species or genus could lead to unintended increases or decreases in others¹⁴². Simplified model ecosystems such as the OMM12 model have shown that bacteriocin production can have broad effects on neighbouring strains, and removal of a single species, in this case, a bacteriocin-producing strain, leads to increased abundance of numerous others¹⁴³.

[H3] Delivery to the gut.

Therapeutics for human or animal consumption must be carefully formulated to be delivered in an active form to the correct anatomical site for function. For example, a small-molecule antibiotic must survive harsh environments during gastrointestinal transit to be absorbed and function systemically to prevent or treat infection. Bacteriocins have long been thought to be proteolytically degraded during gastrointestinal transit¹⁴⁴ and therefore must be protected by encapsulation for applications directed at the large intestine. Recent evidence has challenged this dogma and shows that some bacteriocins, such as nisin, may be more resistant than previously thought, with detectable intact nisin surviving *in vivo* gastrointestinal transit in pigs, and microcin J25 resisting degradation during simulated human digestion^{37,38}. Despite this, many bacteriocins can be readily degraded by proteases in gastric transit^{145,146} and peptide delivery to the gut faces challenges of producing sufficient quantities to administer to large animals, and the degree of purity required for *in vivo* studies. Fortunately, bacteriocins can also be deployed in the form of a probiotic or live-biotherapeutic agent that arrives at the desired destination and delivers a bacteriocin dose *in situ*. Researchers recently showed that genetically modified *E. coli* Nissle 1917 expressing microcin 147 in a preclinical mouse model significantly reduced carbapenem-resistant *Klebsiella pneumoniae* after one week oral administration of 10⁸ cells/day without alteration of the resident microbiota³⁴. *E. coli* expressing the unmodified enterocins A/B and hiracin JM79 have also been shown to reduce vancomycin-resistant *Enterococcus* when provided in drinking water *ad-libitum* (5x10⁸ CFU.ml⁻¹) in a vancomycin-resistant *Enterococcus*-colonised mouse model¹⁴. Live bacteriocin producers can also be used to treat or modulate microbiomes outside the gut, such as the use of a *L. lactis* strain producing lactacin 3147 in a liquid paraffin-based emulsion that was used to treat bovine mastitis (10⁹ CFU.5 ml⁻¹ dose) as successfully as the standard antibiotic treatment¹⁴⁷.

Bacteriocin producers that are closely related to the intended target species can have the added benefit of potentially being able to occupy the same niche, potentially providing future colonisation resistance against pathogens. Bacteriocin-21-producing *E. faecalis* were found to evict their drug-resistant relatives in the mouse intestine when provided in drinking water *ad libitum* (5×10^8 CFU.ml⁻¹) without perturbation of the overall community⁵⁰. However, the plasmid-encoded nature of this bacteriocin resulted in conjugation to native enterococci providing immunity and the capacity to produce the bacteriocin. These data facilitate a scenario where a multi-drug resistant strain could gain another tool to aid colonisation and therefore must be cautiously considered when developing bacteriocin producers as targeted live-biotherapeutics.

Other methods for ensuring unscathed bacteriocin delivery to site of action include through formulation with other compounds that increase peptide stability under biologically relevant conditions¹⁴⁸. Silica mesoporous matrices have been utilised as protective delivery vehicles for bactofencin A and nisin A resulting in active peptides that are resistant to trypsin¹⁴⁹ and pepsin¹⁵⁰ degradation, respectively. Hydrogels have been developed to ensure sustained release of bacteriocins over time, including an injectable polysaccharide gel containing nisin A active for up to 10 days¹⁵¹, and a two-phase release gel of subtilisin developed for inhibition of *Gardnerella vaginalis*, which causes bacterial vaginosis¹⁵². Formulation of bacteriocins into nanoparticles to increase the antimicrobial activity or widen the spectrum of the resulting nano-conjugate is a growing trend for food preservation and clinical application (reviewed in Ref. ¹⁵³). Recently, a mix of five bacteriocins adsorbed on alginate nano-particles was found to be 500-fold more active than the peptides against clinically relevant *E. coli*¹⁵⁴, and Microcin J25-chitosan nanoparticles were active against enterotoxigenic *E. coli* in addition to ameliorating lipopolysaccharide-induced cytotoxicity of macrophages and downregulating lipopolysaccharide-stimulated proinflammatory cytokines¹⁵⁵. Emerging technologies such as

these should continue to improve and develop bacteriocin candidates for application in food preservation and in clinical settings.

