Updated scheme for classification of bacteriocins in Gram-positive bacteria and comprehensive overview for Lactobacillaceae

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Summary

Many Gram-positive bacteria, particularly those within the family Lactobacillaceae, possess significant biotechnological potential across food, feed, supplement, pharmaceutical, and agricultural sectors. Numerous species produce bacteriocins or ribosomally synthesized antimicrobial peptides, some of which undergo post-translational modifications, that may confer a competitive advantage in complex microbial communities. These beneficial properties, along with their capacity to produce such bioactive molecules, make Lactobacillaceae a valuable microbial group with broad application potential.

This review provides a comprehensive overview of bacteriocin classes within Lactobacillaceae, detailing their classification, mechanisms of action, and genetic organization. We propose a unified classification system, including an extension of existing classes and a standardized, source-based nomenclature for RiPPs and unmodified bacteriocins from Gram-positive bacteria to enhance clarity and consistency. While proposed for Lactobacillaceae, our proposal fits within the broader scope of the field for the development of community standards in line with the scope of for example the "Minimum Information about a Biosynthetic Gene cluster" (MIBiG) database.

We also systematically reviewed all reported bacteriocins within Lactobacillaceae, identifying 474 individual bacteriocins to date. Despite advances in genome and peptidome technologies, most remain only partially characterized. Notably, the majority of producing strains originate from food-related niches, highlighting the untapped potential of species from underexplored environments.

Given the growing threat of antibiotic resistance and the demand for natural food preservatives, there is an urgent need to fully characterize these bioactive molecules and to discover new ones from lesser-studied Lactobacillaceae species for future applications.

Introduction

Lactobacillaceae are a family of Gram-positive lactic acid bacteria that, following a recent reclassification, now encompass over 30 genera (1). Their preferred habitats are diverse and range from the human vagina (2), human and animal guts (3), and fermented foods (4), to honeybees (5), a variety of sources that is reflected also in a wide application range. This family includes many strains that are among the best documented for their safety and for their extensive history of research focused on their beneficial properties (**Figure 1**). Many taxa within this family have a 'Generally Regarded As Safe' (GRAS) status in the USA (6) and/or a 'Qualified Presumption of Safety' (QPS) status in Europe (7), making them popular subjects of research directed towards food safety and human health (4, 8). Of note, the Food and Drug Administration (FDA) typically grants GRAS status to

strains, while the European Food Safety Authority (EFSA) grants QPS status on species level with some exceptions. Their beneficial properties are often attributed to their ability to produce antimicrobials with lactic acid often postulated as one of the key antimicrobial molecules (9). Lactic acid acts through a dual mechanism. It lowers environmental pH, which shifts the lactate-lactic acid equilibrium toward the protonated, uncharged form (lactic acid) that can passively diffuse across membranes and interfere with cellular processes, and it directly alters membrane permeability (9). Other molecules of interest include hydrogen peroxide although its antimicrobial role is debated in the vaginal environment due to the low amount of oxygen present in the niche (10), and also antimicrobial peptides, i.e. bacteriocins (9) which is the focus of this review.

Bacteriocins have been defined as “ribosomally-synthesized antibacterial peptides that either kill or inhibit the growth of closely related bacteria” (11). While this definition is still widely used, it has been challenged by examples of both unmodified and modified peptides synthesized by bacteria or archaea with the capability to either kill or inhibit distantly related bacteria and even fungi (12, 13). Therefore, an updated definition should describe bacteriocins as bacterium-produced ribosomally synthesized peptides with antimicrobial activity. These molecules may remain unmodified or undergo post-translational modifications by dedicated processing enzymes that introduce functional groups before the bacteriocins are secreted or displayed on the cell surface (14) (***Figure 2***A). The latter post-translationally modified peptides are also referred to as ribosomally-produced and post-translationally modified peptides (RiPPs) (15). Currently, there is no universal term for unmodified peptides wherefor a bioactivity has been shown (such as antimicrobial or other activities) and therefore, we will use the term ‘Ribosomally-synthesized Unmodified Peptides’ (RuPs) as a collective term. In addition, the term ‘Bioactive Ribosomally-synthesized peptides’ (BRPs) will be used as collective term encompassing bioactive RiPPs and RuPs. Here, bioactive refers to peptides that have been experimentally demonstrated to exert a measurable biological effect on cells, organisms, or molecular pathways, either in vitro or in vivo, irrespective of whether their native physiological role is fully understood. In Box 1, we have listed the definitions for these mentioned terms. Many BRPs have antimicrobial properties, but it is important to highlight that not all BRPs have been demonstrated to possess antibacterial activity (**Figure 2B**). Other documented biological, in vitro activities include antiviral (16), antifungal (12), immunomodulatory (17) and anticancer properties (18, 19), as well as analgesic (pain-relieving) (20) and spermicidal properties (19, 21). However, many functions likely remain undiscovered, as current phenotypic screening tools are limited and primarily

biased toward detecting antimicrobial properties, with generally only a limited number of potential target micro-organisms screened. Within the field of food safety, significant attention has been given to identifying novel bacteriocins capable of inhibiting *Listeria monocytogenes*, the causative agent of one of the most serious foodborne infections, listeriosis, characterized by relatively low morbidity but high mortality (22). As a result, *Li. monocytogenes* is classified as one of the top contributors for hospitalizations and deaths by both the Centre for Disease Control (CDC) and EFSA (22, 23). In 2023, EFSA even mentioned *Li. monocytogenes* as one of the most severe zoonotic pathogens as they exerted the highest case-fatality and hospitalization rates (22). As a result, bacteriocins have been predominantly characterized with respect to their anti-*Listeria* activities or the focus has been on producing species involved in food fermentations, such as the anti-listerial **plantaricin BN** produced by *Lactiplantibacillus plantarum* (24). However, research into the broader biological activities of BRPs outside of antimicrobial activity is expanding. For example, it was recently demonstrated that the antimicrobial **plantaricin EF** produced by *Lp. plantarum* also interacts with the intestinal epithelium leading to an increased fortification of tight junctions (25), a characteristic specifically promising when using the live strain as a gut-targeted probiotic. Here, we argue that expanding screening protocols may uncover many more diverse functions. Given the increasing awareness of antimicrobial resistance and the urgent need for alternative strategies, there is a growing interest in probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (26), and live biotherapeutics, defined as "living microbes (e.g., bacteria, yeast), that are not vaccines, and are used to prevent, treat, or cure human diseases" (27). There is particular interest in strains with specific antimicrobial properties for food supplements and pharmaceutical applications. This has resulted in a shift towards screening more Lactobacillaceae species associated

BOX 1: Definitions and proposal for novel terms

Ribosomally-synthesized and post-translationally modified peptides (RiPPs): As the name suggests, RiPPs are ribosomally synthesized peptides that are subsequently enzymatically modified. The initially produced peptide acts as a precursor molecule or pro-peptide and is post-translationally modified into its final form by processing and maturing enzymes. Peptides processed by general, non-pathway-specific modification enzymes are not considered RiPPs, even though they are ribosomally synthesized and post-translationally modified.

Ribosomally-synthesized and Unmodified Peptides (RuPs): A proposed term for ribosomally synthesized, unmodified peptides that can exhibit diverse biological activities. This includes peptides where the cleavage of the leader peptide is performed, but without further pathway-specific post-translational modifications, thereby excluding all peptides belonging to RiPPs, but including unmodified bacteriocins (i.e. class II) and similar peptides with a more diversified activity profile or unknown bioactivity.

Bacteriocin: an overarching term referring to ribosomally synthesized peptides produced by bacteria with antimicrobial activity. These peptides may or may not harbor post-translational modifications. As a result, bacteriocins can be classified as RiPPs or RuPs.

Bioactive Ribosomally-synthesized Peptides (BRP): ribosomal peptides encompassing both modified and unmodified peptides with a wide range of activities. These include both RiPPs and RuPs.

Nonribosomal peptides (NRPs): peptides produced through a nonribosomal pathway, typically by enzymatic assembly lines referred to as nonribosomal peptide synthetases. These are distinct from BRPs and thus also RiPPs, RuPs and bacteriocins.

Antimicrobial peptides (AMP): an overarching term which encompasses all types of peptides with antimicrobial activity regardless of their origin (plants, animals, bacteria and humans) and types (BRPs and NRPs).

with the human and animal microbiome for their BRP- and specifically bacteriocin-producing capabilities (28, 29). Ideally, these strains would be screened against a wide variety of pathogens that inhabit the mucosal surfaces where Lactobacillaceae also naturally occur (see **Figure 1**), such as the gut (3), vagina (2), skin (30), and nose (31). However, the phenotypic wet-lab screening of these isolates is labor-intensive. Therefore, cutting-edge genome- and other 'omic' based approaches in combination with other technological advancements such as high-throughput robotics are needed to drive this field forward. For example, based on chemical antimicrobial screening estimations, screening hundreds to thousands of strains is needed to find a single promising BRP hit (32). This candidate hit can then be developed as a purified peptide-based drug as has been proposed for nisin (33) and duramycin (34), or as producing strains in the form of a live biotherapeutic product or probiotic. The live applications pose formulation challenges but have the added benefit of offering *in situ* peptide production and a potentially multifactorial mode of action, since other metabolites produced by the live microbe can also potentially act on the pathogen or the host. Currently, research on bacteriocins from Lactobacillaceae is fragmented, with studies focusing on a limited number of strains and phenotypes with only limited evidence to complement claims of novelty. Specifically, contradictions in the literature highlight the need for a critical review of existing evidence and a comprehensive framework with a unified classification system to reduce confusion. Here, we propose an updated classification based on work of the teams of Wilfred A. van der Donk, John C. Vederas, Marco J. van Belkum and Oscar P. Kuipers (15, 35–37).

We aim to combine this updated classification with a systematic overview of the currently known RiPP and unmodified bacteriocin classes within Lactobacillaceae, to promote more targeted applications with bioactive ribosomal peptide (BRP)-producing Lactobacillaceae. In this review, we will use the term bacteriocins for BRPs with documented antimicrobial activity, and the more general terms RiPPs and RuPs when the documented function is more diverse or unknown to date. For each class, examples of relevant bacteriocins are discussed, including their known modes of action and genetic and chemical structures, focusing on BRPs and particularly bacteriocins originating from Lactobacillaceae where possible. When no examples were available for Lactobacillaceae, examples from closely related lactic acid bacteria families such as Enterococcaceae and Streptococcaceae were given. The classes described are based on Alvariez-Sieiro, *et al.* (36), but extended with additional classes where *in silico* data strongly supports their presence, potential functions, and diverse properties.

Ribosomally-synthesized and post-translationally modified peptides

RiPPs are gene-encoded peptides synthesized through transcription and ribosomal translation according to the central dogma of molecular biology that are subsequently enzymatically modified by pathway-specific modification enzymes. Although this term is generally used for specialized peptides in bacteria, they are also produced by archaea (38) and eukaryotes, including fungi (39) and plants (40).

The initially produced peptide usually acts as a precursor molecule or pro-peptide and is typically post-translationally modified into its final form by processing and maturing enzymes (15) (**Figure *2***A). Modification enzymes introduce functional groups such as lanthionines to form a pre-peptide, while maturation enzymes generally form the mature peptide through cleavage of the leader peptide linked to peptide secretion (41). These introduced functional groups can be very diverse and play a significant role in determining their function. In bacteria, the pro-peptides are typically encoded from a single structural gene within a larger biosynthetic gene cluster that usually encodes other proteins and enzymes necessary for transport, regulation, immunity, modifications and processing (42). The pro-peptides consist of a N-terminal leader and/or C-terminal follower peptide that functions as a signal peptide that guides the peptide to the processing and maturing enzymes, and the peptide backbone that is processed into the mature, active peptide. This leader is often removed during export of the pre-peptide (43).

