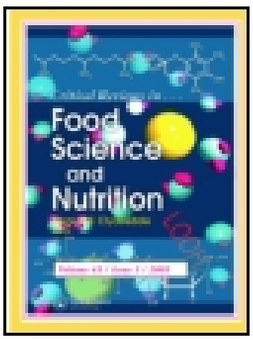

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Bacteriocins as food preservatives: Challenges and emerging horizons

Mr. Eldin Maliyakkal Johnson[a,b], Dr. Yong-Gyun Jung[c], Dr. Ying-Yu Jin[d], Dr. Rasu Jayabalan[b], Dr. Seung Hwan Yang[e], and Professor Joo Won Suh[a,f]

[a]Centre for Nutraceutical and Pharmaceutical Materials, College of Natural Science, Myongji University, Yongin, Korea; [b]Food Microbiology and Bioprocess Laboratory, Department of Life Science, National Institute of Technology, Rourkela, Odisha, India; [c]Interdisciplinary Program of Biomodulation, College of Natural Science, Myongji University, Yongin, Korea; [d]Myongji University Bioefficiency Research Centre, College of Natural Science, Myongji University, Yongin, Korea; [e]Department of Biotechnology, Chonnam National University—Yeosu Campus, Yeosu, Korea; [f]Division of Bioscience and Bioinformatics, College of Natural Science, Myongji University, Yongin, Korea

ABSTRACT

The increasing demand for fresh-like food products and the potential health hazards of chemically preserved and processed food products have led to the advent of alternative technologies for the preservation and maintenance of the freshness of the food products. One such preservation strategy is the usage of bacteriocins or bacteriocins producing starter cultures for the preservation of the intended food matrixes. Bacteriocins are ribosomally synthesized smaller polypeptide molecules that exert antagonistic activity against closely related and unrelated group of bacteria. This review is aimed at bringing to lime light the various class of bacteriocins mainly from gram positive bacteria. The desirable characteristics of the bacteriocins which earn them a place in food preservation technology, the success story of the same in various food systems, the various challenges and the strategies employed to put them to work efficiently in various food systems has been discussed in this review. From the industrial point of view various aspects like the improvement of the producer strains, downstream processing and purification of the bacteriocins and recent trends in engineered bacteriocins has also been briefly discussed in this review.

KEYWORDS

Bacteriocinogenic culture; lantibiotics; induction factors; heterologous expression; biosafety

1. Introduction

Food preservation is targeted towards preserving the original nutritional value, flavor, and organoleptic property of food by preventing the spoilage of food and also inhibiting the growth of potential food borne pathogens (De Martinis and Franco 1998). The increase in population and globalization of food trade has demanded for the large scale production of food products, distribution from centralized production facilities and storage of the products until delivery to the end users. These have posed the challenges of maintaining proper food safety and quality until it reaches the consumer and also throughout its proposed shelf life period. Also the recent increase in the concept of fresh-like food and minimally processed food have fuelled the search for innovative ways to preserve food by inhibiting food borne pathogens and microbial spoilers ensuring the safety, quality, freshness, and organoleptic properties of the food. Generally the preservation process is composed of thermal treatment, drying, salting, cold storage, and/or usage of modern preservation techniques like canning, pasteurization, addition of chemical preservatives to delay the spoilage of food, and to increase the shelf life of the intended food product.

Because of the stringent food legislation, safety standards, and consumer demands towards preservatives free food products, the usage of classical preservative techniques like salting, smoking, and usage of preservatives like benzoic, sorbic, acetic, lactic acids, etc. in the food have been discouraged. Also because of the proven adverse effects of these preservation techniques like the allergic reactions to some of these chemical preservatives and the threats of formation of carcinogenic end-products like nitrosamines from nitrites (Kashani et al. 2012) alteration of the sensory properties of the food and destruction of nutrients available in food by such harsh physical and chemical treatments. These have attracted interest towards an alternative natural biopreservation technology. The usage of non-pathogenic microorganisms and /or their metabolic products and natural products of biological origin to ensure the safety and to improve the shelf life by inhibiting the spoilage of food is gaining more attention in the recent days. The selection, improvement, and production of useful microorganisms and bio-products, their technological improvement for application in food industry have catered to the raising demands for such biopreservation strategies.