[H2] Clinical application.

In addition to few commercial bacteriocin products on the market, none are currently approved for human therapeutic use. Research and development for clinical application of bacteriocins has been chronically underfunded, with 70 novel antimicrobials under investigation in 2020, of which none were bacteriocins¹⁵⁶. Despite this lack of considerable investment, many preclinical and some clinical studies have been performed demonstrating that bacteriocins, and particularly lantibiotics, exert antimicrobial activity *in vivo* in tolerable quantities (Table 1). Several bacteriocins and their producers have been investigated for the oral treatment of *Clostridioides difficile* infection and associated diarrhoea. Preclinical trials have assessed OG-716 (mutacin derivative) for treatment of *Clostridioides difficile* infection and associated diarrhoea when administered via oral gavage (20mg/kg⁻¹ bodyweight) in golden hamsters, finding the peptide to be safe and effective at preventing relapse^{107,157}. A bacteriocin-producing *Bacillus velezensis* strain as a live biotherapeutic for the treatment of *C. difficile* infection has been proven effective preclinically in mice (single oral dose of 5x10⁸ CFU) and in miniature swine (single oral dose of 5x10⁸ CFU, and 28 days administration of 5x10⁹ CFU in capsules), and recently concluded a phase I clinical trial¹⁵⁸. The semi-synthetic thiopeptide LFF571 (derived from GE2270A) has undergone phase I and II clinical trials (oral administration of 200 mg four times per day for 10 days) for *C. difficile* infections and was determined to be comparable to vancomycin treatment^{159,160}.

Beyond *C. difficile* infection the lantibiotic NAI-107 (microbisporicin) has undergone a series of preclinical rodent studies for severe anti-microbial resistant infections with promising results¹⁶¹. In animal studies, nisin and lactacin-3147 have shown promise in the

treatment of mastitis¹⁴⁷, and lantibiotic-producing *Staphylococcus hominis* ShA9 decreased *S. aureus*, improving atopic dermatitis when delivered to the arms in a lotion (10⁶ CFU.cm⁻² applied twice daily for one week) and meeting primary and secondary endpoints in a phase I clinical trial¹⁶². Mutacin 1140-derived topical treatments (5 or 10 mg/kg/day for 7 days) have been proven safe with and more effective than vancomycin for MRSA infection in mice, and have also been effective when administered intravenously(10 mg/kg/day for 6 days)¹⁶³. Fewer trials have been performed with class II bacteriocins, but recently the effect of an Abp-118-producing *L. salivarius* live biotherapeutic on recurrent acute otitis media (middle ear infection) in children was examined¹⁶⁴. The treatment was proven highly effective (84% reduction in episodes over 6 months of daily consumption of 10⁹ CFU) and as one of the most common infections in children, a commercial bacteriocin therapy could greatly reduce antibiotic prescription.

[H2] Beyond antimicrobials.

Thiostrepton, derived from *Streptomyces* spp. has long been used in veterinary medicine for the topical treatment of acute otitis media but has never been approved for human use, nor have any other thiopeptides¹⁶⁵. However, it is one of several bacteriocins gaining interest beyond antimicrobial application. Thiostrepton selectively and potently represses the cancer-associated transcriptional activator and therapeutic target, FoxM1 (Ref. ¹⁶⁶). Diverse bacteriocins have been studied *in vitro* and *in vivo* for chemotherapeutic treatment of different cancer types including nisin¹⁶⁷, microcin E492⁵⁵, and enterocin LNS18¹⁶⁸ with none having yet progressed to phase I clinical trials. Moli1901 (duramycin/lancuvotide) has undergone phase I and II clinical trials as a therapeutic for cystic fibrosis as a host chloride channel activator, though with limited efficacy^{169,170}. Bacteriocins are not immune to the pitfalls of drug development and can be abandoned in early clinical trials due to poor performance or

difficulties in production. Ultimately, the pharmacokinetic and pharmacodynamic attributes of bacteriocins under investigation are critical limiting factors in enabling them to become therapeutic agents. Vastly increased funding of discovery, characterisation, preclinical and human trials is required to adequately advance suitable bacteriocin candidates to the frontline of clinics which currently continues to be sorely lacking.