Ribosomally-synthesized unmodified peptides

In contrast to RiPPs, RuPs are ribosomally-produced unmodified peptides. RuPs with antimicrobial activity are typically referred to as non-modified (or unmodified) bacteriocins with leaderless bacteriocins being a class of a true unmodified bacteriocin. These peptides lack a leader peptide and as a result, a clear mechanism of guiding the export from the cell, making them highly unusual and as a result, elusive (44). While they tend to be referred to as “unmodified”, lacking extensive post-translational modifications, most still require leader peptide cleavage for activation and might harbor more traditional modifications such as disulfide bonds which are catalyzed by primary enzymes without the need for dedicated modification enzymes. Similar to RiPPs, the pro-peptides are typically encoded from a single structural gene within a larger BGC that usually encodes other proteins and enzymes (42). Besides the leaderless peptides, the pre-peptides belonging to this class tend to have a leader and core peptide region, where the leader consists typically of one of two types: a double glycine leader or a signal peptide (sec-dependent) leader. Firstly, the double glycine leader (i.e. GG-bacteriocins) harbors a LxxxxLxxxxGG motif which typically guides the bacteriocin to a

dedicated transporter (45). These transporters then typically harbor a peptidase moiety (typically a C39 peptidase motif) which cleaves of the leader during export. In addition, we have identified putative examples of peptides wherefore cleavage of the leader is catalyzed by a stand-alone enzyme. Secondly, the signal peptides also referred to as sec-dependent leaders do not rely on dedicated transporters, but instead use the general Sec secretion pathways to export peptides out of the cell (46). Due to the widespread abundance of these leader sequence motifs, they are exploited within genome mining approaches as an efficient way to identify novel putative pre-peptides. Therefore, genome mining tools have implemented this approach, including Bagel4 (47) and antiSMASH (48).

Regulation, self-immunity and resistance of bacteriocins

The production of specialized metabolites including BRPs redirects energy from primary metabolism, making their production energetically costly (49). Therefore, their production is usually regulated. Production is often triggered by increasing cell densities when nutrients are reaching depletion (50, 51). Additionally, some bacteriocins are self-regulating, acting as both bacteriocins and pheromones, as seen in **plantaricin A** from *Lp. plantarum* C11 (52). In addition to regulation based on quorum sensing, production can also be induced by various triggers associated with competition (53, 54). While most studies focus on biotic stress responses inducing bacteriocin production, only limited examples can be found in literature where abiotic (or environmental) stress factors have a clear impact. For instance, acidification of the environment was identified as a trigger for lacticin 481 production in *Lactococcus lactis* (55). Meng et al. (2021) showed that the addition of acetate can increase bacteriocin activity up to three-fold for certain bacteriocin-producing Lactobacillaceae (56), while osmoregulation triggered the production of the bacteriocin microcin PDI by *Escherichia coli* (57). In general, the regulatory pathways governing bacteriocin expression exhibit high diversity, often encompassing multiple different regulation pathways. They remain poorly characterized for most bacteriocins (58). Improving our understanding of these regulatory factors is needed to optimize expression under laboratory conditions and facilitating production and purification.

The regulatory pathways of bacteriocins also play a key role in the protection of the producer strain by conferring immunity and protection against its own antimicrobial action. The bacteriocin cluster generally includes genes encoding for immunity proteins that may also give cross-immunity against other bacteriocins (59, 60). A variety of immunity systems have been described, such as (i) active export of the bacteriocins (e.g. export of nisin by NisFEG in *L. lactis* and related species) (61), (ii) production of small immunity proteins that block the insertion of the bacteriocin into the membrane by binding the bacteriocin or shielding the target (e.g. NisI, the immunity protein of nisin in *L. lactis*)

(62), (iii) inherent immunity due to adaptation in membrane, cell wall or receptor structure (e.g. zoocin A produced by *Streptococcus equi* where the length of the peptidoglycan cross-bridge is changed for producer strains) (63), (iv) inactivation through bacteriocin modification (e.g. microcin C7 produced by Enterobacteriaceae where the peptide is acetylated, inactivating the peptide) (64) and, (v) degradation of the bacteriocin (as proposed for Abi proteins - membrane-bound metalloproteases - often found encoded downstream of structural genes within the BGC) (65).

While bacteriocins have specific advantages over classical broad-spectrum antibiotics such as their generally narrower activity spectra, it is important to realize that resistance does exist and can develop (66), despite some claims that there is no resistance development for bacteriocins (67). In general, 'immunity' is the term used for mechanisms associated with bacteriocin production and usually encoded within the bacteriocin gene cluster, while 'resistance' involves mechanisms unrelated to production. 'Insensitive' is the term applied to bacteria outside of the activity spectrum of the bacteriocin. A number of mechanisms can drive the emergence of resistance to bacteriocins in non-producers. Insensitivity is not easily transferable as this generally encompasses intrinsic characteristics of the species or strain, such as cell wall structure or composition (59, 68). An example of acquired resistance are mutations that downregulate proteins such as the mannose phosphotransferase system (Man-PTS), a common bacteriocin target. For instance, lactococcin A specifically targets lactococcal Man-PTS (69). Some nisin-resistant strains of non-nisin producing *L. lactis* have also been shown to harbor a nisin resistance gene that is able to cleave the C-terminal tail of nisin, thereby decreasing its activity 100-fold (70). Another nisin resistance mechanism involves a dedicated two-component nisin transport system (NsrFP) unlike the three-component transport system associated with nisin production (71). Especially when using a producing strain as probiotic or live biotherapeutic, it is crucial to assess the risk of spreading resistance or immunity mechanisms. Assessing the mobility of these genes and resistance development should thus be an essential part of any risk assessment. This can be done *in silico* by for example using ISEscan (72) or MGEfinder (73) to screen for mobile elements or through comparative genomics to identify putative resistance genes as done for Lcn972 by Escobedo and colleagues (74). However, to the best of our knowledge, this is generally not included in risk assessments of probiotic candidates.

General classification of bacteriocins from Gram-positive bacteria

Bacteriocins of Gram-negative bacteria must pass a lipid-rich outer membrane upon secretion rather than the thick peptidoglycan cell wall in Gram-positive bacteria, they typically express different types of bacteriocins. While both groups follow the same general biosynthetic pathway, the differences have led to a split classification for bacteriocins (35, 36, 75). Bacteriocins produced by Gram-positive

bacteria are generally divided into four main categories or classes depending on their molecular weight, temperature stability and modifications as illustrated in **Figure *3***. Class I includes small heat-stable post-translationally modified peptides (<10 kDa); class II includes small heat-stable unmodified (<10 kDa) peptides; and class III are large, heat-labile unmodified bacteriocins (>10 kDa) (36). Further classification into subclasses incorporates other characteristics like specific modifications, intrinsic functions, characteristic sequence motifs, modes of killing, molecular targets, and immunity mechanisms. Of note, these classifications are subject to constant change and debate, as new BRPs and analytical techniques are discovered. In Table S1, we provide an overview of all Lactobacillaceae bacteriocins characterized in literature up to 2024, including their origin, level of evidence, and up-to-date classification.

Class I: heat-stable and modified

Class I contains the heat-stable modified small peptides (< 10 kDa) (36). They are further divided in subclasses based on their chemical structure and functional groups as introduced by their modification enzymes. These include lantibiotics, i.e. lanthipeptides with antimicrobial activity (Class Ia), cyclic peptides (Class Ib), sactibiotics, i.e. sactipeptides with antimicrobial activity (Class Ic), linear azol(in)e-containing peptides (Class Id), glycocins (Class Ie), and lasso peptides (Class If). Examples for each of the compounds are shown in **Figure *3***B, with the examples documented in Lactobacillaceae shown in bold-black when peptides have been characterized. When examples are predicted from Lactobacillaceae genomes, they are shown in bold-grey.

Lanthipeptides (Class Ia) are characterized by lanthionine or methyllanthionine residues (see **Figure *3***B) that are post-translationally synthesized by combining either a dehydrated serine or threonine with a cysteine residue to create a ring (36). Lanthipeptides are extensively studied for their diverse activities, encompassing antimicrobial (76), antiviral (16), antifungal (12), anticancer properties (18), anti-nociceptive and anti-allodynic (i.e. pain oversensitivity) effects with potential in pain management applications (77). Therefore, we prefer to refer to them as RiPPs rather than bacteriocins to reflect their diverse pharmacological potential. Lanthipeptides can be further classified into four subclasses based on their modification enzymes which differ in their mechanism of dehydrating the alcohol residue of serine and/or threonine (**Figure *4***) (78, 79). In the first subclass that we term class Ia-I lanthipeptides, dehydration is performed by a LanB-like dehydratase that catalyzes glutamylation of the hydroxyl group, followed by glutamate elimination and subsequent cyclization by a LanC-like cyclase (80). Nisin is the archetypical example of a class Ia-I lanthipeptide which forms a complex with the peptidoglycan precursor lipid II and can also form pores in the membrane, hence impeding growth of its target bacteria by both inhibiting bacterial cell wall synthesis and disrupting the membrane potential (76). Years of detailed studies on nisin biosynthesis

have revealed many mechanisms that provided insights into lanthipeptides and lantibiotics (33). In class Ia-II lanthipeptides, the dehydratase and cyclase domains are combined in a single LanM-like enzyme that dehydrates through an initial phosphorylation step prior to elimination (81). The dehydratase domain within this class does not show sequence homology to the LanB of class Ia-I. Similarly, class Ia-III and Ia-IV lanthipeptides-processing enzymes also catalyze a dehydration reaction but instead of a dehydratase domain they harbor a lyase and kinase domain. The difference between these two enzyme classes is located within the cyclase domain where class Ia-IV harbors Zn-ligand domains, while these are not present in class Ia-III enzymes (78). Class Ia-III lanthipeptide processing enzymes can introduce an unusual labionin group into the lanthipeptide, a catalytic group characteristic for the carbacyclic lantibiotics named labyrinthopeptins (20, 82). Class Ia-V lanthipeptides are also referred to as lanthidins and the first discovered examples include lexapeptide identified from *Streptomyces rochei* Sal35, cacaoidin produced by *Streptomyces cacaoi* and pristinin A3 identified through a *Streptomyces* genome mining approach (83–85). All these Ia-V peptides harbor an N-terminal (N,N)-dimethylation, a C-terminal S-[(Z)-2-aminovinyl]-(3S)-3-methyl-D-cysteine, and (methyl)-lanthionine bonds. Interestingly, the anti-Gram-positive antimicrobial cacaoidin is hypothesized to exhibit a dual mode of action by binding both the peptidoglycan precursor lipid II and directly inhibiting the cell wall transglycosylase thereby blocking cell wall synthesis (86). For the latter activity the additional glycosylated tyrosine residue is suggested to be necessary. Class Ia-V lanthipeptide-processing is catalyzed by three distinct enzymes, a lyase, kinase and cyclase (84, 87). While the exact mechanisms of class Ia-I and Ia-II lanthipeptide processing enzymes have been well studied (81, 88, 89), less is known about the other subclasses. Notably, a majority of the currently identified class Ia-III and Ia-IV peptides lack demonstrated antimicrobial activity (90), and their biological activities remain to be largely explored.

Lanthipeptides that show antimicrobial activity, lantibiotics, are sometimes subjected to additional classification into three types based on the nature of the active peptides. However, further subclassifications are less widespread within the field, resulting in increased confusion and providing no additional information that was not already present in the main classification scheme. As a result, these classifications will not be incorporated in our proposed scheme.

