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Pre-formulation and delivery strategies for the development of bacteriocins as next generation antibiotics

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ABSTRACT

Bacteriocins, a class of antimicrobial peptide produced by bacteria, may offer a potential alternative to traditional antibiotics, an important step towards mitigating the ever-increasing antimicrobial resistance crisis. They are active against a range of clinically relevant Gram-positive and Gram-negative bacteria. Bacteriocins have been discussed in the literature for over a century. Although they are used as preservatives in food, no medicine based on their antimicrobial activity exists on the market today. In order to formulate them into clinical antibiotics, pre-formulation studies on their biophysical and physicochemical properties that will influence their activity *in vivo* and their stability during manufacture must be elucidated. Thermal, pH and enzymatic stability of bacteriocins are commonly studied and regularly reported in the literature. Solubility, permeability and aggregation properties on the other hand are less frequently reported for many bacteriocins, which may contribute to their poor clinical progression. Promising cytotoxicity studies report that bacteriocins exhibit few cytotoxic effects on a variety of mammalian cell lines, at active concentrations. This review highlights the lack of quantitative data and in many cases even qualitative data, on bacteriocins' solubility, stability, aggregation, permeability and cytotoxicity. The formulation strategies that have been explored to date, proposed routes of administration, trends in *in vitro/in vivo* behaviour and efforts in clinical development are discussed. The future promise of bacteriocins as a new generation of antibiotics may require tailored local delivery strategies to fulfil their potential as a force to combat antimicrobial-resistant bacterial infections.

1. Introduction

Antimicrobial resistance (AMR) is a major contributing factor to mortality and morbidity across the world with major public health and societal implications [1,2]. While antibiotics have revolutionised medicine, AMR poses a major threat to hinder and even reverse this progress. AMR occurs due to genetic factors intrinsic to bacteria; cellular level resistance with mutations and horizontal gene transfer (HGT) of resistance determinants, and community-level resistance including biofilms or persister cells [3]. The rise in AMR has been accelerated by the inappropriate use of antibiotics in agriculture, food and pharmaceutical/medical sectors [4]. The World Health Organisation (WHO) has attempted to focus research towards novel therapeutics as the number of antimicrobial agents in the clinical pipeline is not sufficient to mitigate this problem and the rate of development of novel antimicrobials is not fast enough. They are also steering research towards systems to effectively monitor the rising levels of AMR [5,6]. One potential new class of

antibiotics are antimicrobial peptides (AMPs), and in particular, a class of AMPs called bacteriocins [7-9]. AMPs are gene-encoded polypeptides that are ribosomally produced by nearly all organisms from bacteria to plants [10]. They are small (30–60 amino acids), mostly cationic, hydrophobic or amphiphilic peptides [11-13]. Certain Gram-negative bacteriocins resemble eukaryotic defensins, where ribosomally produced bacteriocins exhibit narrow activity ranges, and non-ribosomally produced bacteriocins demonstrate wider activity ranges to bacteria and fungi [14]. There are a variety of databases showcasing documented AMPs [15-17]. For example, LAMP2, set up in 2013 by Dr. Wu Hungyu [18,19], APD3, set up in 2015 by Dr. Guangshun Wang [20,21], and DRAMP, the database established by Heng, Zheng *et al.*, [16,22]. Koo *et al.* reported 34 AMPs in preclinical trials in 2019 and 27 in clinical trials. There have been 10 AMPs discontinued during drug development due to failure at phase I or II of clinical trials [23]. Despite the high number of AMPs in development, only a few AMPs have reached the market e.g. daptomycin, gramidicin, bacitracin, caspofungin,

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dalbavancin, PAC-113 (P-113) and bilracidin. The molecular weight of these are all low, < 2 kDa, and the stability of the formulations is limited [24–28].

A bacteriocin is an AMP produced by bacterial strains [29]. The producer bacterium has specific immunity against the bacteriocin it produces. Bacteriocins are categorised in terms of their chemical/biochemical composition and whether the producing bacterium is Gram-positive or Gram-negative. Gram-positive bacteriocins are divided into three major classes, by Rea *et al.*: Class Ia, Ib and Ic – the lantibiotics, the labrithopeptins and the sactibiotics respectively; Class II – unmodified, heat stable, positively charged bacteriocins which can be subdivided further into four different classes; class IIa- pediocin-like bacteriocins, class IIb- two-peptide unmodified bacteriocins, class IIc- circular bacteriocins and class IId - unmodified, linear, non-pediocin-like, single peptide bacteriocins (other) and Class III - bacteriolysins [30]. Rebuffat *et al.* best describe Gram-negative bacteriocins which are segregated into three categories, colicins, microcins and phage tail-like bacteriocins [31].

Several bacteriocins have been identified with activity against clinically relevant Gram-negative and Gram-positive bacteria. These include bacterial strains that show resistance to commonly used antibiotics, such as *C. difficile*, methicillin-resistant *S. aureus* (MRSA), *S. pneumonia* and vancomycin-resistant enterococci (VRE) [9,32–34]. Thus, bacteriocins may be a potential alternative to traditional antibiotics. There are a variety of different modes of action employed by bacteriocins to inhibit competing bacteria. Briefly, this can involve the binding of bacteriocins to the pyrophosphate moiety of lipid II at the cell wall of Gram-positive cells, ultimately leading to the disruption of cell wall biosynthesis (Fig. 1a). Other modes for Gram-positive bacteriocins involve pore formation with subsequent leakage of intracellular components (Fig. 1b and c). Gram-negative bacteriocins typically utilise translocation across the cell membrane (Fig. 1d). The different modes of action employed by bacteriocins are best described in a recent review by Meade, *et al.* [14].

Dicks *et al.* provides an overview of bacteriocins' potential in medical applications, assessing their capability as spermicides/contraceptives and for treating upper respiratory tract infections, urogenital tract infections and systemic infections. They also address issues such as

resistance and immunity [35]. Some 230 identified bacteriocins with a wide range of antimicrobial effects and properties are documented in Bactibase, a free-to-access online database set up by Dr. Hammani [36]. As bacteriocins exhibit antimicrobial activity against clinically relevant pathogens that have become resistant to current antimicrobial therapeutics, they have potential for development into clinical applications. Although bacteriocins are used in food preservation [29,37], no bacteriocin is currently commercially available as an antibiotic.

This review aims to elucidate trends in research on the physico-chemical and biophysical properties of bacteriocins and ascertain research directives for their subsequent clinical development. Strategies that have been suggested to date for the formulation of bacteriocins are discussed and essential parameters that are currently lacking for their development into medicines for antimicrobial-resistant infections are identified.

2. Bacteriocins

While the mechanism of antimicrobial activity and structure–function relationship are critical to the performance of bacteriocins, they are beyond the scope of this review and are best described in a recent review by Cotter *et al.* [38]. Analysis of the bacteriocin database Bactibase showed that the number of Gram-negative bacteriocins that have been discovered is much lower than the number of Gram-positive bacteriocins with only 21 of the 230 bacteriocins documented being produced by Gram-negative bacteria. This proportion of Gram-negative versus Gram-positive bacteriocins is reflected in the bacteriocins that have generated significant academic research and published data, selection shown in Table 1. The criteria for defining significant academic research and published data is based on those bacteriocins with a significant amount of published data on, for example, their molecular weight, classification, antimicrobial activity, thermal and pH stability, solubility and/or enzymatic degradation.