Bacteriocins are ribosomally synthesized smaller polypeptide molecules that exert antagonistic activity against closely related and unrelated group of bacteria (Table 1). Among the various gram positive bacteria, the bacteriocins produced by

CONTACT Dr. Rasu Jayabalan rasuayabalan@gmail.com Department of Life Science, National Institute of Technology, Rourkela, Odisha, India.; Dr. Seung Hwan Yang ymichigan@jnu.ac.kr Department of Biotechnology, Chonnam National University—Yeosu Campus, Yeosu, Korea.; Joo Won Suh jwsuh@mju.ac.kr Division of Bioscience and Bioinformatics, College of Natural Science, Myongji University, Yongin, Korea.

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Table 1. Characteristics of bacteriocins from different group of microbes.

Characteristics	Archaea	Gram negative bacteria	Gram positive bacteria
Source organism and antibacterial activity spectrum	Archaeosins are the bacteriocins produced by archaea. They are known to have wider spectrum of activity across species and domains present in the extreme environments.(Atanasova, Pietilä, & Oksanen, 2013)	Gram negative bacteria produce bacteriocins which have narrow antibacterial activity spectrum.	Gram positive bacteria produces bacteriocins which have wide antibacterial activity spectrum against some food spoilage organism and are nontoxic to the non-target eukaryotic cells and are hence regarded as safe for food applications.
Identified bacteriocins	Halocin S8 from halobacteria, a short hydrophobic peptide with 36 amino acids is the first discovered archaeosin.	Colicins from *E.coli*, Klebicins of *Klebsiella pneumonia*, marcescins of *Serratia marcescens*, alveicins of *Hafnia alvei*, cloacins of *Enterobacter cloacae* and pyocins of *Pseudomonads* are the representatives of gram negative bacterial group.	Most of the bacteriocins are produced by Lactic acid bacteria which are rendered "GRAS" status. Nisin A by *Lactococcus lactis, Cytolysin* by *Enterococcus faecalis*, Pediocin by *Pediococcus acidilactici*, Lactococcin from *Lactococcus cremoris* and a vast repertoire of bacteriocins are produced by this group of bacteria.
Structure and mode of action	They are generally divided into two groups (i) Protein halocins (30–40 kDa) like H1 and H4 (Meseguer & Rodriguez−Valera, 1985; Shand & Leyva, 2007) (ii) Microhalocins (smaller than 10 kDa) like H6/H7, R1, C8, S8 and U1 (Shand & Leyva, 2007) The halocin H6/H7 are shown to inhibit the growth by disrupting Na^+/H^+ antiporter causing cell lysis. These archaeosins are produced in stationary phase. They are encoded in megaplasmids.(Meseguer & Rodriguez−Valera, 1985) The halocin H6/H7 are shown to inhibit the growth by disrupting Na^+/H^+ antiporter causing cell lysis. These archaeosins are produced in stationary phase. They are encoded in megaplasmids.(Meseguer & Rodriguez−Valera, 1985)	The bacteriocins have relatively larger structure. They are divided in to three types: (i) Microcins which are less than 20 kDa in size (ii) Colicin-like bacteriocins (CLBs) 20 to 90 kDa in size (Cascales et al. 2007) (iii) Tailocins which are high molecular weight bacteriocins with multi subunits resembling the tail like structure of bacteriophages.(Ghequire et al. 2014) The CLBs are composed of a receptor binding domain, a translocation domain and a cytotoxic domain. Thus the bacteriocins are bound and imported in to cell membrane and exercising the cytotoxic activity by nucleases/pore formation. (Cascales et al. 2007)	They greatly differ in their structure and their mode of action. (See section 2)
Stability and other desirable characteristics	Protein halocins are generally more sensitive to environmental stress. Microhalocins have the ability to withstand low salt concentrations, heating, long-term storage. (Meseguer & Rodriguez−Valera, 1985; Pašić, Velikonja, & Ulrih, 2008; Shand & Leyva, 2007)	They are generally heat labile with the exception of Microcin V produced by *E.coli*	They are generally heat stable and are functional at a wide range of pH.