Head-to-tail cyclized peptides (Class Ib) (sometimes referred to as cyclic peptides) are peptides in which the N- and C-terminal ends are linked by a peptide bond, rendering these peptides very stable and insensitive to exopeptidases that are often present in habitats such as human and animal hosts (36). Of note, these peptides are often wrongly referred to as cyclotides, a different class of RiPPs originating from plants with distinct biosynthetic machinery and structure. To avoid confusion, the term ‘head-to-tail cyclized peptides’ is therefore preferred over cyclic peptides. In some cases,

cyclization seems to be performed within the cell preceding export as is the case for **leucocyclicin Q** produced by *Leuconostoc mesenteroides* TK41401 (91), while in others the circularization event seems to be coupled to (or immediately following) transport out of the cell. This is the case for garvicin ML produced by *Lactococcus garvieae* (92). The exact mechanisms are still under investigation, but these differences could in the future be used for further classification of this peptide class. However, we suggest to further divide head-to-tail cyclized bacteriocins into subclasses based on their biochemical properties and secondary structure as has previously been postulated (93). To this regard, class Ib-I includes head-to-tail cyclized peptides with high pI values (often with cationic islands), typically harboring a saposin-like fold, consisting of four amphipathic α-helices that are arranged to form a small hydrophobic core. An example of this class is **plantacyclin B21AG** produced by *Lp. plantarum* B21 targeting *Li. monocytogenes* and *Clostridium perfringens* (94), which is the only head-to-tail cyclized peptide of Lactobacillaceae for which a crystal structure was obtained (95). In contrast, subclass Ib-II peptides are characterized by low pI values of the mature peptide and an alternative α-helix structure often referred to as a helical bundle structure. The archetypical example of this class is **acidocin B (class Ib-II)** produced by *Lactobacillus acidophilus* M46 targeting *Li. monocytogenes* and *Clostridium sporogenes* (96) wherefore a solution of its structure was obtained with NMR (97). As a result of head-to-tail cyclized peptide structure, they generally target the membrane of sensitive cells, disrupting their membrane integrity. For example**, gassericin A (class Ib-II)** originally identified from *Lactobacillus gasseri* LA39 targets the cytoplasmic membrane of targets such as several *Bacillus* and *Staphylococcus* strains leading to an efflux of potassium ions (see **Figure *5***) (98). Other examples include **plantaricyclin A** (predicted as a Class Ib-I based on pI) from *Lp. plantarum* NI326 targeting several *L. lactis* strains and the common food spoilage pathogen *Alicyclobacillus acidoterrestris* (99), **leucocyclicin Q** (predicted as a class Ib-I) from *Le. mesenteroides* TK41401 targeting several enterococci, streptococci and lactobacilli (100) (**Figure *4*Figure *3***B), and **reutericin 6** (also referred to as gassericin A, class Ib-II) produced by *Limosilactobacillus reuteri* LA6 targeting isolates of *L. bulgaricus* (101). Interestingly, their similarity to circular human peptides and hormones (102) warrants further investigation into their functions beyond antimicrobial activity, as they may hold promise for other therapeutic applications.

Class Ic or sactipeptides are characterized by a sulphur-to-alpha-carbon group formed by a specific sactionine synthase (36, 103). This enzyme belongs to the S-adenosylmethionine enzymes containing a typical [4S-4Fe] conserved region (103). The exact chemical reaction by sactionine synthase is unknown. Within the class, there is high diversity leading to a variety of activities, including antibacterial, spermicidal (21) and hemolytic activity (104). Most characterized sactipeptides originate from *Bacillus* sp., such as thuricidin CD produced by specific strains of *Bacillus thuringensis*

(105). To our knowledge, no sactipeptides have been characterized in Lactobacillaceae, but some sactipeptide-like clusters have been identified in genomes of some *Lp. plantarum* strains (106, 107) and *Ligilactobacillus ruminis* strains, although the latter seemed to be incomplete (108). Much remains to be discovered in Lactobacillaceae on this interesting subclass. Efforts into the discovery of novel sactipeptides and elucidation of their BGCs can be implemented in updated genome mining tools, allowing a more efficient discovery and to novel examples.

Class Id or Linear azol(in)e-containing peptides (LAP) (36) are non-macrocyclic peptides that harbor heterocyclic azol(in)e rings formed by ATP-dependent cyclodehydration followed by dehydrogenation in a flavin mononucleotide-dependent manner (109). The rings are dependent on the substrate used, which is either a cysteine, serine, or threonine residue, forming a thiazol(in)e or oxazol(in)e group, respectively. These groups are generally introduced by enzymes encoded within the cluster as a YcaO-cyclodehydratase, often accompanied by partner proteins that recognize the leader peptide and a dehydrogenase oxidizing the azoline to azole groups (110). Many LAPs are known to inhibit ribosomal translation thereby inhibiting growth of target micro-organisms. Travin et al. (2020) have explored the genomic landscape of LAPs across bacterial genomes by identifying *ycaO* containing BGCs to identify novel translational inhibitors. They identified a novel family of highly similar LAP clusters in Lactobacillaceae with members identified in *Lactobacillus gallinarum, Lactobacillus helveticus, Lactobacillus intestinalis, Lactobacillus kimbladii* and *Lactiplantibacillus pentosus* (110). The respective LAP produced by these clusters were named lactazolicins according to the general guidelines for LAP nomenclature (37). These **lactazolicins** are synthesized by a common set of genes, which includes the modification machinery and export pump homologs of phazolicin and three additional genes: a second *yca*O gene lacking the C-terminal region encoding the PxP-motif, a RiPP recognition element (RRE)-containing gene (protein of unknown function) and a gene with no known homologs. The additional genes are predicted to introduce additional modifications to the precursor peptides (110). The authors hypothesized that **lactazolicins** influence translation due to their positively charged, amino acid-rich N-terminal region, which might allow interaction with phosphate groups of rRNA molecules. However, to the best of our knowledge none of these putative LAP clusters or their compounds have yet been characterized *in vitro*.

Glycocins (Class Ie) (36) are small, glycosylated peptides where either a monosaccharide is covalently linked with a cysteine side chain (S-glycosylated) or a serine/threonine side chain (O-glycosylated). **Glycocin F** (also referred to as **plantaricin KW30**) (111) and **glycocin ASM1** (also referred to as **plantaricin ASM1**) (112) are produced by specific strains of *Lp. plantarum.* These are good examples of bacteriostatic, non-lysing glycocins, containing a characteristic N-acetylhexosamine S-linked to the C-terminal cysteine and a N-acetylglucosamine β-O-linked to a serine residue (113, 114). The latter

glycosylation is located on the loop connecting the two disulfide bridged helices. **Glycocin F** and **glycocin ASM1** only differ by 5 amino acid residues and exhibit activity against members of *Levilactobacillus brevis* and *Lactobacillus delbruecki* but likely with closely related *Lp. plantarum* strains as their targets (115). However, based on *in silico* modeling, **glycocin ASM1** seems to lack secondary structure compared to **glycocin F** (111, 112, 114). Very little is known about the target and activity mechanism of glycocins (36). **Glycocin F** and **glycocin ASM1** have been investigated for their potential antiviral activity *in silico* with protein folding and interaction models where they were identified to bind viral proteins of SARS-CoV-2 (116).

Finally, class If (36) or Lasso peptides are peptides that harbor an unusual lasso structure characterized by a closed ring structure of varying complexity that embraces the C-terminal moiety (117). They show diverse activities including antimicrobial (118), antiviral (119), and anticancer (120). To our knowledge, no Lactobacillaceae-derived lasso peptides have been described in the literature. However, a systematic screening performed by Zhang et al. (2023) did show the presence of putative clusters within members of the *Paralactobacillus* genus.

Due to the discovery of novel modified classes of bacteriocins, the class I group is continuously expanding (35). A systematic biosynthetic screening of lactic acid bacteria revealed clusters annotated as RRE-containing, rSAM modified peptides and others, suggesting that additional clusters falling outside of the current classification are likely present (121). Within the Lactobacillaceae family, screening our *in-house* data has also identified other putative clusters, including putative thiopeptide clusters. We propose classifying the thiopeptide example as class Ig (see **Figure *3***). Thiopeptides (class Ig) are characterized by a six-membered nitrogen heterocycle, which is either a piperidine, dehydropiperidine or imidazopiperidine (35, 122). The structure usually also includes azol(in)e rings and dehydrated amino acids. There is to date only a single characterized example, the thiopeptide **lactocillin** produced by the human vaginal isolate *L. gasseri* JV-V03 (123). Lactocillin has activity against a variety of pathogens, including *Staphylococcus aureus, Corynebacterium aurimucosum* and *Gardnerella vaginalis (123).* Typically, antimicrobial thiopeptides interfere with the 50S ribosomal subunit or elongation factors (122).

To our knowledge, no bacteriocins from other novel classes have been found in Lactobacillaceae. Classes such as lipolanthines, lanthipeptide variants that harbor an unusual avionin moiety (124) (visualized in **Figure *3***B) and an N-terminal guanidino fatty acid; and botromycins, macrocyclic amidines first identified in *Streptomyces bottropensis*, with additional modifications, including a decarboxylated thiazole ring at the C-terminus and β-methylated amino acid residues, show the complexity that RiPPs can achieve through modifications. Due to the constant discovery of novel

clusters and classes, partly enabled by improved mining tools, we propose adding an additional class Iz to encompass all other modified peptides that currently fall outside of the classification scheme (similar to class IId). This approach allows for future subdivision into additional classes (classes Ih-y) when more examples are described in the literature.

Class II: heat-stable and unmodified

Class II bacteriocins are heat-stable, small peptides (<10 kDa) that are largely unmodified, excluding the usual leader peptide cleavage and disulfide bridges (36). This class is widespread in Lactobacillaceae. Within this class, four main subclasses have been distinguished: (IIa) pediocin-like peptides, (IIb) two-component bacteriocins, (IIc) leaderless peptides and (IId) others that do not fit the previous three subclasses (36).

Class IIa is composed of pediocin-like peptides and contain the consensus sequence 'YGNG(V/L)' in the conserved N-terminal region known as the 'pediocin box' and at least one cysteine-cysteine disulfide bridge (36, 125). This box was first identified in the bacteriocin **pediocin** expressed by the members of the *Pediococcus* genus, hence the name (125), but it also occurs in *Leuconostoc, Latilactibacillus* and *Lacticaseibacillus* species. In general, the N-terminal region consists of a S-shaped fold often displaying a three stranded β-sheet that forms when in contact with membranes (126). These regions also contain the stabilizing disulfide bond(s). The C-terminal region seems to be more diverse but generally forms an amphipathic α-helix and hairpin-like structure. These bacteriocins typically exert their bactericidal effect by generating pores and permeabilizing membranes (36, 126, 127). However, this pore formation often depends on specific host Man-PTS receptors on the target's membrane (128), meaning that bacteriocins of this class typically have a narrow and specific host range (126). By directed mutagenesis, the target-cell specificity has been located within the C-terminal hairpin (126). One of best studied bacteriocins of this class is **sakacin P** produced by *Latilactobacillus sakei* (126, 127, 129, 130). Its strong antimicrobial activity against the food pathogen *Listeria monocytogenes* makes it of interest for food safety. The production of **sakacin P** is controlled by an autoinduction mechanism, regulated using a peptide pheromone likely functioning as a cell-density sensor (129). Other examples of this class of bacteriocins of Lactobacillaceae include **leucocin A** produced by *Leuconostoc gelidum* UAL187 targeting *Li. monocytogenes*(131), **curvacin A** by *Latilactobacillus curvatus* LTH1174 also targeting *Li. monocytogenes* (132) and **mesentericin Y105** produced by *Le. mesenteroides* Y105 also targeting *Li. monocytogenes* (133).