For bacteriocins that exhibit antimicrobial activity toward clinically relevant pathogens, a minimum inhibitory concentration (MIC) is essential to determine the required dosage concentration. Purified and formulated bacteriocins should have their MIC determined and

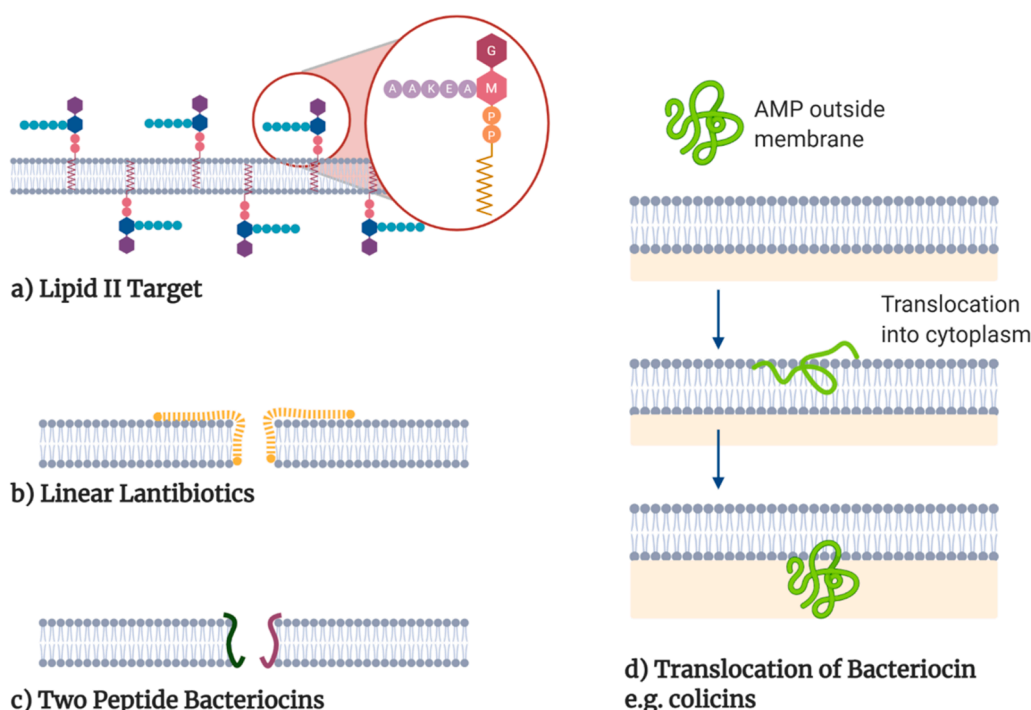


Fig. 1. Modes of action typically employed by bacteriocins to inhibit competing bacteria, figure created using Biorender.com.

Table 1

A collection of physicochemical data presented in the literature for a variety of documented bacteriocins, as available in December 2020.

Bacteriocin*	pI	Solubility	Stability		Solution/pH	Thermal	Ref
			Susceptible to	Not Susceptible			
(Gardimycin) Actagardine	3.85	Insoluble in H ₂ O, MeOH, DMSO. Soluble in acetonitrile:water (7:3), trifluoroethanol: water (9:1)	N/a	N/a	2.2–8.5, optimum ≤ 6	48 hr at room temp, decomposition at 260 °C	[53,54 55]
Acidocin 8912	9.87	N/a	T, Pep, CT, ProK	N/a	N/a	Up to 120 °C for 20 min	[56]
BacCH91	7.92	N/a	N/a	UL StpA/B/C (V8) PD, T, CT, Pep, ProK, ELT, CpsG	Active pH 3–6	80 °C (up to 1 hr)	[57]
Bactofencin A	10.59	PBS, aq.	T, ProK	N/a	N/a	N/a	[58]
Bavaricin-A	9.13	N/a	Pep, T, ProE, ProK,CT	H ₂ O ₂	Active pH 5–9.7 only	100 °C for 60 min	[59]
Boticin B	3.64	N/a	N/a	N/a	N/a	Heat stable	[60]
Bovicin HJ50	8.34	Hydrophobic, soluble in n-propanol	T, SubT, ProK	N/a	N/a	N/a	[61]
Carnocin UI49	6.41	Hydrophobic	T	N/a	N/a	<60 °C (pH 6.5)	[62,63]
Cinnamycin (Lanthiopeptin)	8.05	EtOH, MeOH, DMF, DMSO. 5 mg/ml in H ₂ O, 10 mg/ml in DMSO	N/a	N/a	Acidic conditions	Decomposition at 245 °C	[64]
Curvacin-A	9.37	Low (aq.)	ProK, T	Pep, BSA, RNase	Low stability	Below 60 °C, 60–100 °C (30 min)	[65,66]
Divergicin A	9.67	Very hydrophobic	N/a	N/a	Rapidly degrades in ACN, stable in EtOH	N/a	[67]
Duramycin (B) (Leucopeptin)	8.04	N/a	N/a	N/a	N/a	Resistant to thermal degradation	[68,69]
Enterocin A	8.98	N/a	T, Pep, Pap (85% degradation)	N/a	Active pH 2–8, 10 (12 hr)	100 °C at pH 2 and 4	[70]
Enterocin E-760	8.91	N/a	CT, ProK, Pap	LZ, LP	Stable pH 5–8.7	100 °C for 5 min	[71]
Enterocin NKR-5-3B (Ent53B)	10.47	N/a	T, CT	N/a	Human serum (24 hr), HD ₅₀ 6.9 μM (cyclic)	Cyclic bacteriocin-high, Linear bacteriocin-lower (particularly freeze thaw)	[72]
Enterocin P	8.22	N/a	P	N/a	pH 2–11 (24 hr, 25 °C)	60 min (100 °C), 15 min (121 °C)	[73]
Enterolysin A	9.64	N/a	ProK	N/a	N/a	< 50 °C, no activity > 80 °C	[74,75 76]
Fulvocin-C	3.75	N/a	N/a	RNase, DNase, T, CT, Pro	Active in Chloroform, EtOH, Acetone, Diethylether & Ethyl Acetate.	80 °C for 30 min max.	[77]
Halocin-C8	4.4	N/a	ProK	T	Can be desalted and subjected to organic solvents without losing activity	100 °C for 1 hr	[78]
Hominicin	3.85	N/a	ProK,	T, CT, Pep, LP	Active pH 2–10	Up to 121 °C for 15 mins	[79]
Lacticin 3147 Ltn α	5.44	N/a	CT, T, ProA, ProK	Pep	N/a	Heat stable, at a range of pH	[40,80]
Lacticin 3147 Ltn β	8.51	N/a	CT, T, ProA, ProK	Pep			
Lacticin 481 (DR)	7.35	N/a	Pro, Fic, ProK, CT	T	Opt. pH 6 stable pH 2–10	1 hr at 100 °C (pH 4.5, 7)	[81 82]
Lactocin-S	7.10	Hydrophobic – theoretical	P	T	pH 4.5–9, not active > pH6. oxidative instability	50% after 1 hr at 100 °C	[83–87]
Lactococin MMFII	7.25	N/a	ProK, T, Pap	LZ, LP, GA, α A	Active pH 5–8	Below 70 °C (30 min)	[88]
Leucocin-A (UAL 187) (unpurified)	8.77	N/a	T, CT, Pap, Pep, P	LZ, LP, PhLP	Stable at pH 2–3 (<5)	Boiling 20 min, at pH 2/3 only	[89]
Leucocin-B (Tal1a)	8.77	N/a	Pro, ProK	LZ, Cat, CHCl ₃	pH 2–9, optimum 2–4	100 °C (30 min), active after 121 °C (15 min) at pH 2–4 only	[90]
Mersacidin	3.85	MeOH, CH ₃ CH	N/a	N/a	pH 5–7 at ambient temp.	Ambient temperature	[41]
Mesentericin Y105	8.77	N/a	Pro, ProK, T, CT, Pep	N/a	Low pH only	100 °C for 120 min (pH 4.5)	[91,92]
Microcin J25	5.36	H ₂ O, MeOH, up to 800 μg/ml	ProK, ELT, Pan	CT, Pep, P (S. aureus V8), T	pH 2–12 & 8 M urea	120 °C for 15 min	[93–98]
Mutacin-2	8.06	N/a	T	N/a	pH 4–10 (24 hr)	Active after boiled up to 30 min	[99]
NAI-107 (peptide1)	5.66	N/a	N/a	N/a	pH 2–6, plasma half-life 14 hr (IV)	N/a	[100,101]
NAI-107 (peptide 2)	5.66	N/a	N/a	N/a	pH 2–6, plasma half-life 14 hr (IV)	N/a	[100,101]
Nisin A	8.52	pH 2 highest	CT, Nis, SubPep, Pan, Pep	T	< pH 6, optimum pH 2	Heat stable up to 121 °C, 15 min	[102–106]

(continued on next page)

Table 1 (continued)