[H1] Conclusions

Bacteriocins are a diverse group of antimicrobial peptides produced across a number of bacterial phyla, and ongoing discovery efforts continue to reveal fresh biological insights. These discoveries include not only novel peptide families but also instances of structurally similar peptides and biosynthetic machinery present in taxonomically unrelated species. This phenomenon may shed light on aspects of horizontal gene transfer and the evolution of antimicrobial peptides, akin to the convergent evolution observed in Eukaryotic defensins¹⁷¹.

Bacteriocin discovery has been transformed in recent years from the labour-intensive process of identifying producer isolates *in vitro* to the *in silico* genome mining that can yield hundreds of related peptide sequences within seconds. Machine learning has become an invaluable tool in detecting bacteriocin sequences within vast genomic datasets, and with continuous advancements in computing power and artificial intelligence training, we can expect the identification of even more novel peptides and perhaps even novel bacteriocin classes in the future. Despite the growing number of known peptides, our understanding of their mechanisms of action lags behind. Investigating the cellular targets of bacteriocins is crucial for choosing the correct antimicrobial for specific pathogenic species and for understanding resistance mechanisms. This will also enable the rational design of bacteriocins through peptide bioengineering, potentially leading to the development of highly targeted and potent therapeutic agents.

Bacteriocins hold great promise in complex microbial communities such as the gut microbiome with their ability to selectively target specific pathogens or pathobionts without disrupting the overall community structure and function. However, the potential unintended consequences of such interventions must be carefully considered to avoid disruptions to microbial ecosystems that could lead to undesirable health effects. One substantial challenge is translating laboratory discoveries into clinical applications. Bacteriocins offer a viable alternative or adjunct therapy in the treatment of infections, especially against multidrug-resistant pathogens. Realising these opportunities will require substantial funding and rigorous clinical studies. In addition to their antimicrobial properties, structurally related peptides have been identified that lack antimicrobial activity but possess other valuable bioactivities, such as pain relief or anti-inflammatory effects¹⁷². These findings expand the horizon of bacteriocins beyond antimicrobials, conceivably offering multi-purpose therapeutic agents.

In conclusion, bacteriocins have the potential to act as precise and versatile tools in an ongoing battle against antimicrobial resistant microorganisms. As we continue to explore their intricacies, we pave the way for a future where these remarkable molecules could have a pivotal role in safeguarding our health and well-being. Although challenges lie ahead, the potential for bacteriocins as tools to eliminate clinically relevant microorganisms and precisely shape microbial communities is an exciting and promising avenue for research.

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Author contributions

I.S. researched data for article.

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Competing interests

The authors declare no competing interests.

1206 Table 1. Suggested updated bacteriocin classification scheme with examples*.

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Bacteriocin class	Subgroup	Defining features) and conserved enzyme (if known/present)
I	Bottromycin	Macrolactamidine, YcaO
I	Cyanobactin	N-terminal proteolysis enzyme, PatA
I	Darobactin	Unusual double ring structure from tryptophan tryptophan,-lysine or -arginine linkages, rSAM
I	Epipeptide	D-amino acids, rSAM
I	Glycocin	S- and O-glycosylation of Serine/Threonine
I	Lanthipeptide, type I	Methyllanthionine and/or lanthionine residues
I	Lanthipeptide, type II	Methyllanthionine and/or lanthionine residues
I	Lanthipeptide, type V (lanthidin)	Methyllanthionine and/or lanthionine residues
I	Lasso peptide	Macrolactam with threaded C-terminal tail
I	Linaridin	Dehydrobutyrine, no lanthionine
I	Linear azole or azoline containing peptide (LAP)	Azol(ine)s, YcaO
I	Pantocin	Glu-Glu crosslink, PaaA
I	Pyritide (including thiopeptides)	Six membered nitrogenous heterocycle
I	Sactipeptide	Intramolecular sulfur-to-alpha carbon thioether (sactionine) crosslink
I	Circular	Covalently linked N- and C-terminal residues re in circular peptide backbone
I	Microcins with non-ribosomal siderophore	Serine-rich C-terminus with a non-ribosomal siderophore-type modification
II	Pediocin-like	Contains YGNGVXC motif
II	Linear, two-component	Two peptides, both required for activity
II	Linear, non-pediocin, non-two component	Lacks defining features of other groups
II	Defensin-like	Conserved disulphide pattern of Eukaryotic def
II	Leaderless	Leaderless peptides with or without four-helix