Subclass IIb bacteriocins consist of cooperating peptides that are only fully active when coupled (36). The two participating peptides are generally rich in amino acid residues that promote helix-helix

interactions such as glycine and alanine (134). Van der Waals interactions occur between predominantly GXXXG motifs, where the glycine can be replaced by a serine or alanine residue, that attract the two peptides to each other. While individual peptides as a separate unit are generally inactive, the two peptides function as one strong antibacterial unit when combined (135). This is usually also reflected within the gene cluster as the peptide precursor genes are always co-located. For those representatives where a mode of action has been studied, the co-operating peptides form dimeric transmembrane complexes that assemble upon interacting with a host cell receptor (36, 136). These interaction pairs can be quite selective in their targets and, depending on the peptide pair, are postulated to target an alternative receptor (134). Unfortunately, for most known class IIb pairs, physical interaction with the postulated receptors has not yet been demonstrated, but targets have been suggested based on mutation analysis of putative receptors. For **plantaricin E/F** produced by *Lp. plantarum* C11 (137), a magnesium/cobalt efflux protein was identified as a potential target of Lactobacillaceae with mutations in this protein resulting in a resistant phenotype (138). Similarly, Lactococcin G has been postulated to target the peptidoglycan synthesis-associated membrane-bound undecaprenyl pyrophosphatase enzyme in *L. lactis* (139) and **plantaricin J/K** produced by *Lp. plantarum* strain C11 has been suggested to target a putative amino acid-polyamine-organocation protein in Lactobacillaceae (140). The permeabilization of the membrane upon insertion into the membrane leads to a disruption of the proton motif force and the release of specific intracellular small molecules (135). For example, **plantaracin J/K and E/F** both release monovalent cations, whilst they do not permeabilize the membrane for divalent cations. These **plantaricins** do not only target closely related strains but also target pathogens, for example **plantaricin J/K** was shown to be active against several *Staphylocccus* species, including strains of *Staphylococcus citreus, Staphylococcus muscae, Staphylococcus simulans* and *Staphylococcus carnosus* (13). Like **sakacin P**, expression of the **plantaricin** class IIb bacteriocins are induced by a peptide pheromone, which interacts with a histidine protein kinase involved in regulation and harbors membrane permeabilizing strain-specific activity (141, 142).

The third subclass or subclass IIc bacteriocins (36) are bacteriocins that lack an N-terminal leader peptide. Usually, the leader serves as a recognition site for modification enzymes, peptidases and transporters, protects the producer by keeping the bacteriocin inactive within the cytoplasm and ensures a tertiary structure suitable for enzyme-peptide interaction (143). Due to the lack of such a leader, leaderless peptides do not undergo modification and rapidly fold into their active formation. These characteristics make these compounds incredibly interesting and diverse, but this class is not well understood to date. Members of subclass IIc harbor a common formylated methionine at the N-terminus of the peptide. The role of this formylated methionine is still elusive, but it has been shown

to not affect bioactivity, since non-formylated bacteriocins appear to exhibit identical activity (144). Leaderless peptides can also exist as two (145) up to even four (146) peptide leaderless bacteriocins. However, these should not be misinterpreted as two-peptide bacteriocins class IIb, since these leaderless peptides do not have a leader peptide, and each peptide has activity by itself. The modes of action and targets of leaderless peptides can be very diverse. In general, they do not require a receptor for their antimicrobial activity, with some members such as lacticin Q produced by *Lactococcus* QU 5 targeting Gram-positive bacteria (such as *B. coagulans* and *Lc. lactis*) acting through membrane permeation, with the formation of pores dependent on certain physiological features of the membrane (147) or without pore formation such as shown for the broad spectrum aureocin A53 produced by *Staphylococcus aureus* targeting other *Staphylococcus simulans* such as strain 22 (148). Nevertheless, there are also examples of leaderless peptides that do require certain receptor molecules, such as LsbB produced by *L. lactis* which has been suggested to target the Zn-dependent protease of other competing *L. lactis* strains, determined through mutation analysis of this gene, knock out and knock in experiments (149). Class IIc seems quite diverse and is expected to be a very large but a still enigmatic group due to the lack of a leader sequence, complicating their discovery through genome mining as one cannot screen for typical leader motifs. For Lactobacillaceae, to our knowledge, few examples of class IIc peptides have been identified to date, including two single peptide leaderless peptides, named **Weisselicin Y and M** isolated from the same *Weissella hellenica* QU 13 strain targeting *Bacillus* species (150). While this subclass IIc is clearly a distinct group, some members do show some structural similarities to the modified circular peptides (class Ib) as both harbor the so-called saposin fold consisting of amphipathic α-helices that are arranged to form a small hydrophobic core (143). This similarity potentially resulted in conflicting classifications where the class IIc has been inaccurately referred to as the class Ib circular bacteriocins (134).

Finally, subclass IId contains all bacteriocins that do not fit the preceding subclasses, non-pediocin-like mainly single peptide bacteriocins (36). This is a very diverse and heterogeneous group due to the nature of the classification, including bacteriocins with a variety of structures, immunity mechanisms and mode of actions. Relevant examples of this class include the 'type bacteriocin for this class', lactococcin 972 produced by *L. lactis* IPLA 972 targeting other lactococci (exhibiting inhibitory activity by binding lipid II and thereby inhibiting cell wall synthesis but to the best of our knowledge no know pathogens) (151), the narrow spectrum lactococcin A produced by and active against lactococci targeting the Man-PTS but to the best of our knowledge with no demonstrated activity against known pathogens (152), and enterocin B produced by *Enterococcus faecium (*strains active against other enterococci, lactobacilli and food-borne pathogens such as *Listeria*

monocytogenes (153). Examples of this class from Lactobacillaceae seem relatively widespread across genera (see Table S1), including examples from *Lacticaseibacillus* (154), *Lactiplantibacillus* (155), *Latilactobacillus* (156), *Leuconostoc* (157), *Ligilactobacillus* (158) and *Levilactobacillus* (159) species. One such example includes LSEI_2163 (or m2163) produced by *Lacticaseibacillus paracasei* ATCC 334 with documented antimicrobial activity against several *Listeria* species and anticancerous activity in the human colorectal cancer cell line SW480 (154, 160). Our in-house analysis has also predicted putative class IId clusters in other genera, including *Lactobacillus* species (unpublished data).

Class III: large, unmodified proteins

While class I and II bacteriocins are all characterized by their typically small sizes (<10 kDa), there are also class III bacteriocins that are significantly larger and often act as an active enzyme. Class III bacteriocins have been identified as large (>10 kDa) heat-labile proteins. Subclass IIIa comprises bacteriolysins such as **helveticin J** (161) (and **34.9** (162)) produced by *L. helveticus* 481 (and 34.9) active against other lactobacilli (163), zoocin A produced by *Streptococcus zooepidemicus* 4881 against other streptococci (164) and enterolysin A produced by *Enterococcus faecalis* LMG2333 active against several lactobacilli and enterococci (165). Generally, these enzymes function as lytic enzymes and cause the lysis of bacterial cells by cleaving peptidoglycan crosslinks. However, exceptions to the rule exist, such as **helveticin 34.9** that is proposed to both inhibit the cell division process and form pores in target microorganisms such as closely related lactic acid bacteria and pathogenic *S. aureus* (162). In contrast, Subclass IIIb contains non-lytic antimicrobial proteins. Their antimicrobial activity appears to be mainly mediated by the inhibition of fundamental cellular processes, such as mannose uptake (166) and can be very bacteriocin specific. **Caseicin 80,** produced by *Lc. casei* B80 is active against sensitive *Lc. casei* strains (167), and **helveticin M**, produced by *L. crispatus* K313, is active against *S. aureus, S. saprophyticus* and *Enterobacter cloacae* (168). **Helveticin M** is named as such because of the limited sequence identity to **helveticin J** (35% identity) (168), despite the normal practice of naming compounds according to their producer species. However, the sequence identity to known helveticin compounds is low (35%). Therefore, we have renamed it here **crispanobacin M** (see the section: Revised nomenclature for novel BRPs). It has been shown to act in a bacteriostatic manner against Gram-positive and Gram-negative bacteria such as *S. aureus* and *Enterobacter cloacae* by disruption of the cytoplasmic cell membrane, cell wall and outer cell membrane (168). **Caseicin** has been shown to partially inhibit the biosynthesis of proteins and DNA but not RNA biosynthesis in target organisms such as *Lc. casei* B 109 (167, 169). However, this effect has been proposed as a secondary effect to the compound, with currently no clear information on the effective target.

Class IIIc is a more recent class that includes phage-derived proteins or multiprotein complexes, such as tailocins. These are phage-derived tail-like bacteriocins, previously found in organisms such as *Listeria monocytogenes* targeting closely related *Listeria* strains (35). This class of bacteriocins cause cell death by inserting themselves into the cell membranes of sensitive cells. In Lactobacillaceae, this class seems to be largely overlooked with only one, recently published example, CM175 produced by *Pediococcus pentosaceus* CM175 active against *Li. monocytogenes* (170).

The elusive class IV: large modified proteins

A class IV classification has been proposed many years ago by Klaenhammer (1993) (171) but remains quite controversial. The class IV bacteriocins encompassed a class of proteins (>30 kDa) that are covalently bound to a lipid or carbohydrate moiety. This class emerged following the observation that for certain large bacteriocins, their activity is abolished not by protease activity, but rather by glycolytic and lipolytic treatment (171). Depending on the authors, this class is either incorporated or discarded. The cause of controversy is obvious with **plantaricin S** produced by *Lp. plantarum* LPCO10, which was claimed to be a macromolecule-protein complex in crude extracts (172), but was later purified as a small peptide (173). This led to the belief that this class might not exist and that the examples disclosed in literature may be due to problems with the extraction process such as for **plantaricin S**. Nevertheless, other examples that have been postulated to belong to this class of lipid- and carbohydrate-bound proteins are for example **pediocin N5p** produced by *P. pentosaceus* which is active against other pediococci and *Leuconostoc* species, wherefore activity was abolished after lipase treatment (174). Another key example is **leuconocin S** produced by *Leuconostoc paramesenteroides* targeting the pathogens *Li. monocytogenes* and *Yersinia enterocolitica* in which activity is abolished after amylase treatment suggesting a glycosylated nature (175). As evidence for this class remains limited with the above-mentioned examples lacking sufficient characterization, specifically lacking elucidation of the chemical structure, we will not include this class in our updated scheme. However, as lipidation and glycosylation is not uncommon for proteins, we do envision a class IV in the future when sufficient evidence is provided, potentially subdivided depending on the nature of the adaptation. Focused characterization of **pediocin N5p** and **Leuconocin S,** would readily clarify whether these examples are in fact glycosylated/lipidated.

BOX2: Systematic review methodology

A systematic literature search was conducted using PubMed and Web of Science to identify publications describing novel bacteriocins from Lactobacillaceae. The searches were performed on April 3, 2024 and April 5, 2024, for PubMed and Web of Science, respectively. Full records were exported using EndNote Desktop. The total number of records obtained from both databases was 4,636. The following search strings were used:

In PubMed, yielding 1,739 results:

("Lactobacillaceae"[Mesh] OR lactobacillaceae OR lactobacil*[tiab] OR "lactic acid bacteria"[tiab]) AND ("Bacteriocins"[Mesh] OR Ripp*[tiab] OR bacteriocin*[tiab] OR "antimicrobial peptide"*[tiab] OR lantipeptid*[tiab]* OR lanthipeptid*[tiab] OR lantibiotic*[tiab] OR lanthibiotic*[tiab]) AND (characterized[tiab] OR characterisation[tiab] OR characterization[tiab] OR identified[tiab] OR identification[tiab] OR defined[tiab] OR elucidat*[tiab] OR describ*[tiab] OR investigat*[tiab] OR analyz*[tiab] OR determine*[tiab]) NOT review

In Web of Science, yielding 2,897 results:

TS=(Bacteriocin* OR RIPP* OR "antimicrobial peptide" OR "bactericidal peptide" OR lantipeptid* OR lanthipeptid* OR lantibiotic* OR lanthibiotic) AND TS=(lactobacillaceae OR lactobacil* OR "lactic acid bacteria") AND TS=(characteriz* OR characterisation OR characterization OR identified OR identification OR describ* OR investigat*) NOT Review Article

Deduplication: Deduplication was performed using EndNote following the methodology described by (191). The number of records after deduplication included 467 in PubMed and 2,892 in Web of Science, totaling 3,359 records. An additional four duplicates were identified and removed using Rayyan, leaving 3,355 unique records.

Update and additional sources: The search was updated in January 2025 to include papers published between April and December 2024, adding 75 new publications to Rayyan. Additionally, manual searches in Google Scholar identified further relevant publications that were not captured in the initial database searches.