Bacteriocin*	pI	Solubility	Stability				Ref
			Susceptible to	Not Susceptible	Solution/pH	Thermal	
Nisin Z	8.51	pH 3 (75 °C), higher solubility than nisin A at pH 5	CT, ProK, ActE	T, Pap, Pep, LP, αA	pH 3, stability reduced above and below	Heat stable up to 121 °C, 15 min	[105,107,108]
Nukacin ISK-1	8.53	N/a	P, CT, Pep, Pan, Fic, Pap, ActE	LZ, LP, RNase RNaseA, αA, T, ProK	Acidic conditions	60% at 121 °C, 20 min (pH 3–8)	[109]
Pediocin PA-1/AcH	8.85		Pep, Pap, T, Protease IV/XIV/XXIV/K, CT, Fic	RNaseA, LZ, LP,	Active pH 2–10	100 °C (60 min), 121 °C (15 min)	[110–112]
Penisin (Elgicins)	7.8	N/a	N/a	ProK, T	Stable at pH 2–12	Up to 100 °C, no more	[113]
Pep5	11.08	N/a	T, CT, Pro	Pep, αA, RNase, DNase, LZ, LS, CPD, PhLP	Stable in aqueous, pH 2–8	80 °C for 1 hr, > 3 months at 4 °C, lyophilisation	[114]
Plantaricin 432	N/a	N/a	CT, T, ProK, Pep, Pap	N/a	Active pH 1–10	Active at 80 °C, 50% activity at 100 °C for 60 min and 25% activity after autoclaving (121 °C for 15 min)	[115]
Plantaricin C19	9.87	N/a	N/a	N/a	Active pH 5.5–7 only	7 °C to 37 °C (192 hr)	[116 116]
Plantaricin F	10.64	N/a	Pep, Pro, T, ProK	α-A, UV	pH < 4.5 only, optimal pH 3.5	100 °C for 30 min, 85 °C 1 hr. Activity lost 90 min at 35–100 °C	[117,118]
Plantaricin E	12.08	N/a	N/a	N/a	N/a	N/a	
Plantaricin W α	7.25	N/a	N/a	N/a	Active from pH 2–6, loss above pH 7	Tested from 4 °C to 100 °C, stable up to 121 °C for 15 min	[119,120]
Plantaricin W β	10.63						
Salivaricin 9	8.52	N/a	ProK, PD, Sal9	Cat, LY, detergents	Acidic/Neutral, pH 2–10	90 °C – 100 °C	[121]
Serracin-P	5.8	N/a	ProE	Pap, LP, T, RNase	N/a	Not heat-stable was destroyed at 50 °C (10 min).	[122]
Subpeptin JM4-B	7.30	N/a	ProK, T	ProE, Pep	Active pH 2–8	20 °C to 120 °C for 30 min	[123]
Thuricin CD α	3.85	N/a	N/a	Pep, CT	Active pH 2–9	Up to 85 °C, activity lost at 100 °C	[43,124]
Thuricin CD β	3.85	N/a	Pep, CT	N/a			
Thuricin-17	3.55	N/a	ProK, P	Cat, αA	pH 1.25–9	100 °C for 15 min	[125,126]
Thuricin-S	3.55	N/a	ProK	Pep, T, Pap	Stable pH 3–10.5	100 °C, 10 min	[44]
Variacin	6.47	N/a	ProE, ProK, Fic	Cat	Stable pH 2–10	Up to 115 °C	[127]

**n/a indicates no data available.

αA: α-Amylase, **ActE:** Actinase E, **Cat:** Catalase, **CPD:** Carboxy peptidase, **CpsG:** Catepsin G, **CT:** α-Chymotrypsin.

DNase: Deoxy-ribonuclease, **EIT:** Elastase, **Fic:** Ficin, **GA:** Glucoamylase, **LP:** Lipase.

LS: Lysostaphin, **LY:** Lyticase, **LZ:** Lysozyme, **Nis:** Nisinases, **P:** Protease, **Pan:** Pancreatin.

Pap: Papain, **PD:** Peptidase, **Pep:** Pepsin, **PhLP:** Phospholipase, **Pro:** Pronase, **ProA:** Pronase A.

ProE: Pronase E, **ProK:** Proteinase K, **RNase:** Ribonuclease, **Sal9:** Salivaricin 9, **Stp:** Staphopains.

SubPep: Subtilo peptidase, **SubT:** Subtilisin, **T:** Trypsin, **UL:** Urealysin.

* 19 bacteriocins presented in Table S1 were excluded due to a lack of published data.

compared to the Clinical and Laboratory Standards Institute (CLSI) or the European Committee for Antimicrobial Susceptibility Testing (EUCAST) standard breakpoints. EUCAST specify determination of MIC using EUCAST disk diffusion methodology and subsequent inhibition zone diameters, whereby Mueller-Hinton (MH) agar or MH agar for fastidious organisms (MH-F) is typically utilised. Preparation and storage of media, inoculum, turbidity standards, agar plate inoculation, disk application, incubation including conditions of incubation, examination of plates, measurement and interpretation of zones and quality control conditions are strictly defined (most recently updated in January 2021, V9.0) [39]. Most reported activity assays are performed using the well diffusion assay method with the cell-free supernatant or the purified bacteriocin resuspended in broth [40–44], indicative of their era of publication, as these modes of MIC determination were standard at the time, while newer standardised methods enable better comparative inhibition concentrations. Some re-suspend the bacteriocins in different buffers containing solubilising agents such as Tween 20/80, typically at concentrations of 0.1% [45–48]. While some bacteriocins show potent activity against a range of bacteria, including antimicrobial-resistant bacteria, there is only one bacteriocin mimic, brilacidin, in clinical trials at present [49]. It has been well established that formulation strategies at the early stages of drug product development can be crucial for successful outcomes during preclinical and clinical phases of

development, particularly for molecules with challenging physicochemical properties. In the next section, the physicochemical properties of bacteriocins and their potential influence on the progression of bacteriocins to clinical trials is critically assessed.

3. Physicochemical properties of bacteriocins

Bacteriocins, like any peptide or protein, can aggregate or unfold during formulation, in storage and *in vivo* [50]. This is known as physical instability as the chemical composition of the bacteriocin is unchanged, but its physical state changes. Chemical instability involves the production of new chemical entities through the formation or breakage of covalent bonds within the bacteriocin [51]. This includes enzymatic degradation of proteins and bacteriocins, which can occur during production or *in vivo* [52]. Bacteriocins can display low bioavailability or bioactivity depending on their permeability, stability and solubility *in vivo*. The physical and chemical stability data, available from the literature on bacteriocins is summarised in Table 1 and analysed in the subsequent discussions. The classification of these bacteriocins, their molecular weight, and bacteria against which the bacteriocins are active are available for reference (supplementary information).

3.1. Physical stability

Physical instability in the form of aggregation and unfolding depends on the inherent properties of the peptide. It can result from changes in factors such as pH, ionic strength and temperature, causing a loss in solubility and subsequent loss in activity and biocompatibility. These factors are discussed below with respect to bacteriocins [128,129].

3.1.1. Solubility

Low solubility is a major issue in the drug development process, reducing both *in vitro* and *in vivo* bioactivity [130]. Almost 70% of new drug candidates suffer from the problem of poor water solubility [131,132]. Antimicrobial therapeutics are no exception to this, with traditional small-molecule antibiotics exhibiting poor water solubility, such as tetracycline and ciprofloxacin [133]. Salt formation, if the drug is ionisable or formulation strategies such as the addition of solubilisers, nano-sizing, metastable crystalline/amorphous solid forms or the use of more soluble prodrugs can help to overcome these solubility challenges for small molecules. Careful selection of buffers and solubilising and stabilising additives are typically used to keep peptides and proteins stable in solution or with an additional lyophilisation step, in a reconstitutable solid form, before and after administration. Adequate solubility in physiological media is a prerequisite for good activity.

While the majority of the reported literature on bacteriocins discuss and present their stability in terms of solution, thermal and chemical stability, very few report quantitative solution concentration data, Table 1. Quantitative solubility data is desired as it indicates the concentration of dissolved drug i.e. the amount of bacteriocin which would be in systemic distribution *in vivo*. Depending on the properties of the bacteriocin (e.g. isoelectric point, hydrophobicity/hydrophilicity, amino acid composition), certain pH values at different physiological locations in the body may cause the concentration of dissolved peptide to go below its MIC. This would lead to loss of activity and could cause an unwanted immune response in patients due to precipitation of aggregated peptide. Out of the bacteriocins listed in Table 1, only 11 have reported solubility data. Out of this cluster of 11 bacteriocins, quantitative data was only found for nisin A/Z, microcin J25 and cinnamycin [64,96,103–106]. Qualitative data alone was reported for the remaining 8 bacteriocins. Four studies reported conditions such as ‘low aqueous solubility’ for curvacin A, and ‘hydrophobic’ properties for lactocin-S, divergicin A and carnocin UI49 [63,66,67,85]. Four bacteriocins (actagardine, bovicin HJ50, bactofencin A and mersacidin) reported certain organic solvents that solubilise the bacteriocin [41,55,58,61]. The grand average of hydropathy values (GRAVY) for each bacteriocin in Table 1 was analysed and it was found that 54.9% of these bacteriocins are hydrophobic (positive GRAVY index), and 45.1% hydrophilic (negative GRAVY index) (Fig. 2), an important consideration in terms of their development into medicines. A list of the GRAVY index values is available (supplementary information) [36].