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1210 * Bacteriocins can be generally classified into two major groups; post-translationally modified
 1211 (class I), and non-significantly-post-translationally modified peptides (class II). Both groups
 1212 are further subdivided on the basis of conserved features unique to the subgroup. Within class
 1213 I bacteriocins groups can be defined according to a specific modification that is installed by
 1214 one or more modification enzymes.

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 1218

Table 2. Bacteriocins and producers undergoing investigation for therapeutic application.

Form	Bacteriocin	Class	Producer	Clinical trial phase (completed)	Treatment and animal model (if applicable)	Administration	Result	Company
Peptide	NVB333 (Ref. ¹⁸⁷)	Lantibiotic	<i>Actinoplanes liguriae</i> NCIMB41362	Preclinical	Mouse thigh infection model, MRSA disseminated	Intravenous	Equal efficacy to vancomycin	Novacta
Peptide	NVB302 ¹⁸⁸	Lantibiotic	<i>Actinoplanes liguriae</i> NCIMB41362	Phase I	<i>Clostridioides difficile</i> infection	Oral	Not reported ¹⁸⁹	Novacta
Peptide	Mutacin 1140 and analogs ¹⁶³	Lantibiotic	<i>Streptococcus mutans</i> JH1000	Preclinical	Mouse MRSA skin and systemic infection	Intravenous or Topical	Effective intravenous treatment	Sano Chemicals
Peptide	OG716 (mutacin 1140 derivative) ¹⁰⁷	Lantibiotic	<i>Streptococcus mutans</i>	Preclinical	Hamster model of <i>Clostridioides difficile</i> infection and associated diarrhoea	Oral	100% survival and no relapse	-
Peptide	NAI-107 (Microbisporicin) ¹⁹⁰	Lantibiotic	<i>Microbispora corallina</i>	Preclinical	Acute severe infections in mouse and rat models	Intravenous	Lower effective dose than best alternative, rapid bactericidal activity	Naicons SRL, Sentinelia Pharmaceuticals
Peptide	Moli1901 (Duramycin/Lancuvotide) ^{169,170}	Lantibiotic	<i>Streptomyces cinnamomeum</i>	Phase I and Phase II	Cystic fibrosis symptoms	Intranasal, inhalation	Well tolerated, no difference from placebo	Lantibio/AOP Orphan Pharmaceuticals
LBT	Sh-lantibiotic α/β (Ref. ¹⁶²)	Lantibiotic	<i>Staphylococcus hominis</i> ShA9	Phase I	Atopic dermatitis	Topical	Achieved primary safety endpoint and decreased <i>Staphylococcus aureus</i>	-
LBT	Abp-118 (Ref. ¹⁶⁴)	Pediocin-like	<i>Ligilactobacillus salivarius</i> PS7	Phase I	Recurrent acute otitis media	Oral consumption	Significant decrease (84%) in AOM episodes	ProbiSearch