Screening and selection: Rayyan.ai was used for manual screening of the collected papers. After screening for relevance, 397 publications were identified as the first to report novel bacteriocins (in total 474) from Lactobacillaceae.

Trends in Lactobacillaceae bacteriocin research

Using all the knowledge summarized above, we conducted a systematic review by searching PubMed, Web of Science, and Google Scholar for all bacteriocins, including both modified and unmodified bacteriocins, characterized in Lactobacillaceae up to December 2024 followed by a manual review using the reviewing tool Rayyan (176) (See Table S1 and Box 2). A total of 474 bacteriocins were found across 17 Lactobacillaceae genera, with the majority of clusters originating from strains isolated from food sources (289/474 or 60.9%) (**Figure *6***A). This is likely due to the success of the lantibiotic nisin, one of the oldest and best characterized antimicrobial compounds, as a preservative in the food industry, leading to an interest in the discovery of novel anti-food pathogen (e.g. anti-*Listeria*) preservatives (33). While the food focus remains, in recent years bacteriocins from other environments have increased in popularity, such as from animal and human-microbiomes, supporting the trend towards the use of BRPs, specifically bacteriocins and their producer strains in therapeutic applications.

Another major observation from our analysis was that we were unable to predict a bacteriocin classification for 286 'novel' bacteriocins (**Figure *6***B), which largely coincided with their level of evidence (**Figure *6***C). This level of evidence is a measure for the strength of proof for these bacteriocins, ranging from unequivocal strong evidence (level 1), where the entire biosynthetic pathway is reconstructed using purified biosynthetic enzymes outside a living cell and the peptides are produced in their active form, to weak evidence (level 9), where the bacteriocin is only weakly characterized based on antimicrobial activity, sometimes after partial extraction of the compound from the producer strain, but without any indication on structural properties. This level of evidence classification scheme is largely based on the level of evidence as introduced within the MIBiG database to retain standardization within the field (177). Almost half of all bacteriocins (235/474 or 49.6%) appear to be only identified through activity-based or basic proteomic techniques (e.g. SDS-page-based size determination), coinciding with an evidence level 9, what we have defined as the weakest included level of evidence (see **Figure *6***, Table S1 and Table S2). While such basic analyses were the state-of-the-art at the advent of bacteriocin research, about 100 articles with evidence level 9 have been published in the past decade, even as next-generation genome sequencing and MS-based techniques have become more affordable and accessible. In addition, we also noted that that the majority of studies on bacteriocins did not complement their peptide characterization with genomic data attempting to identify the BGC cluster responsible for production (348/474 or 73.4%), so they only received an evidence level of 7 or higher (7-9). We argue that this should become common practice in the current genomic era. Due to the relatively low level of evidence for most bacteriocins, which lack proper characterization and thus cross-study comparability, we think it likely that the number of duplications is high. This is particularly apparent from the number (n = 118) of peptides identified from *L. plantarum,* which likely includes many duplicates. Dereplication has previously been performed for some bacteriocins, such as **Pediocin PA-1** (128), which has been later shown to be identical to **Pediocin SJ-1** (178), **Pediocin AcH** (179), **Pediocin LB-B1** (180), and **BacST202Ch** (181) (see Table S1).

Revised nomenclature for novel BRPs

Table S1 reveals several naming inconsistencies for published bacteriocins and antimicrobial RiPPs, such as the case of **helveticin M**, which was discovered in *Lactobacillus crispatus* rather than *L. helveticus* and was named solely based on its low sequence identity (35%) to **helveticin J** (182)**.** Since helveticin J frequently appears as a common hit when screening genomes with the genome mining tool Bagel4 (47), peptides with limited sequence similarity are often labeled as "helveticin-like" in the literature (183). Such terms can lead to confusion in classification and highlight the need for standardized naming conventions. Especially in the current era of data science, a unified

nomenclature suits within the broader field of natural products research and is in line with the scope of the MIBiG database for standardization within the field. Therefore, we propose a general nomenclature scheme which includes a species determined prefix followed by infixes that denote classification and biological activity.

Here, we propose a source-based naming for new bacteriocins, using a prefix derived from the producing strain's species (e.g., *L. helveticus* → **helveti-cin**), or, when clearer, from the strain's genus to ensure consistency. This approach aligns with current common practice. Importantly, if a bacteriocin is later identified in a second species but is identical to one previously named, it should retain the original name assigned from the first species. When multiple bacteriocins can be produced by the same species, numbering, strain names, or suffixes should be used to distinguish them, as is already the common practice. Where relevant, bacteriocin names should be further clarified by incorporating functional descriptors as infixes, enhancing both classification and interpretability. We propose the use of two infixes, one indicating the classification of the BRP and the other denoting the primary biological activity (**Figure *7***). The activity-based infix should reflect the main function described in the original publication introducing the BRP. In cases where multiple activities are reported, the infix **-mu-** (for multifunctional) may be used, however we do not envision renaming when additional activities are identified after the name has already been given and is widely accepted.

One example where the addition of a general prefix could clarify is for the unmodified bacteriocins **lactacin F (class IIb)** and **acidocin A (class IIa)** from *L. acidophilus* which should be renamed **acido-dua-ba-cin F and acido-pe-ba-cin *SD1*** (184, 185), respectively, following the convention established by **acidocin B** (186). Here, the -dua- and -pe- infix refers to their classification and the -ba- infix refers to its anti**ba**cterial activity (see the next paragraphs for further clarification). We have adapted this accordingly in our overview table under the column 'Proposed Renaming_convention'. Of note, all bacteriocins and thus the examples given here, would in theory receive the -ba- (or -fu-) infix as these signify their antimicrobial activity.

For example, the bacteriocin **plantacyclin B21AG** could be renamed to **planta-cy-ba-cin B21AG** (94), where **planta-** is the species-specific prefix, -**cy-** denotes its classification as a Class Ib bacteriocin, and -**ba**- identifies its antibacterial activity '. Similarly, **plantaricin C** (187) could be renamed to **plantalanbacin C** (for class Ia), where **-lan-** indicates its classification as a Class Ia lantibiotic (Table S1). The commonly used term "lactocin", a broad label for bacteriocins from *Lactobacillus* species, should be replaced with more species-specific names. For instance, **lactocin 705** (188) would

become **casei-dua-ba-cin 705,** reflecting its origin from *Lacticaseibacillus casei* (casei-) and its classification as a two-component type bacteriocin (-dua-) with antibacterial activity (-ba-) (190).

While the proposed naming system enhances clarity for novel bacteriocins and is extendable to other non-antimicrobial bioactive peptides, it is not intended to retroactively rename well-established compounds. For instance, antimicrobial glycocins such as **glycocin F** and **ASM1** should retain their original names, rather than being renamed as **planta-gly-ba-cin F** or **ASM1,** respectively. Similarly, the extensively studied lantibiotic nisin should not be renamed as lacto-lan-ba-cin, as altering widely recognized names would be counterproductive and potentially confusing. The examples provided earlier serve primarily to illustrate how the proposed nomenclature would apply to newly discovered or less-characterized peptides. However, to promote transparency and consistency, manuscripts should include minimal information aligning with the new framework when referencing existing bacteriocins. This includes specifying the peptide's biochemical structure and primary activity ("type"). We have encountered the need for clarity and standardization firsthand during global hackathon efforts to update the MiBIG database, where consistent annotation of BRPs (and other secondary metabolites) was essential for accurate data integration and interpretation. For novel bacteriocins, this classification should be embedded directly in the name. For existing compounds, their type should be clearly stated upon first mention. For example, nisin should be introduced as a "lanbacin-type bacteriocin originally obtained from *L. lactis* (Class Ia-I antimicrobial lantibiotic)".

Finally, many assigned names (see Table S1) consist of arbitrary numbers and/or letters. We discourage this practice due to the lack of informative value these designations provide. However, such designations can be useful, and even recommended, for peptides identified with very low levels of evidence (i.e., evidence level >7), where the arbitrary name serves as an indicator of their preliminary status. Once sufficient characterization is achieved, these peptides should be renamed according to the proposed nomenclature system. The original arbitrary identifiers may still be retained as suffixes or secondary labels to preserve traceability. For example, the peptide **m2163**, produced by *Lc. paracasei* ATCC 334 and characterized with strong evidence (Level 2) via heterologous expression (160), should be renamed **para-si-ba-cin m2163**, reflecting both its origin, classification (class IId) and antibacterial activity while retaining the original identifier for reference.

For more information about our renaming efforts, we refer to Table S1. Updated nomenclature is provided only for those bacteriocins with a predicted class supported by a medium to strong level of evidence (≤7), and these revised names should be used going forward. Except for the well-researched bacteriocins for which renaming would be counterproductive which are marked with an

asterisk (*).This includes several bacteriocins from Lactobacillaceae that have already been incorporated into MiBiG database version 4.0 (177). For standardization, we provide an online tool that automatically generates the appropriate nomenclature for known or novel BRPs based on the producing species, predicted class, and biological activity (available through https://lacripper.streamlit.app/).

Conclusion

In summary, this review has provided a comprehensive overview of bacteriocins within the Lactobacillaceae family. We have proposed an updated classification scheme for bacteriocins from Gram-positive bacteria and a novel standardized source-based naming system to enhance clarity and consistency in BRP nomenclature. We explicitly exclude renaming the most well-known bacteriocins such as nisin and **glycocin F**, since these have been extensively studied for decades and renaming them would be counterproductive. Additionally, we recognize that genus and species names are subject to taxonomic revisions; therefore, well-known bacteriocin and more broadly BRP names, should not require constant updates in response to such changes.

We have also provided an overview of the various bacteriocins that have been discovered in genomes from different genera of Lactobacillaceae and that have been characterized to some level. However, well-characterized examples remain limited, with many documented bacteriocins lacking sufficient characterization. Of note, when we performed our systematic review, we noticed that many studies have used primers specific for known bacteriocins to identify such clusters in novel isolates (189, 190). While valid, this significantly reduces the novelty of the findings. However, more recent articles focus on genome mining due to the decreased price of whole genome sequencing and improvements in genome mining tools such as antiSMASH (48) and Bagel4 (47). Consistent with this trend, Zheng et al. predicted 130,000 novel specialized metabolite and peptide clusters in lactic acid bacteria through mining of genomes and metagenome-associated genomes (121). These clusters remain experimentally uncharacterized, underscoring lactic acid bacteria as a largely untapped reservoir of novel BRPs. Our own systematic review presented here has found a total of 474 bacteriocins that are described in Lactobacillaceae (Table S1). This includes fully and partially (e.g. without an identified BGC) characterized bacteriocins, while excluding homology-based predictions. This comprehensive overview could be of significant interest for updates towards databases such as the MiBiG database (177). Our data underscores the need for further research to unlock the full potential of these bioactive molecules and gain insights into the underlying mechanisms of their antipathogenic and other activities, as well as their subspecies diversity. Such

efforts will enable the field to identify interesting strains and molecules for the development of novel microbiome-based treatments, including live biotherapeutics.

Author Contributions

J.D.: Conceptualization, writing – original draft, review and editing, visualization, systematic review. L.M.: writing – review and editing, systematic review. C.H.: writing – review and editing. S.L.: conceptualization, funding acquisition, supervision, writing – review and editing.

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Conflicts of Interest

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Figures and legends

Figure 1: **Applications of Lactobacillaceae and their bacteriocins within animal health, human health, biocontrol, pollinator protection and, food fermentations, preservation and safety.**

Figure 2: A) General biosynthesis pathway of ribosomally-synthesized and post-translationally modified peptides. The pro-peptide is produced through general transcription and translational and consists of a leader and core peptide. Subsequently, the leader guides the pro-peptide towards processing enzymes that introduce a variety of modifications. Finally, the leader of the modified pre-peptide is cleaved releasing the modified core or mature peptide. The cleavage is either performed by extracellular peptidases or during export. ***B)*** **Overview of key properties used for classification of RiPPs**. BGC; biosynthetic gene cluster.