The lack of quantitative solubility data available, the reported low solubility of some bacteriocins in aqueous media and the bioinformatics analysis showing >50% of all bacteriocins in the Bactibase database as being hydrophobic, indicate that there could be challenges in keeping many bacteriocins in solution in the body. It is clear from Table 1 that pH will also play a role in the solubilisation of bacteriocins but the incorporation of solubilisers or complexing agents to improve aqueous solubility could be inherently valuable at early stages of development. Alternative strategies to improve aqueous solubility could be the chemical modification of the bacteriocin but that could also inadvertently affect activity and/or specificity. The reported solubility of some bacteriocins in organic solvents could also enable a wide variety of formulation processes such as encapsulation in PLGA microspheres or solid lipid nanoparticles while retaining activity and high encapsulation efficiencies. Such strategies could be then used for long-acting therapies.

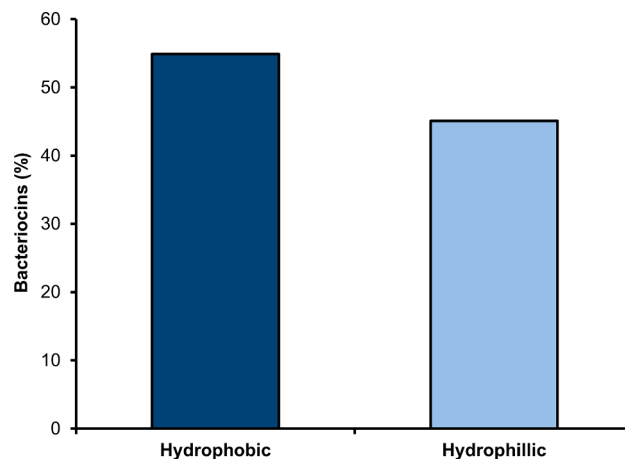


Fig. 2. Graph showing the percentage of all reported bacteriocins that are hydrophobic (54.9%) or hydrophilic (45.1%) based on their GRAVY index. Data sourced from Bactibase[36]

3.1.2. Thermal and pH stability

While bacteriocins often exhibit heat stability, Table 1, it's important to note that it is not a universal property [134]. Bacteriocins that have been found to be thermostable generally exhibit efficacy at a wide pH range [135]. Out of the presented bacteriocins (Table 1), 23 were reported to be stable up to 100 °C (time-dependent), while 8 other bacteriocins showed stability after treatment at 121 °C for 15 min. On the other hand, some bacteriocins such as plantaricin C19 or lactocin S do not report significant thermal stability. Certainly, it appears that the majority of bacteriocins are stable at or above body temperature, 37 °C, indicating that they will not degrade thermally in the body. Several of the bacteriocins are stable over particular pH ranges only, which may dictate what potential routes of administration should be used. For example, instability at low pH means that oral administration through the stomach will be challenging unless formulation strategies such as enteric coatings are used to protect the bacteriocin as it travels through the stomach. Instability at higher pH may compromise solubility, permeability and activity in the small intestine or indeed when the bacteriocin reaches systemic distribution. Low solubility at pH 7.4 may result in precipitation in the body which could induce an immunogenic response. Thus, solubilising strategies and careful dosage regimens would be necessary to ensure good bioavailability, low toxicity and good activity *in vivo* (see section 5).

3.1.3. Aggregation

Understanding how and under what conditions aggregation occurs is important in the development of bacteriocins into antibiotics to ensure the highest efficacy of the bacteriocin and the least toxicity *in vivo*. Biologics in their aggregated state are more prone to elicit immune responses than the parent monomer [136]. It has been reported that crude reuterin 6 self-aggregated, where aggregates were determined to be 200 kDa in size [137]. Other bacteriocins that were reported to aggregate include enterocin AS-37, which forms dimers and higher-order oligomers in aqueous solutions [138–140]. Enterocin P (EntP), another bacteriocin from Enterococci, also has a strong tendency to form aggregates but the size and degree of oligomerisation of these EntP aggregates, found through Western blotting with anti-EntP antibodies, was not presented. Gutierrez *et al.* suggested a considerable reduction in antimicrobial activity in the bacteriocins aggregated state [141]. Enterocin Q (EntQ) was also reported to aggregate when purified, presumably due to hydrophobic interactions. Interestingly, Cintas *et al.*, reported these aggregates to be biologically active utilising an overlay assay [142]. Bhunia *et al.* showed that crude preparations of pediocin AcH tend to aggregate with other proteins and to precipitate during dialysis, but they showed that these aggregates had antimicrobial

activity [111]. Other studies show aggregation of lactacin 481 at neutral or low pH's [143], while lactocin S aggregates with lipid materials and in organic solvents (EtOH), which are required for stabilisation in its purified state [83,85]. Nisin was found to aggregate at high pHs (pH > 7/7.5) with dimers, trimers, tetramers and pentamers observed at pH 11 [103]. Scherer *et al.* studied the aggregation of nisin in the presence of Lipid I, Lipid II and bactoprenol-diphosphate, the lipid responsible for the transport of peptidoglycan monomers across the cell membrane. They found that Lipid I/II induced aggregation, while nisin aggregates with bactoprenol-diphosphate were significantly smaller. They concluded that the membrane permeation capacity of nisin is determined by the size of bactoprenol containing aggregates in the membrane [144]. On the other hand, some bacteriocins, such as plantaricin C19 did not aggregate or associate with other substances under non-dissociating conditions [116]. Microcin J25 which is active at nanomolar concentrations was shown not to form aggregates up to 10 μ M [145]. More experimental detail surrounding the conditions causing oligomerisation, the size of the aggregates, or the inhibitory activity of aggregates for bacteriocins would be valuable to enable rational selection of excipients, e.g. lipids, sugars, polymers, surfactants, etc. to reduce the risk of aggregation, a common approach in the stabilisation of biologics in solution and upon administration [146]. Each bacteriocin may require a specific additive, depending on the mechanism by which it aggregates and its structure. Identification of the conditions under which aggregation occurs would be crucial to identify when and what additive should be trialled to reduce this propensity.

3.2. Chemical stability

Different chemical instabilities such as proteolysis, oxidation, disulfide exchange, de-amidation and hydrolysis will affect bacteriocins, with resulting alterations in efficacy, pharmacokinetics and antigenicity. In the literature, a primary focus is given to proteolysis when discussing chemical degradation of bacteriocins with scant published data on other chemical degradation pathways. Proteolytic enzymes, or proteases, are the enzymes responsible for the break-down of proteins or peptides through proteolysis. Proteases are present in all organisms and have roles in digestion, apoptosis, signal transduction, haemostasis, reproduction and the immune system [147,148].

3.2.1. Enzyme susceptibility

The human degradome contains 588 proteases, of which some are specific and some non-specific [149]. Specific proteases, such as the -angiotensin-converting enzyme, target a unique peptide bond on one protein. Most proteases, however, are non-specific and target multiple substrates [148]. A-chymotrypsin, trypsin and pepsin are non-specific proteases found in the gastrointestinal tract (GI). A-chymotrypsin cleaves bacteriocins at the C-terminal side of the large hydrophobic residues leucine, phenylalanine, tyrosine, and tryptophan, amino acids that regularly exist in bacteriocins, thus increasing their susceptibility to proteolytic cleavage by this enzyme [34]. Trypsin cleaves at the C-terminus of arginine and lysine. Pepsin works by cleaving peptide bonds between adjacent aromatic residues [150]. Pepsin is perhaps one of the most extensively studied proteases for several reasons from its ease of isolation from gastric fluids, to its high proteolytic activity. It is the only proteolytic enzyme in the human stomach, and is present at relatively high concentrations where it varies, depending on digestion, from 0.26 to 0.58 mg/ml [131,151]. Other proteases exist *in vivo* and may also affect the stability of bacteriocins, such as those in the blood (like thrombin) as well as metalloproteases (such as collagenases).