LBT	Multiple ¹⁵⁸	Multiple	<i>Bacillus velezensis</i> ADS024	Preclinical	<i>Clostridoides difficile</i> infection	Oral	Inhibited <i>Clostridoides difficile</i> infection in mice and did not colonise mice or swine after 28 day repeat dose	Adiso Therapeutics
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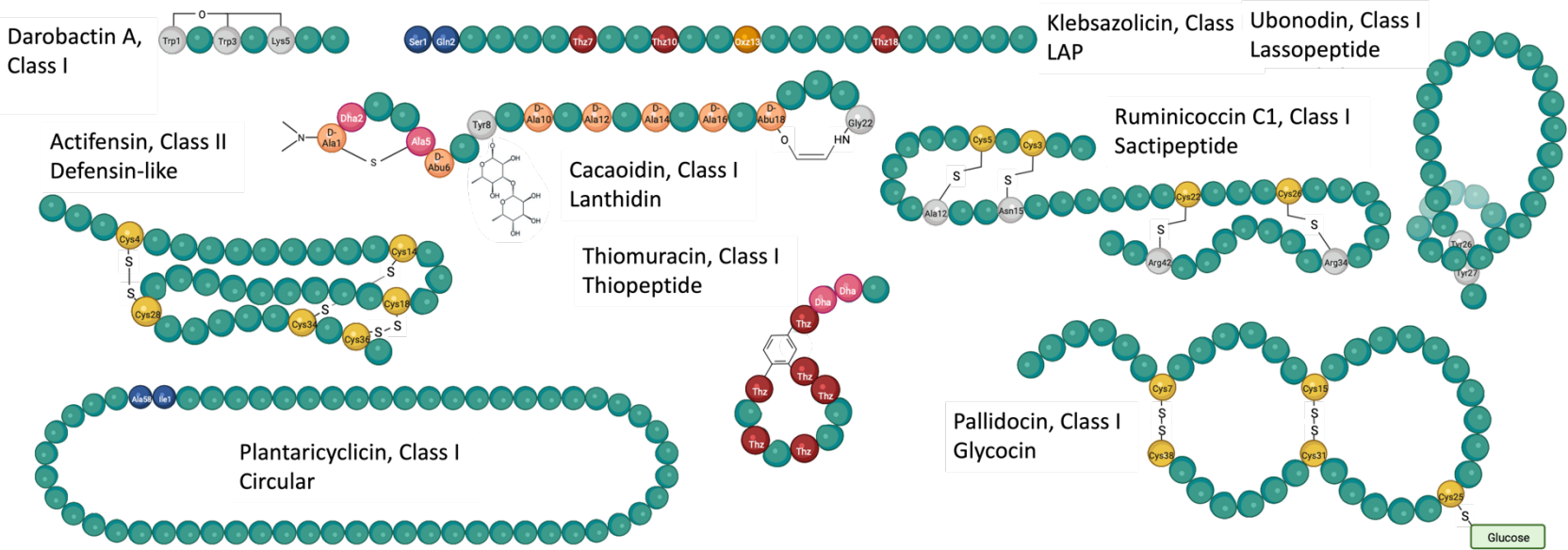
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1221 AOM, acute otitis media, LBT, live biotherapeutic. MRSA, methicillin-resistant

1222 *Staphylococcus aureus*

1223 Figure 1. **The structural diversity of bacteriocins.** Bacteriocins are typically classified into classes and subgroups based on their mechanism of
1224 production and secondary structures (Table 1). Lanthipeptides and other class I bacteriocins may contain complex intra-peptide bonds that are
1225 installed through enzymatic modifications of specific residues in the peptide backbone. These bonds may form small ring structures such as the
1226 C-O-C Trp-Trp ether bond in darobactin A (key residues coloured in grey) and the cacaoidin AviMeCys ring (modified residues coloured in orange).
1227 Multiple intrapeptide crosslinks may be formed following modification within one peptide such as the sacchioine bonds in ruminicoccin C1
1228 (involved cysteine residues coloured in yellow). Class I bacteriocins may also feature posttranslational installation of other moieties, shown here
1229 in glycosylated pallidocin at cysteine-25, and tyrosine-8 of cacaoidin. Modified residues are not always part of ring structures, such as the thiazole
1230 and oxazole residues present in the linear azoline-containing peptide, klebsazolicin. Large ring structures, however, are prominent features of
1231 different subgroups of class I bacteriocins and take different forms such as the loop structure present in the lasso peptide ubonodin that sterically
1232 locked by bulky tyrosine side-chains, or the macrocycle of the heavily modified thiomuracin A. The largest rings are present within the circular
1233 bacteriocins wherein N- and C-terminal residues are covalently linked resulting in an closed loop structure. Class II bacteriocins do not undergo
1234 enzymatic modification and are therefore less enzymatically decorated but may feature disulphide bonds that can also result in intercalated
1235 structures of such as those in defensin-like bacteriocins, like actifensin.