Figure 3: Overview of the current classification of bacteriocins and RiPPs in Lactobacillaceae and related Gram-positive bacteria (A) Classification is generally done in four main classes based on the size of the peptide and the degree of modifications. (B) The main classes are further divided in subclasses (indicated by the letter) based on specific modifications, functional groups or conserved sequences and mode of action as indicated (see text for more information). This figure is based on Alvarez-Sieiro et al., (2016) and Simons et al., (2020). Classes for which examples have been characterized in Lactobacillaceae so far are indicated in bold and classes where BGCs have been identified but the peptide has not been characterized is shown in bold grey.

Figure 4: Proposed classification for Class Ia lanthipeptides. These updated lanthipeptide processing classes are based on the processing enzyme structure and enzymatic mechanism. Lanthipeptide processing enzyme structure is indicated per lanthipeptide class (A) and mechanism of (me-)lanthionine/labionine formation (B). (Me)-lanthionine = methyllanthionine.

Figure 5: Overview of representatives for class Ia, IIa, IIb, IIc and III, including their structure, mode of action and target (13, 33, 98, 127, 182). Mannose-PTS: Mannose phosphotransferase system and APC or Amino Acid-Polyamine-Organocation family of transport proteins. Created with BioRender.com.

Figure 6: Overview figures showing trends in bacteriocin and RiPP research. A) Count of publications of 'novel' RiPP/bacteriocin discovery per year colored per isolation source of the producing bacterium. B and C) Count of 'unique' bacteriocins identified per genus colored per Predicted class (B) or Level of evidence with a value of 1 referring to the strongest evidence (see Supplemental table 1) (C).

Figure 7: Schematic overview of the proposed nomenclature scheme for bacteriocins and RiPPs in Gram-positive bacteria. The example shown (planta-lan-ba-cin C) illustrates how the naming structure integrates taxonomic origin, RiPP class, and biological activity. Abbreviations: LAP – Linear-azol(in)e containing peptides.

Author Biographies

Jelle Dillen is a senior researcher at the Laboratory of Applied Microbiology and Biotechnology (LAMB) under supervision of prof. Sarah Lebeer. He earned his MSc diploma in 2019 under supervision of prof. Rob Lavigne and prof. Joleen Masschelein where his interest in antimicrobial peptides and molecules started. As a result, he started his PhD at LAMB, investigating the role of antimicrobial peptides and molecules in the vaginal microbiome, which he completed it in 2024. Currently, he aims to better understand how these beneficial bacteria can protect against disease and explores their potential as live biotherapeutics or next generation probiotics, with a key focus on assessing their safety, efficacy, and the potential risks related to the spread of antibiotic and antifungal resistance.

Laurence T. Maeyens is a PhD candidate who earned a Master's degree in Bioscience Engineering: Cell and Gene Biotechnology from Ghent University (Belgium), after which he conducted research on the human microbiome and the discovery of novel antimicrobials under the mentorship of Dr. Sarah Lebeer. Currently, he is a PhD student within the Integrated Biomedical Sciences program at the University of Texas at San Antonio under the mentorship of Dr. James F. Nelson and Dr. Shangang Zhao, where he investigates the biological mechanisms driving women's longer lifespan compared to men. He is also exploring the effects of adipose tissues and dietary fatty acids on aging and lifespan regulation.

Colin Hill is a Professor of Microbial Food Safety at University College Cork (Ireland), the former President of the International Scientific Association for Probiotics and Prebiotics (ISAPP) and a Principle Investigator in APC microbiome Ireland. His research interests are specifically focused on molecular microbiology concerning infections, which has led to the discovery of novel antimicrobials such as the RiPP, Thuricin CD. Additionally, he is one of the leading scientists exploring the human virome.

Sarah Lebeer is a research professor in the Department of Bioscience Engineering at the University of Antwerp (Belgium), where she holds a chair in applied microbiology and biotechnology. She earned her PhD in Bioscience Engineering from KU Leuven (Belgium) and has been active in microbiome research since 2004. She coordinated the ERC-funded Lacto-Be project (2020–2025), which explored the evolutionary ecology of lactobacilli and their potential in microbiome-based innovations. Her early work focused on *Lacticaseibacillus rhamnosus* GG as a model strain to study host interactions in the gut, particularly in the context of inflammatory bowel diseases. In 2012, she

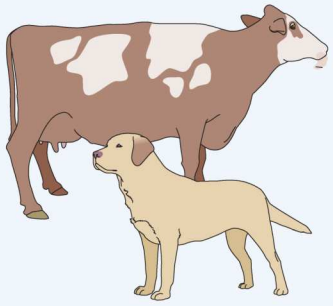
1592 founded her own research group investigating beneficial Lactobacilli across diverse human-
1593 associated habitats. Since 2016, she has served on the academic board of ISAPP. She also leads the
1594 global citizen science program Isala, which explores the vaginal microbiome, one of the most
1595 important natural habitats for Lactobacillus in the human body.

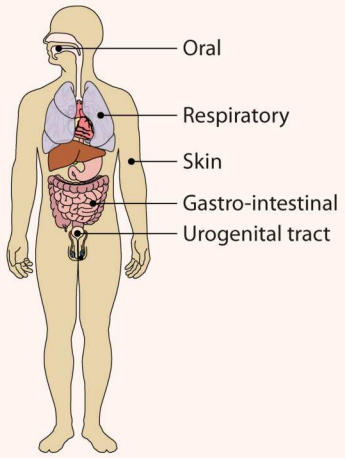
1596

Animal health

Under research:
pediocin PA-1
sakacin P
plantaricin

Lactiplantibacillus plantarum
Pediococcus acidilactici
Latilactobacillus sakei
Limosilactobacillus reuteri





Human health

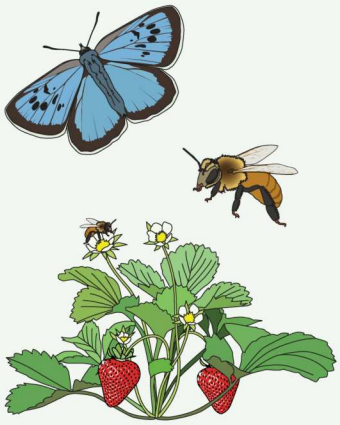
Under research:
pediocin PA-1
sakacin P
plantaricin

Lactobacillus crispatus
Lactobacillus gasseri
Limosilactobacillus reuteri
Lactiplantibacillus plantarum
Lacticaseibacillus rhamnosus

Biocontrol and pollinator protection

Under research:
pediocin PA-1
sakacin P
plantaricin
kunkecin A

Lactiplantibacillus plantarum
Apilactobacillus kunkeii
Lactiplantibacillus pentosus
Limosilactobacillius fermentum

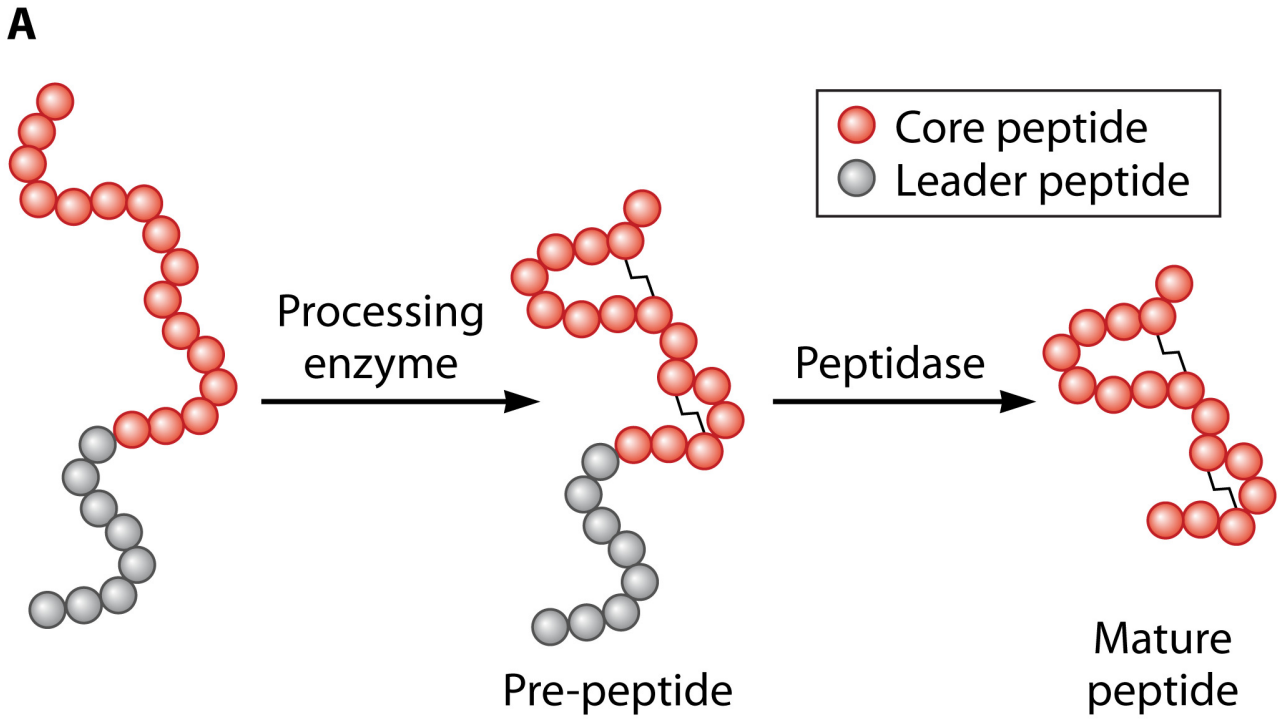


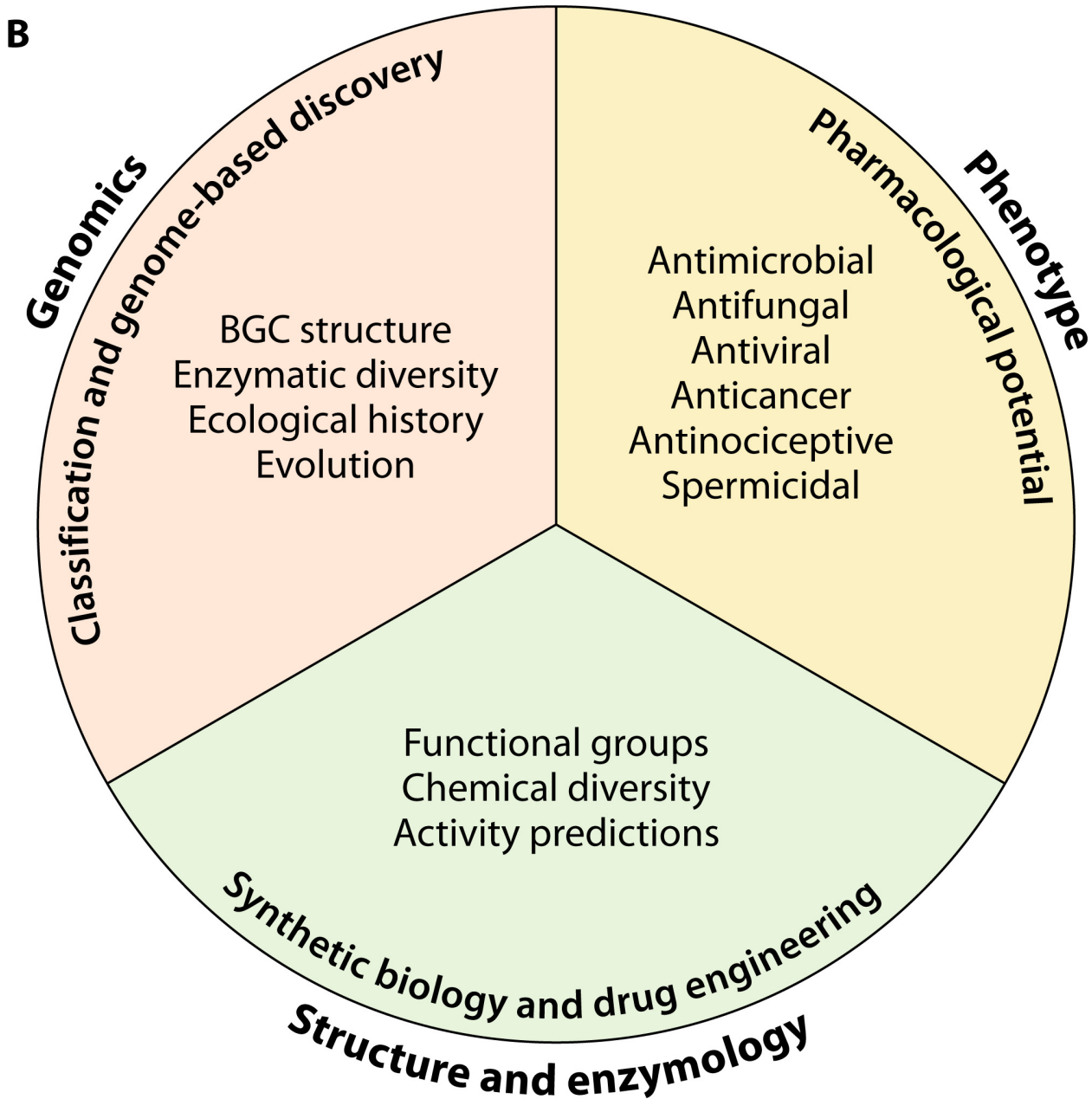
Food fermentation, preservation and safety