In vitro testing of the susceptibility of bacteriocins to enzymatic degradation in the literature utilises a variety of other enzymes, Table 1. Pronase is a trade name that describes a crude preparation of proteolytic enzymes. These enzymes are isolated from a broth culture of *Streptomyces griseus*, during the production of streptomycin [152]. Pronase E comprises multiple proteases and peptidases that hydrolyse

glycoprotein peptide bonds from both termini [153]. Actinases hydrolyse peptides near proline residues [154]. Ficin is a sulfhydryl enzyme elaborated from the fig tree, containing a cysteine residue that shares many properties with papain. It hydrolyses peptide bonds, particularly those following an aromatic residue [155]. Another protease, proteinase K, is a serine protease from the mould *Tritirachium album*, and a member of the subtilisin family. It preferentially hydrolyses aromatic and hydrophobic amino acids [156]. Pancreatin comprises a multitude of digestive enzymes produced by the pancreas, including lipases, proteases and amylases [157]. It appears, upon survey of the literature that bacteriocins are most susceptible to proteinase K (Fig. 3a), with most being tolerant to trypsin (Fig. 3b). It must be noted, however, that certain proteolytic enzymes would preferentially be utilised in *in vitro* studies for various reasons such as ease of availability and affordability, and as such may not fully represent the stability of bacteriocins in this regard.

The only way to prevent enzymatic degradation *in vivo* is to essentially inhibit the proteases (which could lead to other unwanted side effects) or to hide the bacteriocin from the proteases until it is near the target site of action. There is a wide range of delivery systems designed to protect their cargo from enzymatic degradation – PEGylation, encapsulation into a protective shell or matrix or chemical modification, some of which will be discussed in Section 5. Thus, if the bacteriocin under development is prone to protease degradation along the target route of administration, then such strategies should be employed.

4. The cytotoxicity of bacteriocins

The cytotoxicity of a compound is its toxicity towards eukaryotic cells i.e. its ability to cause cell death [158]. For compounds such as bacteriocins to be approved for use in humans, their cytotoxicity must be fully elucidated to ensure that, alongside their efficacy, the peptides are safe for use. Two common *in vitro* assays to determine the cytotoxicity of a substance is to measure its haemolytic activity and cell viability post-exposure [159]. There are several reports of toxicity data (*in vitro* and *in vivo*) in the literature on bacteriocins, Table 2. The high antimicrobial potency of bacteriocins may lead to toxic effects *in vivo* as AMPs also target eukaryotic cells, however the typically small size of most bacteriocins (<10 kDa), alongside their alteration of hydrophobic and hydrophilic patches provide some prokaryotic specificity, an advantage bacteriocins have over other AMPs [14].

Published bacteriocin toxicity studies include models for a range of routes of administration including oral, subcutaneous, intravenous, intranasal and intravaginal delivery, showing promise for their non-cytotoxic effects. All bacteriocins studied *in vitro*, except cinamycin and microcin J25, showed low toxicity at concentrations that were low, but above their active concentrations. Nisin A and pep5 showed toxicity to vaginal epithelial cells and lung fibroblasts, respectively, at high concentrations. These concentrations, however, are above that required for activity and therefore, should not be a cause for concern. Cinnamycin displayed toxicity against both cell lines tested, HeLa cells and human erythrocytes at low concentrations. Thus, cinnamycin may not be feasible for use as an antibiotic in humans. Microcin J25 showed toxicity towards rat heart mitochondria at low concentrations, but not to MDA-MB-435, MCF-7, RBC or IPEC-J2 cell lines. This highlights where microcin J25 should (GI and cutaneous) and should not (systemic) be administered in the body. Further studies are required for bacteriocins that display toxicity to certain cells, like cinnamycin and microcin J25, to elucidate alternative target sites or routes of administration. Colicins and microcins exhibit cytotoxic effects such as pore formation, peptidoglycan producer degradation, phosphatase activity, 16S rRNA targeting activity and specific DNase and tRNase activity [14]. As discussed previously (Section 2) several bacteriocins show low aqueous solubility or are active at particular pH values only. Care must be taken to ensure that the bacteriocin remains in solution and active during these *in vitro* and *in vivo* studies. If solubilising or stabilising strategies

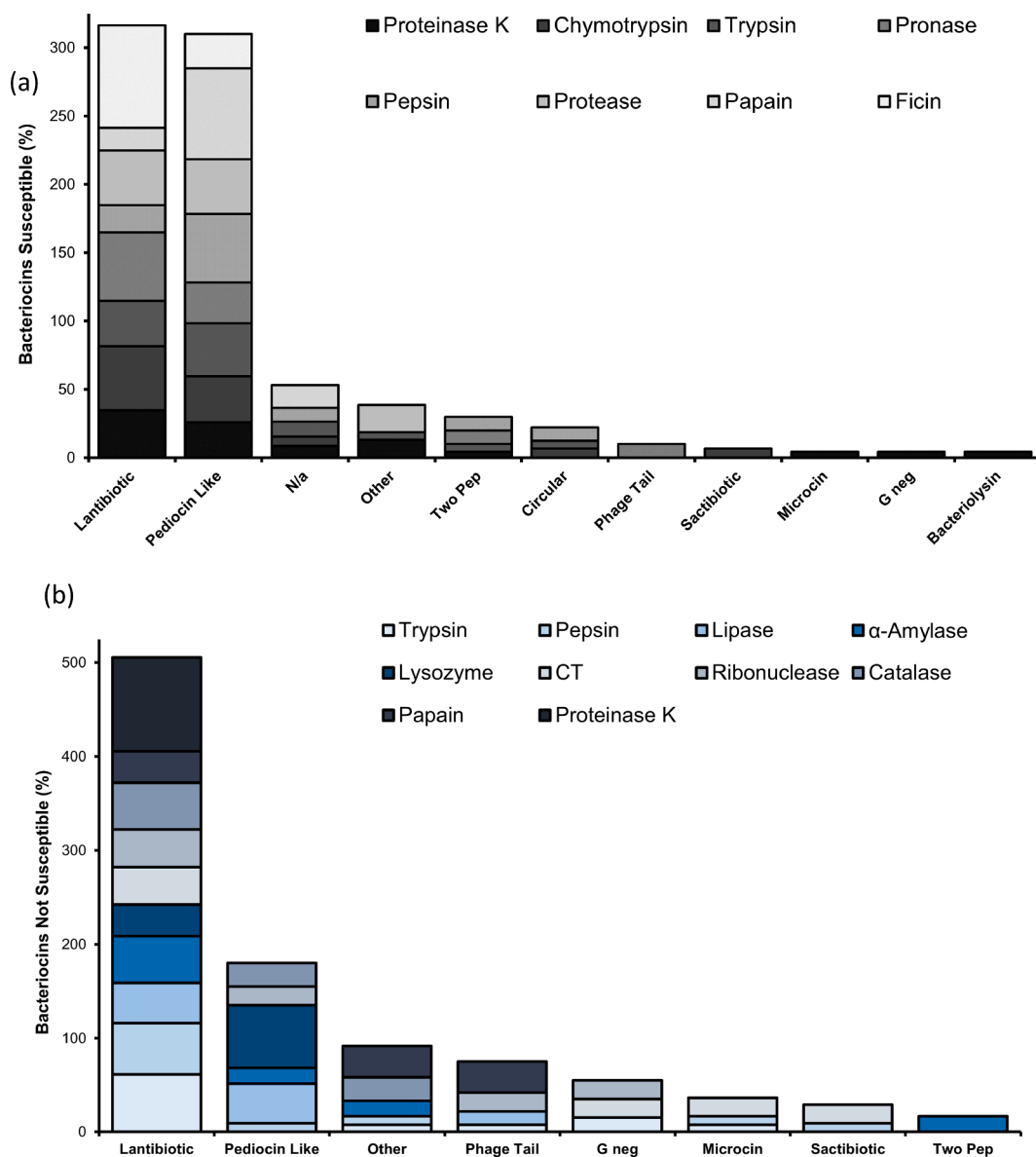


Fig. 3. (a) Susceptibility of the reported bacteriocins to proteases in Table 1 relative to their classification. Only bacteriocins susceptible to > 3 enzymes were included in this analysis and (b) Incidence of bacteriocins reported not susceptible to proteases in Table 1 relative to their classification. Only bacteriocins not susceptible to > 3 enzymes were included in this analysis.

are used to improve the solubility or stability of the bacteriocin, the cytotoxicity of the formulated bacteriocin must then be assessed as it may change the toxicity results due to higher solution concentrations and/or activities. The use of multi-hurdle strategies may help to curb potential cytotoxic effects by lowering concentrations of bacteriocin required, when administered with a traditional antibiotic, for synergistic antimicrobial effect [178].