1237 Figure 2. **Bacteriocins mechanisms of action and resistance mechanisms.** Bacteriocins are heterogenous in function and exhibit various
1238 mechanisms of action. Bacteriocins are typically cationic hydrophobic peptides that are drawn towards anionic bacterial cell surfaces. At the
1239 surface they can interact with specific receptors, such as nisin that complexes specifically to the cell wall precursor molecule lipid II and
1240 subsequently forms pores across the membrane, which leads to cell death. Mechanisms of immunity and resistance protect strains from nisin by
1241 binding, ejecting or proteolytically cleaving the peptides. Pediocin-like bacteriocins (class II) attach to the extracellular surface of the mannose-
1242 PTS core-domain, penetrate the membrane and shift the core domain away from its V-motif, thereby forming a pore in the membrane. PedB
1243 proteins in the targeted bacterium can physically plug the pore formed by pediocin PA-1 and the mannose-PTS system thereby providing cytosolic
1244 immunity. Other peptides target the membrane directly, such as aureocin53 that initially causes Gram-positive membrane thinning by accumulating
1245 in the upper leaflet of the phospholipid bilayer which is mediated by specific alpha-helices of the peptide, then causes poration favoured by other
1246 alpha-helices within the peptide. Bacteriocins can also interrupt essential cellular processes such as outer membrane protein integration of BamA
1247 by darobactin, or inhibition of protein and nucleic acid synthesis of klebsazolicin and capistruin, respectively. Microcin E492 translocates across
1248 the outer membrane using a ‘Trojan horse’ strategy wherein the siderophore moiety is recognised by outer-membrane transporters that facilitate
1249 uptake of the peptide into the periplasm, followed by binding to the mannose-PTS system at the inner membrane and subsequent lethal disruption
1250 of membrane potential. Immunity to microcin E492 is provided by the action of the immunity protein MceB, via an unknown mechanism.