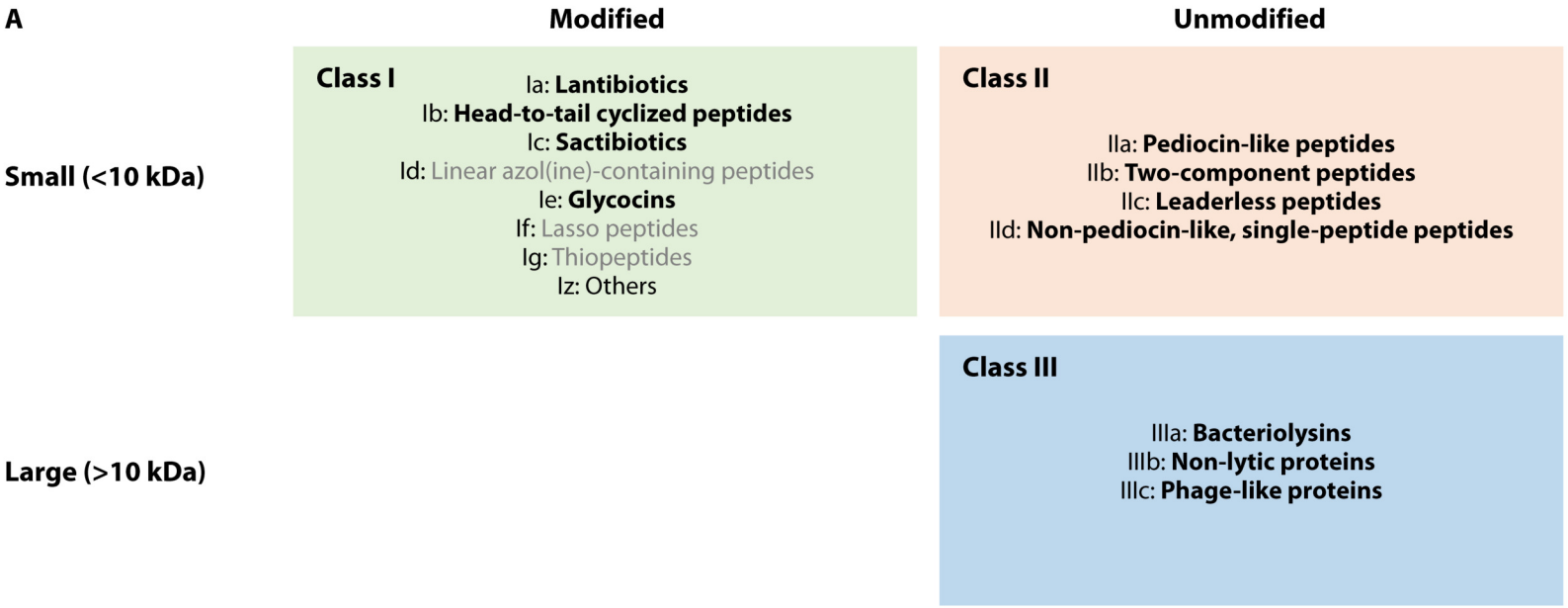
Approved:
nisin
pediocin PA-1

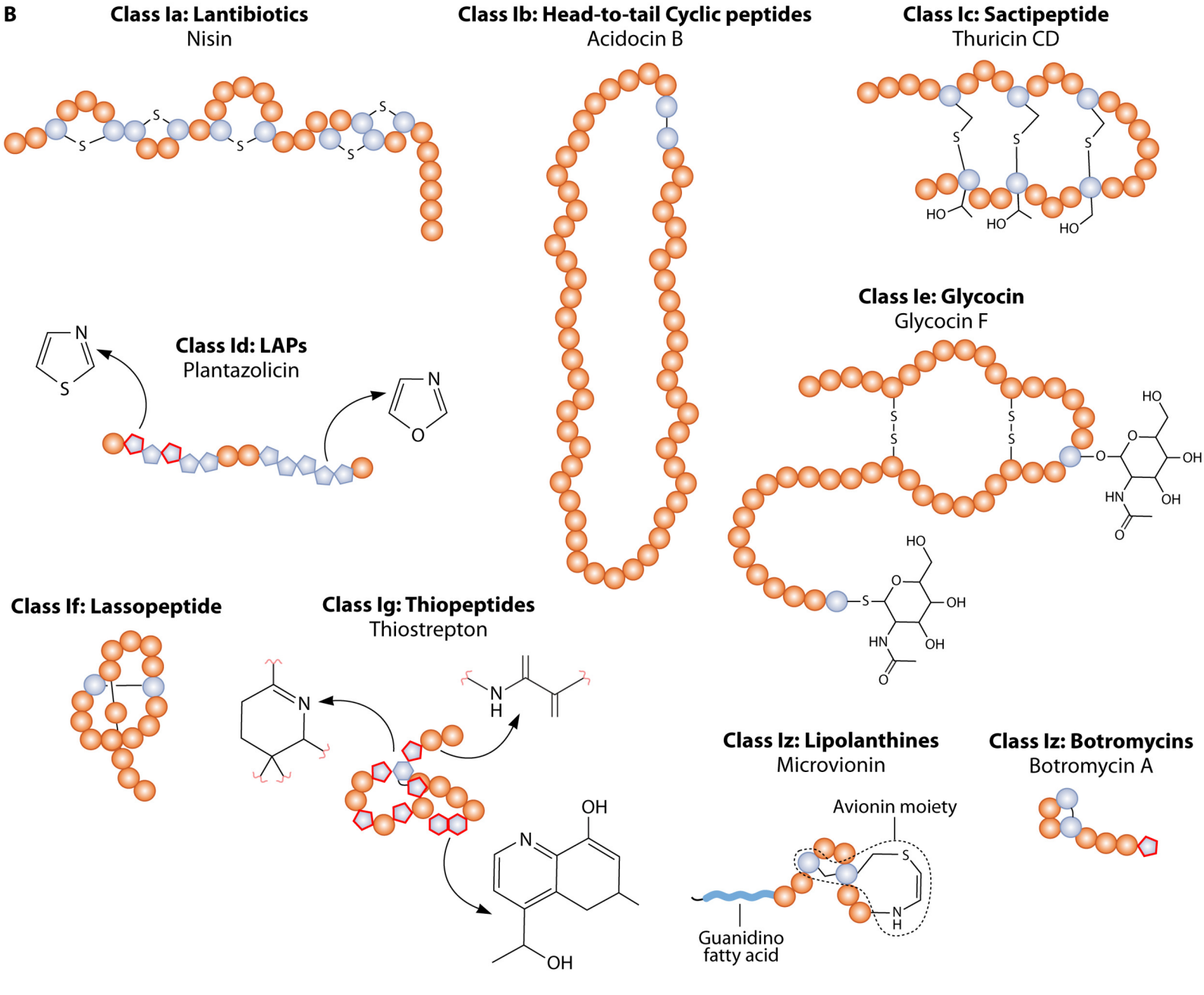
Under research:
sakacin P
plantaricin

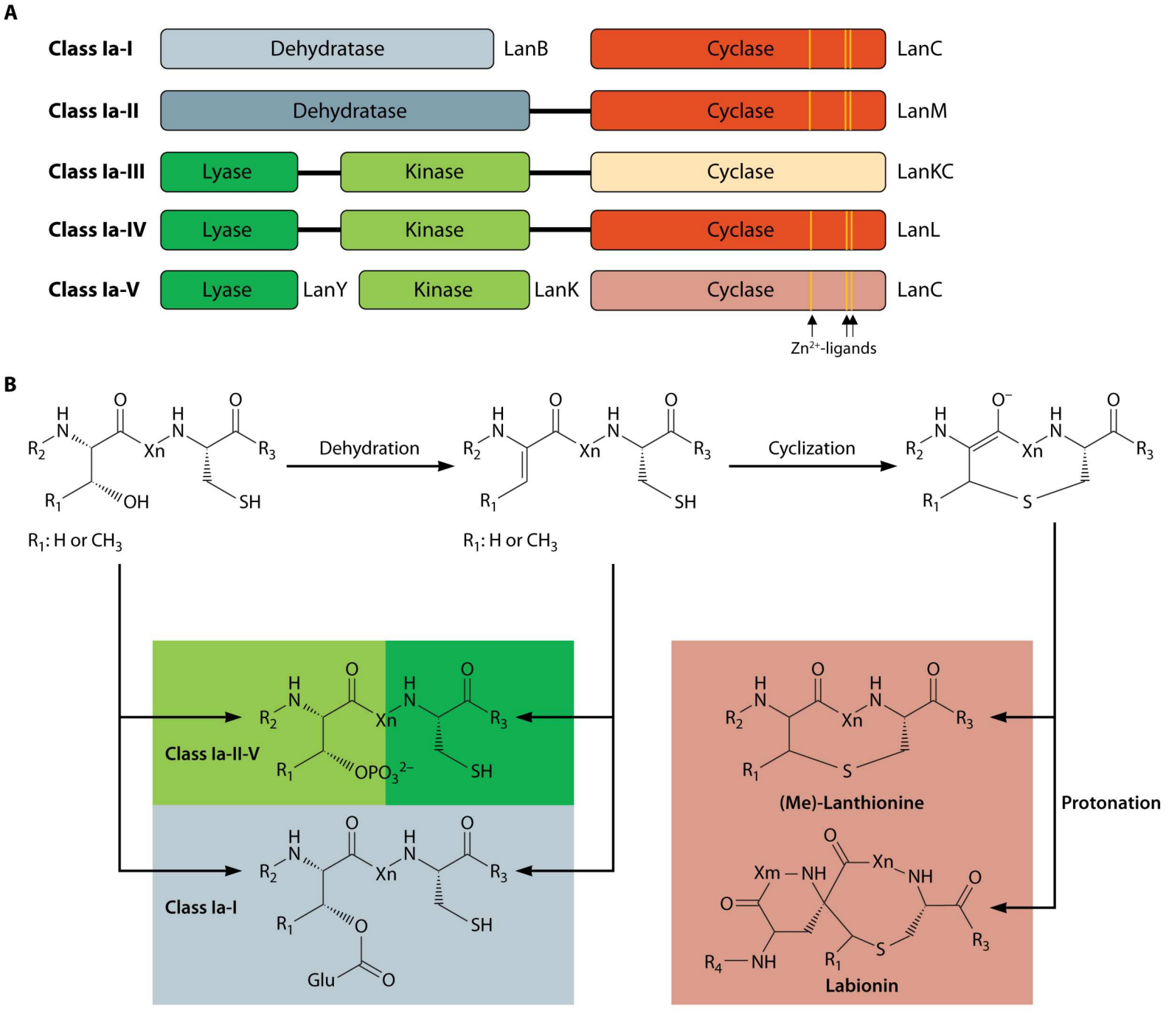
Lactiplantibacillus plantarum
Pediococcus acidilactici
Lactobacillus delbrueckii
Latilactobacillus sakei
Levilactobacillus brevis
Weisella hellenica