5. Drug delivery strategies for bacteriocins

It has been shown thus far, that many bacteriocins will need formulation strategies to improve their physicochemical properties for successful clinical development. Depending on these properties, appropriate routes of administration to increase their bioavailability, attain their therapeutic dose at the site of infection and increase their stability can be selected. Thus, protease degradation, aggregation and/or unfolding can be avoided during production and storage and *in vivo* [179].

5.1. Routes of administration and bioavailability

All routes of administration, from parenteral to oral delivery, present challenges for the delivery of biologics like bacteriocins. The bioavailability of a substance is largely dependent on its solubility and permeability. Good water solubility allows a substance to dissolve *in vivo* and good permeability then allows the substance to diffuse through epithelial layers to get to sites of action [132,180]. For example, for an orally administered molecule to reach systemic circulation, it must first pass through the epithelial lining, lamina propria and the vessel wall, while also surviving a range of pHs and degradation by enzymatic proteases and other metabolic processes [181]. A drug's oral bioavailability is known to decrease when its molecular mass is >700 Da. The hydrophobicity of a drug also affects its bioavailability. The permeability of a molecule is higher the more lipophilic it is [50]. Although the large size (>700 Da) of most bacteriocins may decrease their bioavailability after oral administration, their hydrophobicity may increase their permeation leading to higher bioavailability than first expected. Intestinal permeation can be modelled *in vitro*, *in vivo* and *in situ*. The Caco-2 cell system

Table 2
Toxicity studies reported for the bacteriocins outlined in Table 1.

Bacteriocin	Test Subject	Outcome	Ref
In Vitro			
Cinnamycin	<ul style="list-style-type: none"> • HeLa • Human Erythrocytes 	<ul style="list-style-type: none"> • HeLa cells: toxicity was dependent on temperature, • The concentration of cinnamycin to induce 50% hemolysis of human erythrocytes in 4 min of incubation at 37 °C was 5 µM. 	[160 160]
Enterocin AS48	<ul style="list-style-type: none"> • RAW264 cells 	<ul style="list-style-type: none"> • Scarce toxicity to macrophages found. 	[140][161]
Enterocin S37	<ul style="list-style-type: none"> • Caco-2 TC7 • Caco-2 	<ul style="list-style-type: none"> • No significant effect on differentiated monolayer, no apoptotic affect up to 10 mg/L. • Cytotoxicity low for undifferentiated cells (8 hr, 10 µg/ml), increased toxicity after 24 hr (2–10 mg/L). 	[162]
Lacticin 3147	<ul style="list-style-type: none"> • Equine blood cells • Distal colon model 	<ul style="list-style-type: none"> • Did not exhibit toxicity to equine blood cells. • A decrease in firmicutes and bacterioidetes, and an increase in proteobacter observed in the colon model – indicates a negative effect on gut health. 	[163,164]
Microcin J25	<ul style="list-style-type: none"> • Rat heart mitochondria • MDA-MB-435 • MCF-7 • RBC • IPEC-J2 	<ul style="list-style-type: none"> • Rapid depolarisation of mitochondria membrane in rat heart, as low as 1 µM inhibits ATP production. • Low toxicity to MDA-MB0435, MCF-7 cells (100 µM, >80% viability). • Low haemolytic activity (RBC). Displays anti-apoptotic activity (isolated mitochondria). • No significant effect on IPEC-J2 cells – maintains the cell membrane integrity (up to 256 mg/L). 	[165-170]
Nisin	<ul style="list-style-type: none"> • Caco-2, HT29 • Vero • MRC-5 • Vaginal cells • Sperm 	<ul style="list-style-type: none"> • Low toxicity to Caco-2 and HT29, indicates the potential for use in the GI. • Significant toxicity in vaginal epithelial cells only seen at high concentrations (95 µM). • Toxicity in Vero cells at 0.85–3.4 µM, no effect on mycoplasma/RBC (up to 1 µM). 	[171-173]
Penisin	<ul style="list-style-type: none"> • RBC • HeLa • RWPE1 • RAW 	<ul style="list-style-type: none"> • No haemolysis, even at high concentrations. • No toxicity to HeLa, RWPE1, RAW up to 40 µM (>80% viability). 	[113]
Pep5	<ul style="list-style-type: none"> • Human lung fibroblast 	<ul style="list-style-type: none"> • No haemolysis in sheep or human erythrocytes. • ATP efflux ~ 35% at 1 mM, indicating toxicity to lung fibroblasts. 	[173]
In Vivo			
Actagardine (Gardimycin)	<ul style="list-style-type: none"> • Albino CF mice (m/f) 	<ul style="list-style-type: none"> • No signs of toxicity evident up to 250 mg/kg subcutaneously. • LD₅₀ 3,310 mg/kg subcutaneously. 	[53 174]
Enterocin AS48	<ul style="list-style-type: none"> • BALB/c mice 	<ul style="list-style-type: none"> • Administered at dietary levels up to 200 mg/kg for 90 days, no adverse effects. Very small degenerative changes in the liver. 	[140][161]
Mersacidin	<ul style="list-style-type: none"> • Intranasal – Liver 	<ul style="list-style-type: none"> • Intranasal administration did not cause mucosal lesions or morphological liver changes. 	[175]
NAI-107	<ul style="list-style-type: none"> • CD-1 mice (female) 	<ul style="list-style-type: none"> • Very low acute toxicity IV/SC at very high concentration (200 mg/kg). 	[101]
Microcin J25	<ul style="list-style-type: none"> • Mice • Landace × Yorkshire × Duroc pigs 	<ul style="list-style-type: none"> • Orally administered to mice at concentrations of 4.55, 9.1 & 18.2 mg/kg. 9.1 mg/kg gave low inflammation. 	[165-170]
Nisin	<ul style="list-style-type: none"> • Holtzman rats 	<ul style="list-style-type: none"> • Risk that high dosage mice had increased permeability and an imbalance of intestinal bacteria. • No alteration to the oestrous cycle length or cell morphology, no histopathological lesions in the epithelium when administered intravaginally (14 days). Physiological function of the liver and kidneys were unaffected. • The fertility of treated rats was unaffected. 	[171]
Penisin	<ul style="list-style-type: none"> • BALB/c mice 	<ul style="list-style-type: none"> • Mice survived after 8 days of 80 mg/kg injections subcutaneously. 	[113]
Plantaricin E & F	<ul style="list-style-type: none"> • ddY mice • Digestive tract health 	<ul style="list-style-type: none"> • No mortality even at high concentrations (LD₅₀ > 5000 mg/kg (BW) per orally, 48 hr), with optimal doses being 250 and 500 mg/kgBW for E & F, respectively. • Only small amounts of mononuclear inflammatory cell infiltration. • Suggested benefits in digestive tract health. 	[101,176,177]

is the most characterised *in vitro* intestinal permeation model [182], while *in situ* models such as the intestinal perfusion model elucidate the mechanisms of adsorption and determine diffusion constants [183]. A study performed by Spadoni *et al.* confirmed that a molecule of 4 kDa (Fluorescent isothiocyanate (FITC)-low molecular weight dextran) could cross the gut-vascular barrier whereas one of 70 kDa (FITC-high molecular weight dextran) could not [184]. This contradicts the previous 700 Da cut off discussed by Antosova *et al.* [50]. Kruszewska *et al.* suggested that the lantibiotic B, mersacidin, could be absorbed into the bloodstream from the nasal epithelium in mice, but indicated the need for additional studies [175]. A paper by Dreyer *et al.* presents the bacteriocins nisin A, plantaricin 423 and bacST4SA labelled with NHS fluorescein, where they showed the ability of those bacteriocins to cross epithelial and endothelial cells without changing the integrity of the monolayers or exhibiting toxic effects. They showed that 85% of plantaricin 423, 75% of nisin, and 82% of bacST4SA migrated across a Caco-2 cell monolayer within 3 h. The same studies on HUVEC cells showed migration levels across the cell monolayers of 93% plantaricin 423, 88% nisin, and 91% bacST4SA after 3 h. The hypothesised mode of transport was suggested to be paracellular based on the lack of toxicity observed, or alternatively, transcytosis. It also highlighted the need for increased permeability and cytotoxicity studies, apoptosis assays, haemolysis assays and more research into the mechanism involved in bacteriocins

crossing epithelial cells [185]. A recent review by Dicks *et al.* best describes the permeability of bacteriocins across the gut blood barrier, concluding that bacteriocins may cross the gut blood barrier, although this may only be for a select few (studied by Dreyer), namely nisin A, plantaricin 423 and bacST4SA [186]. Thus some bacteriocins may be transported across the gut-blood barrier but the transport mechanisms are unclear and individual studies will be required for each bacteriocin, based on bacteriocin size, lipophilicity and potential carrier-mediated transport mechanisms.