the lactic acid bacteria (LAB) have attracted tremendous attention among the other bacterial species with respect to utilization in food industry (Cotter, Hill, and Ross 2005b), because, they are recognized with "Generally Regarded As Safe (GRAS)" status by the US Food and Drug Administration (Montville et al. 2013, Chen and Hoover 2003). Due to the proven safety and antagonistic potency against various food spoilage microbes and pathogenic bacteria these bacteriocins have attracted greater attention and application as natural preservative agents in food industry. This revolution has led to the discovery of a repertoire of bacteriocins and preservation technologies for using them in a range of food matrixes. These bacteriocins are distinct from antibiotics in a way that they do not require multi enzyme system for their synthesis in contrast to the latter, further bacteriocins are very effective in a very small concentration and most of the well characterized bacteriocins with proven safety, have hardly any toxicity or side effect causing adverse interactions like that of the antibiotics (Cotter, Ross, and Hill 2013). The bacteriocins have distinct host cell immunity mechanisms and advantageous with respect to mechanisms of target cell resistance and tolerance thus making it a more favorable and safe alternative for wide spread usage in food preservation strategies. (Cleveland et al. 2001, Riley and Wertz 2002a, Nes et al. 2007, Balciunas et al. 2013)

The first bacteriocin discovered is colicins in the year 1925 was isolated from gram negative bacteria *Escherichia coli*, these antibacterial peptides exerted bactericidal effects on only closely related bacteria or narrow spectrum of bacteria by inhibition of cell wall synthesis, permeabilization of the cell membrane, and inhibition of RNase or DNase activity (Cleveland et al. 2001, Riley and Wertz 2002a, Nes et al. 2007, Balciunas et al. 2013). Later the broad spectrum bacteriocins from gram positive microbes belonging to the group lactic acid bacteria which are effective against an array of food spoiling microbes and food borne pathogens were discovered which have greater application in food preservation because of their various advantageous traits than that of bacteriocins from their gram negative counterparts like: (i) the usage of bacteriocin producing strains directly in the food matrix as protective cultures, (ii) the possibility of using LAB as a starter culture in fermented foods, (iii) no rigorous purification procedure is required for bacteriocin preparations from LAB unlike gram-negative bacteria which may contain LPS and other endotoxins causing deleterious effect,

if not removed (Cintas et al. 2001, Riley and Wertz 2002a, Galvez et al. 2008).

2. Classification of bacteriocins from gram positive bacteria

Bacteriocins have been classified into various groups based on their physical properties, stability, chemical structure (see Fig. 1), molecular size, mode of action, and the kind of organism producing them. There has been lot of debate regarding their classification and it has been classified and reclassified in to many groups starting from Klaenhammer (1993) to (Heng et al. 2007) incorporate the similarities and differences that were observed in the new molecules discovered all through the years. In this review the classification proposed according to (Heng et al. 2007) is followed in an attempt to encompass all the bacteriocin molecules discovered and isolated from the gram positive bacteria.

2.1. Class I (The Lantibiotics)

They are post translationally modified peptides (<5 kDa) (Jack et al. 1995) ranging from 19 to more than 50 amino acids and are referred to as lantibiotics (McAuliffe et al. 2001) because they are composed of certain unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine, and dehydroalanine. They are thermostable because of the presence of lanthionines in their structure (Balciunas et al. 2013). The preliminary translation product of lantibiotics is a prepeptide which is composed a leader peptide at the N-terminus and the pro-peptide region. Extensive post translational modifications occur in the pro-peptide region especially in the serine, threonine, and cysteine residues (Brotz et al. 1998) which results in the formation of multiple thioether rings in the lantibiotic structure. These modifications are considered to give thermal stability, resistance towards proteolytic degradation, and antibiotic activity (Brotz et al. 1998, McAuliffe et al. 2001). The leader peptide is composed of 23 to 59 amino acid residues and no amino acid modification occurs in the leader peptide region.

These lantibiotics are further classified in to three subgroups:

2.1.1. Class Ia

They include linear lantibiotics such as nisin which are cationic and are composed of hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid class Ib lantibiotics. e.g., Nisin from *L. lactis* subsp. *lactis*

2.1.2. Class Ib

They have a globular structure with up to 19 amino acid residues and have no net charge or might have a net negative charge (Altena et al. 2000). e.g., Mersacidin from *Bacillus* sp.