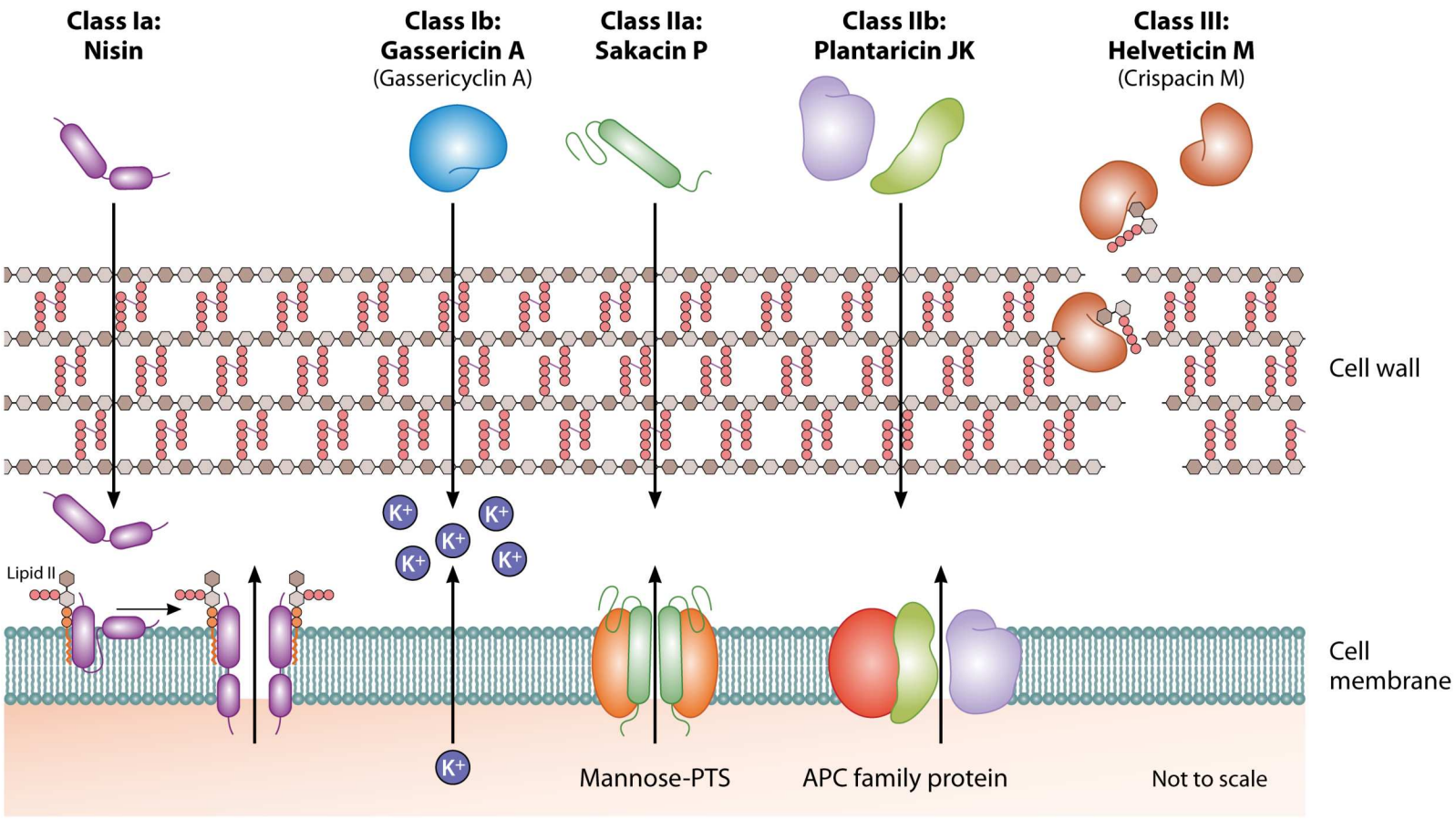

A
Core peptide
Leader peptide
Processing enzyme
Peptidase
Pre-peptide
Mature peptide

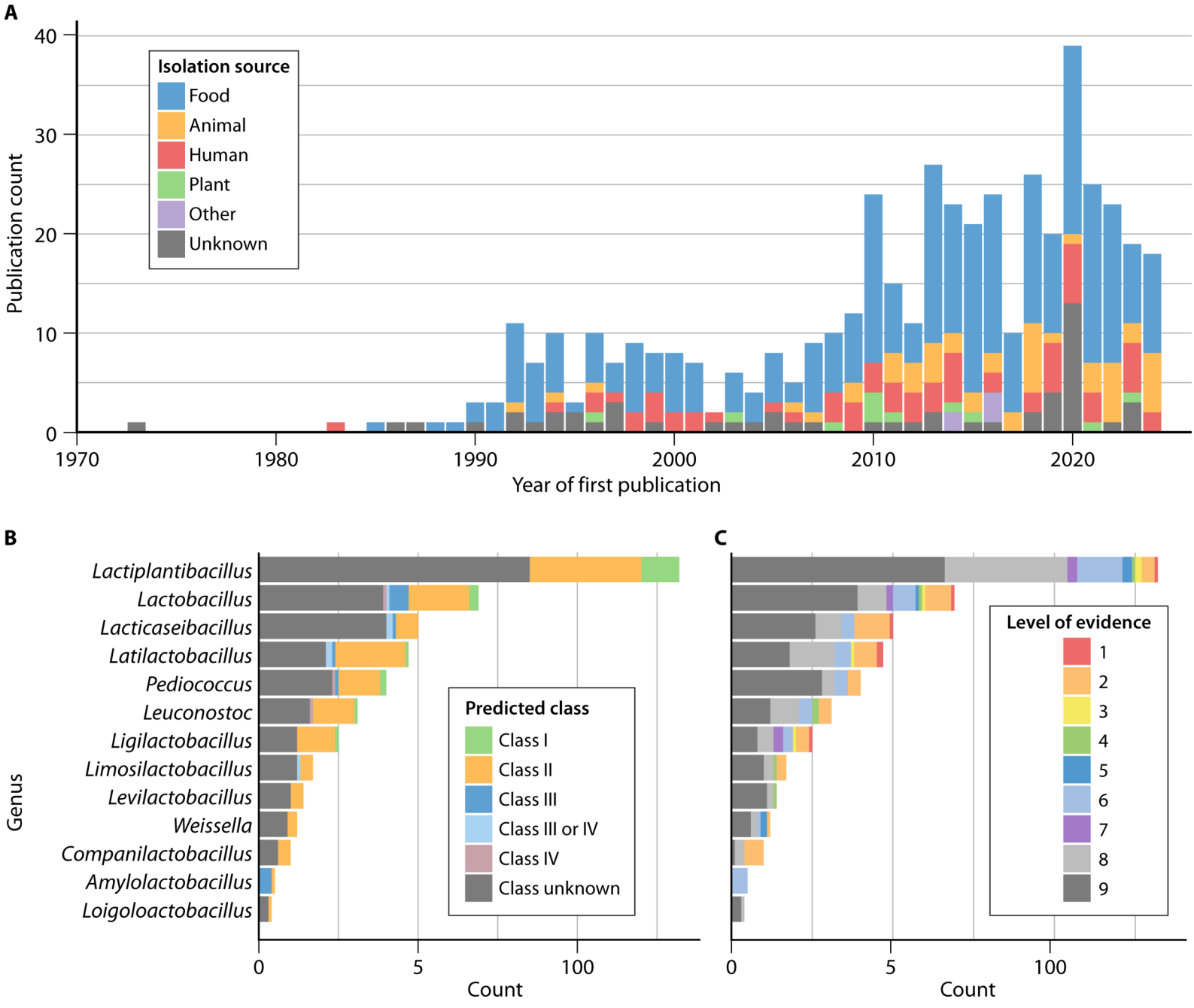

B
Genomics
Classification and genome-based discovery
BGC structure
Enzymatic diversity
Ecological history
Evolution
Phenotype
Pharmacological potential
Antimicrobial
Antifungal
Antiviral
Anticancer
Antinociceptive
Spermicidal
Structure and enzymology
Synthetic biology and drug engineering
Functional groups
Chemical diversity
Activity predictions


A
Modified
Unmodified
Class I
Ia: Lantibiotics
Ib: Head-to-tail cyclized peptides
Ic: Sactibiotics
Id: Linear azol(ine)-containing peptides
Ie: Glycocins
If: Lasso peptides
Ig: Thiopeptides
Iz: Others
Class II
IIa: Pediocin-like peptides
IIb: Two-component peptides
IIc: Leaderless peptides
IId: Non-pediocin-like, single-peptide peptides
Class III
IIIa: Bacteriolysins
IIIb: Non-lytic proteins
IIIc: Phage-like proteins
Small (<10 kDa)
Large (>10 kDa)


B
Class Ia: Lantibiotics
Nisin
Class Ib: Head-to-tail Cyclic peptides
Acidocin B
Class Ic: Sactipeptide
Thuricin CD
Class Id: LAPs
Plantazolicin
Class Ie: Glycocin
Glycocin F
Class If: Lassopeptide
Class Ig: Thiopeptides
Thiostrepton
Class Iz: Lipolanthines
Microvionin
Avionin moiety
Guanidino fatty acid
Class Iz: Botromycins
Botromycin A


A
Class Ia-I
Dehydratase
LanB
Cyclase
LanC
Class Ia-II
Dehydratase
Cyclase
LanM
Class Ia-III
Lyase
Kinase
Cyclase
LanKC
Class Ia-IV
Lyase
Kinase
Cyclase
LanL
Class Ia-V
Lyase
LanY
Kinase
LanK
Cyclase
LanC
Zn2+-ligands
B
Dehydration
Cyclization
R1: H or CH3
R1: H or CH3
Class Ia-II-V
Class Ia-I
(Me)-Lanthionine
Labionin
Protonation


Class Ia:
Nisin
Class Ib:
Gassericin A
(Gassericyclin A)
Class IIa:
Sakacin P
Class IIb:
Plantaricin JK
Class III:
Helveticin M
(Crispacin M)
Cell wall
Lipid II
K+
Cell
membrane
Mannose-PTS
APC family protein
Not to scale


A
Publication count
Year of first publication
Isolation source
Food
Animal
Human
Plant
Other
Unknown
B
Genus
Lactiplantibacillus
Lactobacillus
Lacticaseibacillus
Latilactobacillus
Pediococcus
Leuconostoc
Ligilactobacillus
Limosilactobacillus
Levilactobacillus
Weissella
Companilactobacillus
Amylolactobacillus
Loigoloactobacillus
Predicted class
Class I
Class II
Class III
Class III or IV
Class IV
Class unknown
Count
C
Level of evidence
Count

Short prefix depending on the producer species

Infix A (Classification)

Class I	lan	lanthipeptide
	cy	head-to-tail cyclized peptides
	sa	sactibiotic
	lap	LAP
	gly	glycocin
	las	lassopeptide
	tio	thiopeptide
Class II	pe	pediocin-like
	dua	two-component
	le	leaderless
	si	other unmodified
Class III	ly	bacteriolysins
	no	non-lytic
	phi	phage-like

Infix B (Activity)

ba	antibacterial
fu	antifungal
vi	antiviral
pa	antiparasitic
tu	anti-tumor
li(m)	immunomodulatory
noc	antinociceptive
mi	miscellaneous

Suffix ending with -cin followed by numbering, strain names, or additional suffixes to distinguish them.

To illustrate, class Ia bacteriocin,
plantaricin C ⟶ planta-lan-ba-cin C