Even for local delivery to the gut, activity is not guaranteed – an *L. lactis* (DPC6520) bacteriocin producer survived the gastrointestinal tract but was not effective against *L. monocytogenes* in the colon [187], Table 3. For bacteriocins to act in the gastrointestinal tract, they must be protected from the proteolytic enzymes which may degrade them and from the varying pH of the GI [131]. The pH of the GI varies from pH 1–2.5 in the stomach to pH 7.88 in the small intestine [105,188]. This leads to changes in the stability and solubility resulting in the loss of activity of bacteriocins as they move through the GI. As presented, however, low water solubility has been reported for several bacteriocins with no data available on aqueous solubility for many others, Table 1, so it is unclear if sufficient peptide will dissolve for many bacteriocins to allow for good activity [189]. For local delivery to the GI, the use of a matrix for bacteriocins to protect from pH or enzymatic degradation

Table 3
Summary of reported *in vivo* studies on bacteriocins in infected animal models.

Bacteriocin	Formulation	Route of Administration	<i>In vivo</i> Outcome	Ref
Bacteriocin produced by <i>L. acidophilus</i> CH1	Conjugated to gold NP's	Oral	Sustained reduction in faecal spore shedding after treatment (94.26%). Reduction in intestinal spore load was highest with the conjugated bacteriocin in mice (89.7%).	[199]
<i>L. lactis</i> DPC6520 (Lacticin 3147 producing strain)	Cell suspension in PBS (10 ⁹ CFU/ml)	Oral	Survived the GI, not effective against <i>L. monocytogenes</i> in a mouse model or <i>C. difficile</i> in a human distal colon model.	[187]
Lacticin 3147	Suspension in PBS	SC	Prevented the systematic spread of <i>S. aureus</i> in a mouse model.	[197]
Mersacidin	Suspension unspecified	Intranasal	Eliminated Methicillin-resistant <i>S. aureus</i> (MRSA) in blood, lungs, liver, kidney, spleen and nasal scrapings of an infected mouse rhinitis model.	[175]
Microcin J25	Suspension in aqueous 0.01% Tween 80.	Local (intraperitoneal)	Significant decrease of <i>Salmonella newport</i> in the infected spleen and liver of mice.	[167]
NAI-107	DMSO, β-hydroxypropyl cyclodextrin and glucose solution	IV/SC	Tested against murine septicaemia caused by <i>S. aureus</i> . Protected mice from septicaemia after IV and SC administration.	[101]
Penisin	Suspension unspecified	SC	SC administration led to a 91% decrease in <i>S. aureus</i> MTCC96 load	[113]
Thuricin CD	Enema preparations or suppositories in 10% EtOH	Rectal	95% reduction in <i>C. difficile</i> in the colon of infected mice 1 h post rectal delivery of thuricin-CD	[124]

would improve their activity in the gut. Rectal administration via suppositories or enema preparations presents another option for local activity in the GI, with high efficacy such as that seen by Rea, *et al.* [124], Table 3, although potential issues with patient compliance may pose a challenge to the development and marketing of these formulations. For systemic delivery through oral administration, formulation with protective matrices, solubilising additives and permeation enhancers may be required.

Indeed, the main routes of administration for biologics are frequently intravenous (IV), subcutaneous (SC) and intramuscular (IM) [190,191], due to their challenging stability and permeability properties. IV infusion is the most common form of parenteral administration for biologics, such as antibodies and recombinant protein therapies [192]. IV infusion bypasses many absorption barriers, efflux pumps and metabolic mechanisms, while also achieving therapeutic effective drug concentrations in minimal time [189,192]. There are drawbacks however, IV injections can be painful, expensive and invasive. Another issue is the solubility of a drug in an aqueous solution. This is important to prevent embolism or phlebitis as a result of precipitation [193]. SC administration also possesses drawbacks, such as limitations in volume available for administration (typically < 1 ml to 2 ml), injection site degradation, local side effects brought on by retention of dose at the injection site, as well as increased inter-individual variability in dosages [194]. As such, quantitative solubility testing in simulated body fluids becomes an essential aspect of bacteriocin development. As recorded in Table 1, several bacteriocins exhibit low aqueous solubility and many have not reported whether or not they dissolve in an aqueous media and to what extent. Unlike IV administration, SC administration is more patient-friendly, can be administered by the patient and in comparison to IV infusion, reduces pain and discomfort significantly. IM administration is a more painful injection route due to the high density of muscle tissue and can lead to localised muscle tissue damage [192]. Many approved biologics, including those for managing chronic disease states or symptoms, are currently administered subcutaneously which would be a viable option for bacteriocins [190,195].

Of course, there are other modes of delivery for biologics, e.g. marketed biological agents such as calcitonin, oxytocin and nafarelin are approved for intranasal administration. Irvine *et al.* best describes these routes of administration but a common theme arises whereby the short half-life of biologics *in vivo* increases the necessity for repeated administration or dosages, posing discomfort and poor patient compliance [190]. Local drug delivery (LDD) platforms can offer controlled and prolonged delivery of a drug alongside a range of advantages such as the reduced risk of systemic side effects. They can be administered directly to a site of infection, such as topical, periodontal, dermal or ocular administration [171]. LDD systems also offer advantages such as improved drug bioavailability at the target site and reduced dosage, thus

providing improvements in patient compliance [196]. Studies have shown that when bacteriocins are administered subcutaneously, antimicrobial effects have been detected both locally and systemically, for example, with lacticin 3147 and NAI-107 [101,197]. When NAI-107 was also delivered by IV infusion [101], similar results to its subcutaneous administration were observed. Penisin was also shown to be highly effective upon SC administration, with up to a 91% reduction in bacterial load of *S. aureus* in the thighs of mice [113], Table 3. Upon review of the literature, it would appear that local and SC/IV administration of bacteriocins show more suitability to treat bacterial infections outside the GI tract than oral administration [101,113,167,198].

5.2. Formulation approaches for Bacteriocins: State of the art

While bacteriocins have been discussed in the literature for more than a century now, their development into medicines remains elusive. A myriad of different drug delivery strategies for active pharmaceutical ingredients, from nanoparticles (NP) of different materials to carbohydrate-based structures, tablets, gums and impregnated implants exist [200–202]. Studies of these strategies with bacteriocins are limited and focus on well known, and well-characterised bacteriocins, namely nisin and other LAB bacteriocins [203–210], *S. salivarius* bacteriocins [211–214], enterocins [215–217] and garvicin [178,218–220]. Approaches have involved studies of free bacteriocins in solution [167,175,197], conjugation to gold or silver nanoparticles [221–223], encapsulation in hydrogels [209,224], cyclodextrin complexes or lipid carriers [204,225–229] or adsorption to inorganic matrices [58,102], Figure 4.

Improvements in stability and activity were generally reported from *in vitro* studies of these formulation approaches, Table 4, but only bacteriocin conjugated nanoparticles have been tested *in vivo*, Table 3 [197]. Given that bacteriocins have demonstrated such instability to proteases, low aqueous solubility, a propensity to aggregation and pH-dependent activity, as discussed in Section 3, antimicrobial activities *in vivo* (Table 3) could be improved significantly by the use of the delivery strategies such as those summarised in Table 4 or derivatives thereof.