2.1.3. Class Ic

These are lantibiotics which requires two or more modified peptides to be functional. e.g., lacticin 3147 from *L. lactis* DPC3147

2.2. Class II (The unmodified peptides)

The class II bacteriocins are composed of translationally unmodified small peptides (< 10 kDa)

They are further subdivided in to three subgroups:

2.2.1. Class IIa

This subgroup have a conserved consensus motif YGNGV $(X)C(X)_4C(X)V(X)_4A$ at their hydrophilic N-terminus end

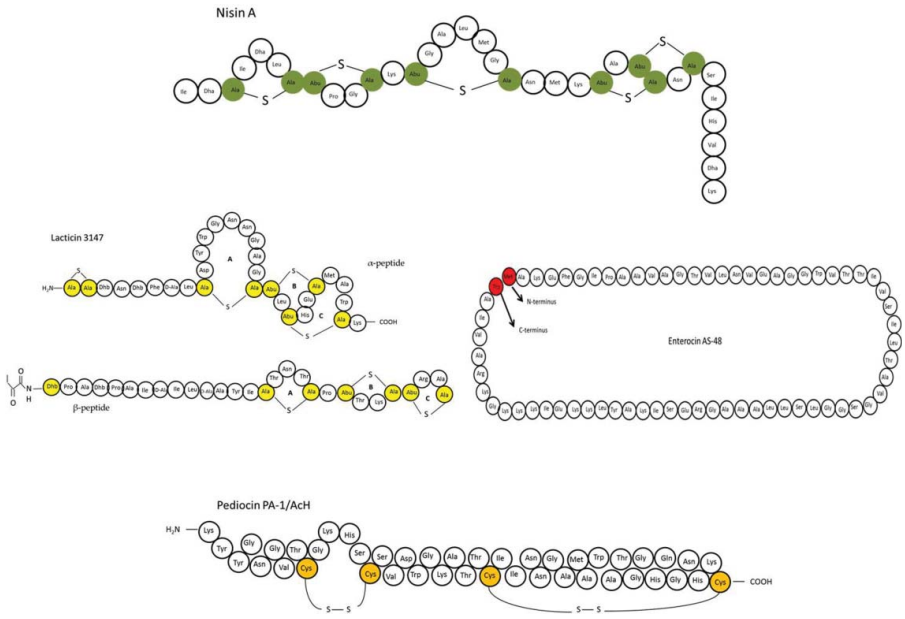

Figure 1. Structure of lantibiotics, nisin A and lacticin 3147 from class I, cyclic bacteriocin enterocin AS-48 and unmodified (class II) bacteriocin pediocin PA-1/AcH (Class IIa bacteriocin). Each ring of individual lantibiotics are represented by capital letters. Dha- dehydroalanine, Dhb- dehydrobutyrine. Enterococin AS-48, which is a 70 amino acid peptide molecule, containing methionine in the N-terminal region is ligated to tryptophan in the C-terminal region forming amide bond. (Adapted from Nishie, Nagao, and Sonomoto (2012)).

with a more varying hydrophobic or amphiphilic C-terminus. They are thermostable and do not contain the lanthionine and their derivatives. They include pediocin like peptides having the N- terminal consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys and have a molecular mass of 3–7 kDa, peptide chain length of 25–58 amino acids and are positively charged. e.g., pediocin PA-1 (pediocins AcH, JD, Bac, 347, mesentericin 5 are all synonymous and are identified as the same bacteriocin (Rodríguez et al. 2002), hence the more widespread name, pediocin PA-1 will be used in this review) from *Pediococcus acidilactici*. This sub group has attracted attention because of their anti-listerial activity which is a potent food contaminant (Ennahar et al. 1999). They are regarded as anti-listerial peptides active against *L. monocytogenes* such as leucocin A and pediocin PA-1 (Hastings et al. 1991). To date more than a dozen of pediocin-like bacteriocins have been isolated (Drider et al. 2006). All these pediocin like bacteriocins consist of a disulphide bridge formed by cysteine residues at their N- terminal region and some may have an additional disulphide bond near the C-terminus like pediocin PA-1, enterocin A, and sakacin G. These disulphide bridges give the stability to the molecule and also enhance the antimicrobial activity (Fimland et al. 2000).