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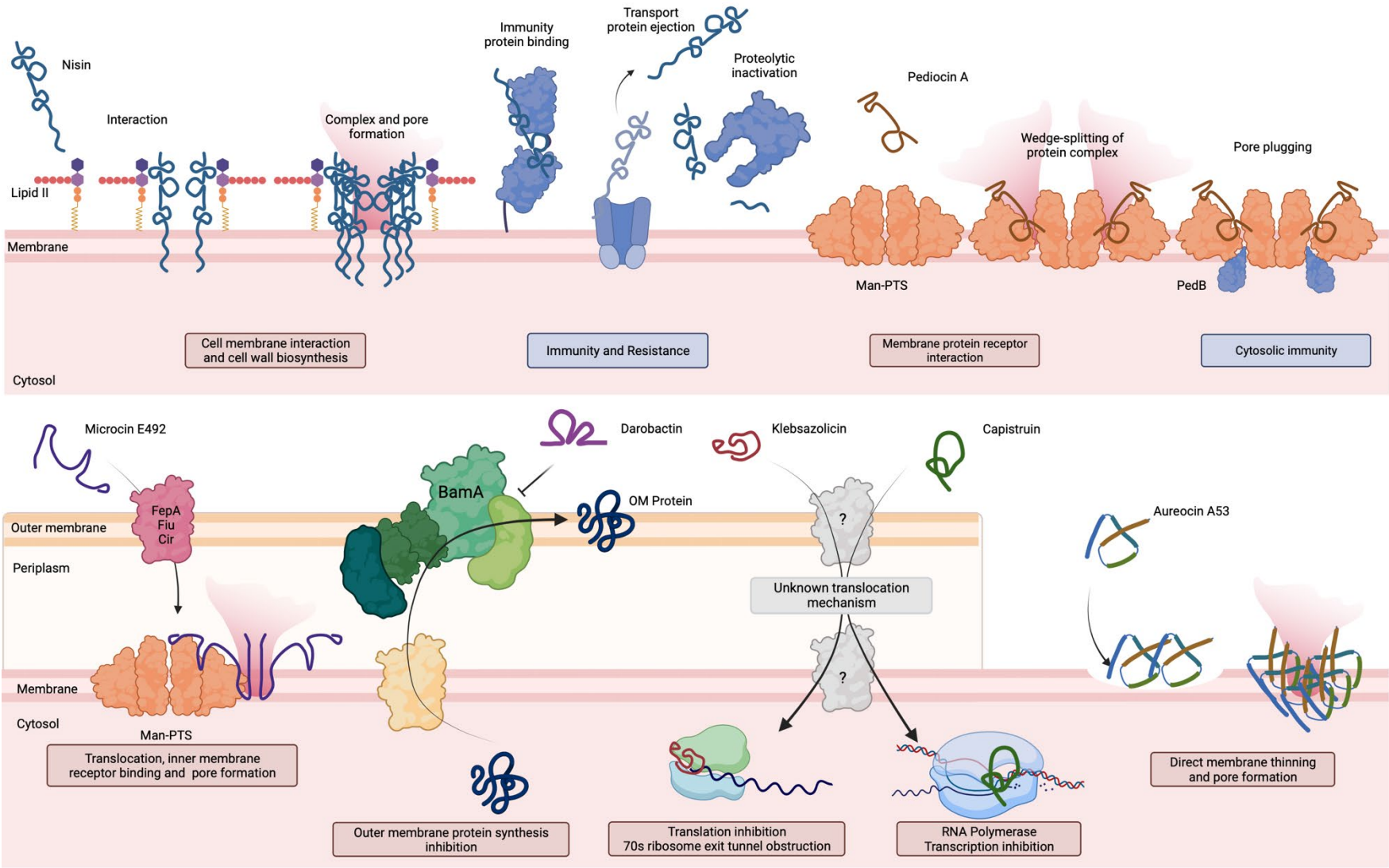
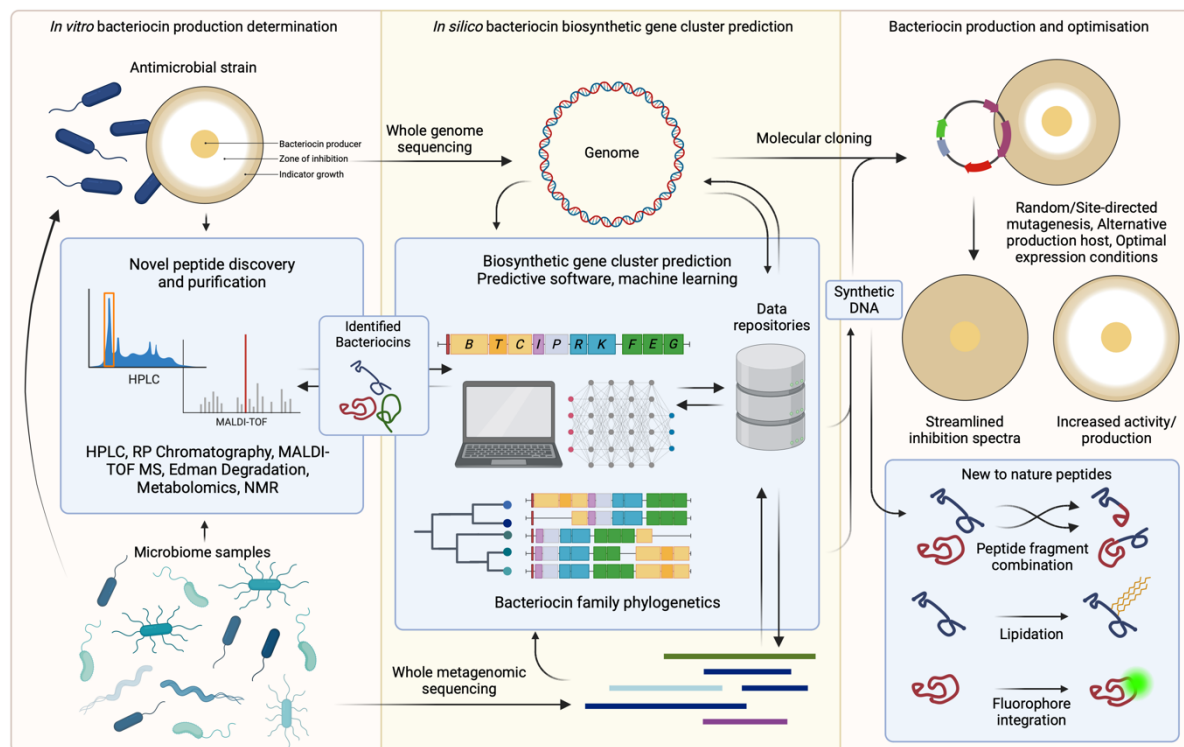