5.3. Semisynthetic bacteriocins

Although natural bacteriocins produced by bacteria have been the focus of this review, recent developments in synthetic/semi-synthetic bacteriocins are worthy of discussion. In November 2011, the semi-synthetic bacteriocin aminoheptylamido-deoxyactagardine B (NVB302) entered clinical trials through Novacta Biosystems Ltd. for the treatment of *C. difficile* infections [234,235]. The clean toxicological profile observed in pre-clinical studies was borne out in healthy

Table 4
Formulation strategies explored for bacteriocins to date.

Bacteriocin	Formulation	Route	Outcome
Bactofencin	Mesoporous matrices (MSE and SBA-15)	Oral	Increased activity compared to free bactofencin against <i>S. aureus</i> [58].
Enterocin (produced by <i>Enterococcus faecium</i> FH99)	Silver NP's	Food Preservation	NP's were found to be active against <i>E. coli</i> , <i>L. monocytogenes</i> and <i>S. aureus</i> at pg/ml concentrations without displaying any toxicity to erythrocytes [216].
Bacteriocin produced by <i>L. acidophilus</i> CH1	Conjugated to gold NP's	Oral	Increased potency against microsporidia. The bacteriocin bound nanoparticles were also found to be non-toxic in mice (Table 3) [199].
Lactacin 3147 Ltn α & Ltn β	Solid lipid nanoparticles	Oral	Increased activity against <i>C. difficile</i> and indicated protection against α -chymotrypsin [230].
Lactocin 705, AL705, nisin	Montmorillonite clay	Food Preservation	Increase in thermal stability for nisin up to 121 °C for 1 hr, with activity conserved for 10 days. The activity was conserved up to 18 months after storage at -20 °C [231].
NAI-107	In DMSO, β -hydroxypropyl cyclodextrin and glucose solution	IV/SC	Protected mice from septicaemia caused by <i>S. aureus</i> when delivered via both routes (Table 3) [101].
Nisin	Silver NP's	Food Preservation	Increased antimicrobial activity against foodborne microbes compared to free nisin [222].
Nisin	Hydrogel (Dextran and alginate)	Local (injection)	Controlled release of the lantibiotic nisin into simulated gastric fluid, along with improved antimicrobial activity against <i>S. aureus</i> 20,231 DSM (up to 10 days) [224].
Nisin	Solid lipid nanoparticles (SLN's)	N/a	Inhibition of <i>L. monocytogenes</i> and <i>L. plantarum</i> was observed over 15–20 days, whereas free nisin only inhibited for <3 days in solution [204].
Nisin	Mesoporous matrices	Oral	Matrices loaded with nisin A were shown to remain active against <i>S. aureus</i> at pH 6.5 [102].
Nisin	Gum Arabic hydrogel microparticles	N/a	Enhanced the heat stability of the bacteriocin [206].
Nisin	Hydrogel capsules(chitosan)	Food Preservation	Activity against <i>B. subtilis</i> was increased. Thermal and pH stability increased, max. activity observed at pH 5–6 [209].
Nisin	Tablet(pectin and HPMC)	Oral	Dissolution up to 100% observed with controlled-release based on the concentration of HPMC [203].
Nisin & <i>Lb. plantarum</i> ATM11 bacteriocin	Gold NP's	Food Preservation	Combined antimicrobial activity against <i>Micrococcus luteus</i> , <i>B. cereus</i> , <i>S. aureus</i> and <i>E. coli</i> was increased [223].
P34	Soybean phosphatidylcholine (PC) liposomes	Food Preservation	Was shown to retain its activity against <i>L. monocytogenes</i> post encapsulation [225].
Pediocin Ach	Lipid carriers (phospholipids)	Food Preservation	Maintained complete activity against <i>L. monocytogenes</i> , <i>L. innocua</i> and <i>L. ivanovii</i> for the first 13 days with a 50% decrease in activity thereafter [232].
Plantaricin 423 and ST4SA	Electrospun hydrogelnanofibers (poly(D, L-lactide) and poly (ethylene oxide))	N/a	Maintained 88% of their activity against <i>E. faecium</i> and were released from the fibres at a high concentration in the initial stages of the experiment [233].
Subtilisin	Hydrogels (polyethylene glycol)	Local (intravaginal)	Sustained release of the bacteriocin, providing up to an 8 log reduction in <i>G. vaginalis</i> [198].

volunteers. No serious adverse effects were reported up to a 1.5 g single dose or a 1 g daily dose (for 10 days). Rather, minor adverse effects were observed, which were independent of dosage. The phase I trials also showed that plasma levels of the lantibiotic were almost non-existent, conversely faecal levels of >1000x the MIC were obtained [234]. Progression of these trials doesn't appear to be reported, however, an international patent was published in 2014 [236].

Intrexon Corp. and Oragenics Inc. have also been involved in the publication of blueprints for further variants of mutacin 1140 such as the variant OG716, a promising progression in bacteriocin research [237,238]. Further developments have also included chemical synthesis of the bicyclic ring of mutacin 1140 by Oragenics Inc. and SynQuest Laboratories. This study presents the first report of a complete chemical synthesis, using orthogonally protected lanthionines, of the bicyclic C/D ring of mutacin 1140, although further characterisation is required [239]. While these recently published studies show incredible potential and progression in bacteriocin research for medical development, the lack of progression to clinical trials presents some uncertainty.

5.4. Clinical development of bacteriocins

The first clinical development of bacteriocins was reported in 1996, by AMBI/Astra. The NY based company planned to initiate clinical testing for *Helicobacter pylori* infection eradication using Ambicin (nisin). They planned to assess the *in vivo* safety and to progress to other tests combining nisin with compounds like Omeprazole, in a multi-hurdle approach, for the treatment of *H. pylori* infections in animals. Phase I clinical trials were reported to have gone ahead, however, phase II trials, scheduled to take place in 1997 were abandoned. Approval was also granted for the clinical phase I trials to treat *C. difficile* and

vancomycin-resistant *Enterococcus* infections to proceed in 1997, however, no information was released. The product 'Ambicin' (a nisin based formulation used for bovine teat sanitisation prior to and post milking, for the prevention of mastitis [240]) was sold to Biosynexus in 2000. While under the hold of Biosynexus Inc., two National Institute of Health (N.I.H) grant applications in 2003 (\$388,596 and \$407,495) were approved for the development of a topical anthrax treatment using nisin [241]. However, no further outcome was reported. Ambicin was discontinued in Japan and the US in 2006.

The bacteriocin Moli1901 (duramycin) was investigated in phase I trials for cystic fibrosis by AOP Orphan Pharmaceuticals AG. Phase II trials, completed in 2009, showed total cumulative aerosolised dose (0.9% saline administered via nebuliser canister) of 12.5 mg to be safe in human adult patients with CF, indicating a positive outcome. While the positive outcome of the phase II trials indicates the potential for further studies into its efficacy in CF patients, none appear to have been reported [242-244].

6. Conclusion and future perspectives

There exists a large panel of bacteriocins that exhibit a wide range of activities against clinically relevant bacteria. As noted by Cotter *et al.* in a Nature review in 2013, a severe lack of sufficient investment in bacteriocins has hindered their development into a new class of antibiotics [245]. In addition, from the data gathered in this review, comprehensive physicochemical characterisation of bacteriocins would enable the selection of formulation strategies that could allow bacteriocins to progress more successfully through the stages of drug development. This is important data, imperative to the appropriate design of dosage forms and to maintain the efficacy of the bacteriocin during production,

storage and after administration. Bacteriocins were found to be generally heat-stable but this is not a universal characteristic. Enzymatic stability varies greatly between bacteriocins but almost all bacteriocins have been reported to be susceptible to at least one protease tested. Quantitative solubility data in aqueous (and organic) solvents as well as permeability data would enable the development of better formulation strategies. Indeed, it would appear that local delivery may prove preferential in the administration of bacteriocins as therapeutics – importantly though, this should not rule out further development for systemic applications, focusing on formulation/delivery strategies to improve solubility, stability and permeability to achieve optimal systemic dosage requirements. The generally positive cytotoxicity profile of bacteriocins and the favourable properties of semi-synthetic/synthetic bacteriocins lends promise to the development of clinical bacteriocin therapies. Unfortunately, at this stage, conclusions are difficult to draw in terms of whether future investment will be made into these antimicrobials for their development into next generation antibiotics, and their use in the mitigation of antimicrobial-resistant infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejpb.2021.05.015>.

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