2.2.2. Class IIb

They are composed of bacteriocins which require the aid of two or more non-modified peptides for eliciting antimicrobial activity. These multicomponent unmodified bacteriocins have 30–60 amino acid residues with molecular mass of 3–6 kDa having net positive charge and high isoelectric points. They may be amphiphilic or hydrophobic structures and attacks the cell membranes of the target strains. Only two component bacteriocins have been discovered among the multicomponent bacteriocins, so far in this group. e.g., enterocin 1071 (A and B) from *E. faecalis* BFE1071 (Nissen-Meyer et al. 2010)

2.2.3. Class IIc

They are single peptide non-pediocin like bacteriocins which are a heterogenous group with a molecular mass of 3.5–7.5 kDa and have diverse mode of action for killing the target cells. e.g., enterocin EJ97 from *E. faecalis* EJ97

2.3. Class III (Large proteins)

They are large proteinaceous (>30 kDa) heat labile bacteriocins (hence poorly utilized in food preservation applications) and are generally termed as bacteriolysins which is basically endopeptidases causing lysis of the cell walls of susceptible strains through their enzymatic action. Their primary structure is composed of two domains a C-terminus substrate binding domain and an N-terminal catalytic domain belonging to M37/M2 endopeptidase family. These two functional domains are connected by a threonine-proline containing linker sequence (Simmonds et al. 1996, Nilsen et al. 2003).

They have been sub divided in to two subgroups:

2.3.1. Class IIIa

They are considered as lytic bacteriocins which acts like an enzyme and they attack the peptidoglycan layer of the cell wall in susceptible gram positive bacterial targets. The resultant cell damage causes exosmosis and cell death. They have a molecular mass of 25–35 kDa and have 200–400 amino acid residues. e.g., enterolysin A from *E. faecalis* (Maliničová et al. 2010, Nilsen et al. 2003)

2.3.2. Class IIIb

They are non-lytic heat labile bactetiocins which have peculiar mode of action against the susceptible bacterial cells. Helveticin J (Joerger and Klaenhammer 1986), streptococcin A-M57 (Heng et al. 2004), and dysgalacticin (Heng et al. 2006) are the bacteriocins belonging to this group. Dysgalacticin is the bacteriocin which is active against *S. pyogenes*, they bind to the phosphoenolpyruvate-dependent glucose and mannose-Phosphate Transport System which hampers the glucose uptake by the cells and starves them and also disturbs the membrane potential which triggers the utilization of stored ATP to counteract the disturbance in membrane potential and hence depleting the energy reserve leading to cell death (Swe et al. 2010).

2.3.3. Class IV (Circular peptides)

These circular bacteriocins are unique from the other linear bacteriocins by their cyclization formed by the ligation of their N-terminus to the C-terminus via an amide bond. They are primarily synthesized ribosomally as linear molecules which are then post translationally cyclized after the cleaving of the leader peptide. This leader peptide is considered to play a pivotal role in the cyclization step (Fernandez et al. 2008). They have a molecular weight of 5.5–7.5 kDa and have a peptide chain length of 35–70 amino acids. The enterocin AS-48 isolated from *Enterococcus faecalis* subsp. *liquefaciens* S-48 is the first isolated bacteriocin of this group that have been purified and studied extensively (Galvez et al. 1989). The AS-48 enterocin molecule is 70 amino acid globular protein composed of five α-helices made up of hydrophobic amino acids which make the core of the globular structure highly hydrophobic. This hydrophobicity of the core plays a crucial role by inserting themselves in to the hydrophobic regions of the lipid bilayer of the cell membrane and elicits its antimicrobial activity by this membrane action (Sanchez-Barrena et al. 2003). The linear form or denatured form of AS-48 molecule has hardly been found to be active than their circular counterpart and are shown to be highly temperature sensitive. Their secondary structure prediction credits the α-helical structure and the saposin-like motif, for their specific interaction with the lipid membrane and thus making these circular structures membrane active (Martin-Visscher et al. 2009). Similar structures are found in gassericin A, which points towards these circular structure accountable for stability and antibacterial activity (Montalbán-López et al. 2012).

Among all these classes of bacteriocins those belonging to class I, II and IV like nisin (available in the market as Nisaplin™ and Novasin™ etc.,), pediocin (commercial preparation Alta™ 2341), and enterocin AS-48 (no commercial preparation available) are widely utilized in food preservation (Table 2), because of their stability to heat and activity at a

Table 2. Bacteriocins used in food preservation and their characteristics.