Figure 3. **Methods of *in silico* bacteriocin identification and translation to *in vitro* production, optimisation and bioengineering.** Until the recent proliferation of large-scale whole metagenomics bacteriocins were mainly identified *in vitro* through screening with agar-based assays. Specific antimicrobial peptides could be identified and purified through a number of techniques including mass spectrometry and HPLC (left panel), and corresponded to a biosynthetic gene cluster identified from a sequenced genome (middle panel, top). Now, large datasets can be more easily generated or sourced from data repositories and mined for bacteriocin gene clusters with improved predictive software, and corresponded with metabolomic data to identify peptides (left panel, bottom, and middle panel) . *In silico* mined peptides and known peptides can be heterologously expressed in desirable hosts to optimise production and/or modified to generate mutant peptides with desirable characteristics like improved activity, or streamlined inhibition spectra (right panel, top). Novel techniques have also been developed to further modify expressed peptides through recombination of peptide fragments generating new-to nature peptides, or modification with compounds of specific properties, such as lipids or fluorophores (right panel, bottom). HPLC, high performance liquid chromatography; RP, reverse phase, MALDI–TOF MS, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; NMR, nuclear magnetic resonance.



Box 1. History of bacteriocin nomenclature.

Searching the literature with the term ‘bacteriocin’ returns more than 11,000 publications, describing a heterogeneous group of compounds that mostly share two general features; bacterial ribosomally-synthesised peptides/proteins that are antagonistic towards other bacteria. These compounds can range from multi-protein contractile nano-machines¹⁹¹ to antimicrobial secreted peptides¹⁹². The lack of a precise definition after a century of bacteriocin research results in a field that can often be contradictory and confusing.

Jacob *et al.* first coined ‘proteins of the colicin type’ as ‘bacteriocins’ following Andre Gratia’s initial work in 1925 describing colicins as the source of strain-specific antagonistic activity between *Escherichia coli*^{193,194} (see the figure). Subsequent publications used ‘bacteriocin’ to describe lytic proteins from Gram-positive bacteria, such as megacin from *Bacillus megaterium*¹⁹⁵. The discovery of nisin was published in the same decade as colicin²

but Gram-positive bacteria-derived antimicrobials were not as widely described as bacteriocins for decades¹⁹⁶. In 1976, Tagg *et al.* acknowledged no universally accepted definition existed, and criticised the use of the term ‘bacteriocin’ for poorly characterised compounds. It was proposed that ‘bacteriocin’ should describe bactericidal compounds containing a biologically active protein moiety¹⁹⁶. The 1993 Klaenhammer scheme expanded this definition, which is the basis for modern bacteriocin classification. Peptides were grouped into class I (post-translationally modified) and class II (unmodified), with larger proteins grouped as class III¹⁹⁷. Research on bacteriocins from Gram-positive bacteria grew considerable, and in 2005 Cotter *et al.* suggested that ‘bacteriocin’ be reserved for peptides rather than heat-labile proteins¹⁹⁸. The term has not ceased to be used for these larger proteins. Other schemes have been proposed including up to four classes, and many bacteriocins are now popularly referred to as RiPPs (ribosomally produced and post-translationally modified peptides)^{3,23,199}. We suggest consistent usage of the two major class scheme for bacteriocins to provide clarity for future researchers navigating the literature.