Bacteriocin class	Bacteriocin	Desirable properties	Producer organism	Target food contaminant	Type of food matrix preserved	Reference
Class Ia	Nisin	Heat stable at 121 °C for prolonged heating at pH 2. Become less heat stable at pH 5–7. Sensitive towards α-chymotrypsin, resistant to trypsin, elastase, carboxypeptidase A, pepsin, and erepsin.	*L. lactis subsp. lactis*	*Streptococcus thermophilus, Lactobacillus spp., L. monocytogenes, L. lactis, S. aureus, Clostridium botulinum, Bacillus cereus*	Processed cheese products, ricotta cheese, pasteurized milk and milk products, canned products, salad dressings, crumpets, sausages, meat products	Meghrous, Lacroix, and Simard (1999)
Class Ic	Lacticin 3147A	Heat stable at 100 °C for 10 min at pH 5 or 90° C for 10 min at pH 7.Stable at room and low temperature, heat stable at 100° C for 60 min or 121° C for 10 min. Most stable at acid and neutral pH.	*L. lactis* *DPC3147*	*B. subtilis, S.aureus, L. monocytogenes Lactobacillus fermentum*	Infant formula, yoghurt, cottage cheese, sausages, ground beef	Ryan et al. (1999)
	Plantaricin C	Resistant to pepsin, proteinase K, α-amylase, and lipase.	*L. plantarum*	*Clostridium, Bacillus, Staphylococcus and also to Listeria monocytogenes*	Dairy products, meat, canned foods	Gonzalez et al. (1996)
Class IIa	Pediocin PA-1	Stable at pH 4 to 6, becomes less stable as pH increases. Heat stable at 80° C for 60 min or 100° C for 10 min. Resistant to phospholipase C, catalase, lysozyme, DNAses, RNAses, and lipase.	*Pediococcus acidilactici*	*Lactobacillus helveticus, Pediococcus pentosaceus, L. monocytogenes*	Cheddar cheese, munster cheese, liquid whole egg, meat products (sausages, meat sticks),	Rodriguez, Martinez, and Kok (2002)
Class IIb	Enterocin 1071	The peptides are heat resistant (100° C, 60 min; 50% of activity remained after 15 min at 121°C), remain active after 30 min of incubation at pH 3 to 12, and are sensitive to treatment with proteolytic enzymes.	*Enterococcus faecalis BFE 1071*	*L. monocytogenes, Listeria innocua, Enterococcus faecalis, Enterococcus faecium*	Fish spread	Balla et al. (2000)
Class IIc	Enterocin EJ97	Very stable under mild heat conditions and is sensitive to proteolytic enzymes	*E. faecalis EJ97*	*Bacillus spp., E. faecalis, L. monocytogenes,L. monocytogenes, S. aureus, Geobacillus Stearothermophilus*	Canned vegetable foods and drinks	Galvez et al. (1998)
Class IV	Enterocin AS-48	Active at pH 9.0 and in combination with moderate heat treatment. Inactivated by heated for 5 min at 65°C in an alkaline (pH 9.0). Compatible with several chemical compounds: EDTA, lactic acid, peracetic acid, polyphosphoric acid, sodium hypochlorite, hexadecylpyridinium chloride, propyl-p-hydroxybenzoate, and hydrocinnamic acid	*Enterococcus faecalis subsp. liquefaciens S-48*	*Bacillus spp., S. aureus, L. monocytogenes, E. faecalis, E. faecium, E. coli Alicyclobacillus acidoterrestris, Bacillus spp., Paenibacillus spp., Geobacillus stearothermophilus, Brochothrix thermosphacta, Staphylococcus carnosus, Lactobacillus sakei and other spoilage lactic acid bacteria*	Ready-to-eat salad, model sausages, canned fruits and vegetables, raw fruits and fruit juices, vegetable sauces, vegetable soups, and purees	Cobo Molinos et al. (2008)

wide range of pH, making it more advantageous to be used in a variety of food matrixes.

3. Ecology of bacteriocin producing gram-positive bacterial species

Microbes produce a variety of defensive metabolites like antibiotics, bacteriocins, organic acids such as lactic acids, bacteriolytic enzymes like lysozymes, protein exotoxins, hydrogen peroxide etc. Among these defensive molecules, bacteriocins are found in large diversity and in abundance in most of the ecological niches (James et al. 2013, Riley and Gordon 1992). These bacteriocins equip the producer bacterial strain with a defensive advantage over the other group of bacteria in the niche by helping them in surviving, outgrowing and eliminating the competing bacterial species in a particular ecological niche, where the competition for nutrition is intense, considering the diversity and the difference in growth pattern among the species (Dykes 1995). The ability to produce bacteriocins and immunity to bacteriocins in the producer strains play a vital role in moulding a bacterial population within an ecological niche (Riley and Wertz 2002b). Thus the interactions of the bacteriocins are very complex and play a significant role in an particular ecological setting and at evolutionary levels in mixed populations, such as in biofilms and food systems (Riley 1998). Apart from the defensive role of bacteriocins they are also known to mediate quorum sensing in an ecosystem (Miller and Bassler 2001), thus the bacteriocins play multiple role depending on the changes in both biotic and abiotic components of the environment. The usage of the bacteriocins is also associated with the risk of the evolution of resistant pathogenic or food spoiler strains but the window of escape is that the resistant strains are known to have a slower growth rate compared to their sensitive precursor (Dykes and Hastings 1998) and the usage of a combination of bacteriocins (Bouttefroy and Millière 2000, Vignolo et al. 2000) and the inclusion of additional hurdles along with the bacteriocin treatment would efficiently control the food spoilage.

The recurring events of listeriosis outbreaks coupled with the inability of the conventional food preservation methods to inhibit the growth of such food pathogens which have the ability to grow at near-freezing temperatures have brought bacteriocins in to lime light in the food preservation arena (Palumbo 1986). The bacteriocin producing strains are also discovered in various fermented foods like cured meats, soybean paste, cheese etc., Pediocin incorporated on to gelatin films when used in the packing of hot dogs have inhibited the growth of *Listeria monocytogenes* (Raloff 1998) and also the usage of bacteriocinogenic strains in sausages have reduced the load of *Listeria* considerably when compared to untreated sausages. Pediocin activity was detected in the treated sausages even after two months of refrigeration showing the competitive advantage of bacteriocin producing strains over the spoilage organisms in the food ecosystem. Also in the case of fermented Spanish-style green olives containing bacteriocins producing *L. plantarum* strain, showed the persistence of this strain in the food matrix for about three months when compared to that of the non-bacteriocin producing strain in the same ecosystem. It was found that the latter was easily outcompeted by the background *Lactobacilli* of the ecological niche (Ruiz-Barba et al. 1994). The lethality of the bacteriocins are not only limited to a narrow spectrum of action against bacterial species like in the case of lactococcins A, B and M which are only active against *Lactococcus* (Ross et al. 1999) but, some of the bacteriocins are also known to have a broad spectrum of activity like nisin A and mutacin B-Ny266, which are known to affect numerous organisms like *Streptococcus, Staphylococcus, Actinomyces, Bacillus, Clostridium, Enterococcus, Lactococcus, Listeria, Mycobacterium, and Micrococcus* (Mota-Meira et al. 2000) and also various gram negative bacteria like *Campylobacter, Neisseria, Helicobacter* and *Haemophilus* (Mota-Meira et al. 2000). The bacteriocins are produced in the gram positive bacteria during the stationary phase of their growth such as in the case of nisin the production begins in the mid-log phase and accumulates to its maximum level during the stationary phase (Buchman et al. 1988). Thus the regulation of the production of bacteriocins is found to be dependent of cell density rather than cell cycle. It is also observed that the synthesis of nisin A is regulated by a protein pheromone, through a two-component signal transduction system, similar to that of quorum-sensing systems. The genes involved in the regulation process are annotated as nisR which is the response regulator and nisK the sensor kinase (De Ruyter et al. 1996). The induction of the nisin transcription by the external addition of nisin to the fermenting medium also supports the notion of the self-regulatory action of the bacteriocin. It is also observed that the nisin expression is directly proportional to the amount of nisin added externally to the production medium.

Several mathematical models have been developed from these ecological traits and they are being used to determine the efficiency and the production of bacteriocins by the producer strains in various food matrixes. (Leroy and De Vuyst 2003, Messens et al. 2003, Leroy and De Vuyst 2005, Leroy et al. 2002). A method using agar plates for determining the effect of various intrinsic factors like pH, concentration of sodium chloride and indicator cell's density on the diffusion and efficiency of bacteriocins were also developed (Blom et al. 1997). Commercial production of bacteriocins requires optimization of process parameters to increase the yield of bacteriocins, such that it would be economically viable. Such production process guided by robust mathematical models predicting the yields at a given physicochemical condition are developed and translated from lab scale optimization studies to industrial scale production process (Dominguez et al. 2007, Messens et al. 2002, Neysens and De Vuyst 2005). It was found that the concentration of the indicator cells and the pH considerably affects the activity of the bacteriocins and production yield in all the bacteriocins types.

4. Genetic localization and the organization of the gene clusters of bacteriocin

Bacteriocins in gram positive bacteria are diverse than gram negative bacteria (Jack et al. 1995, Tagg, Dajani, and Wannamaker 1976). They are unique from gram negative bacteria in two ways: (i) The production of bacteriocin does not cause lethality to the producer strains by virtue of the transport mechanism and the release of bacteriocin, (ii) regulation of the

production of bacteriocins. Also the production of bacteriocins in gram positive bacteria recruits more genes for their production than the gram negative bacteriocins for example the nisin gene cluster is composed of prepeptide (*nisA*), enzymes for the modification of aminoacids (*nisB, nisC*), genes encoding for the cleavage of the leader peptide (*nisP*), secretion (*nisT*), immunity (nisI, nisFEG) and regulation of expression (nisR, nisK) (**Fig. 2**) (Tagg et al. 1976, Ra et al. 1999, Lau, Parsons, and Uchimura 1992, Kaletta and Entian 1989, Engelke et al. 1994, Engelke et al. 1992, Buchman et al. 1988). These gene clusters are found mostly on plasmids and are occasionally found on the chromosome. Some of the gram-positive bacteriocins including nisin are also found to be located on transposons (Dodd et al. 1990).

The genes encoding for bacteriocin production and immunity are usually found as operon clusters (Nes et al. 1996, Sahl and Bierbaum 1998, McAuliffe et al. 2001). For the linear unmodified bacteriocins like the sakacins, carnobacteriocins, and plantaricins the inducing peptides are located on the same gene cluster (Quadri et al. 1997, Brurberg et al. 1997, Anderssen et al. 1998). Three types of localizations of bacteriocin operons have been observed in the producer bacteria:

(i) Chromosomal localization such as in case of Class I lantibiotics like mutacin II and mutacin III produced by *Streptococcus mutans* (Qi et al. 1999a, b) salivaricin A produced by *Streptococcus salivarious* (Ross et al. 1993) and also in heat stable peptide bacteriocins belonging to Class II lantibiotics such as enterocin A and B produced by *Enterococcus faecium*, lactacin F by *Lactobacillus johnsonii* (Allison, Fremaux, and Klaenhammer 1994), plantaricin S (Stephens et al. 1998) and plantaricin A (Diep et al. 1994) by *Lactobacillus plantarum*.

(ii) Plasmid localization of operons are found in lacticin 481 located on 70 kb plasmid (Rince et al. 1997) and the two-component lacticin 3147 located on 63 kb plasmid (Ryan et al. 1996, McAuliffe, Hill, and Ross 2000) in *Lactobacillus lactis*. Numerous non-lantibiotic bacteriocins are also found to be localized on plasmids such as in pediocin PA-1 in a 94 kb plasmid (Marugg et al. 1992) and pediocin AcH (Bukhtiyarova, Yang, and Ray 1994) in an 8.9 kb plasmid, produced by the strains of *Pediococcus acidilactici*. Sakicin A is found to be localized in an 60 kb plasmid as found in *Lactobacillus sakei*, enterocin P which is a two component bacteriocin synthesized by *Enterococcus faecium* (Cintas et al. 2000).

(iii) The lantibiotic nisin which is an widely used bacteriocin in food preservation produced by *Lactococcus lactis* are localized on an conjugative transposon Tn5276 within the chromosome of the producer strain (Van der Meer et al. 1993). The localization of lacticin 481 also produced by *Lactococcus lactis* is found in the transposon Tn5721 located on a 70 kb plasmid. Both the transposon localized bacteriocin genes are flanked by intact insertion sequences (IS) or inverted repeats (IR). Many other bacteriocin genes are also located in proximity to IS or IR suggesting their origin from an ancestral gene on mobile genetic elements or they could be an evolved trait.

(iv) A peculiar genomic localization is found in the non-lantibiotic bacteriocin carnobacteriocin BM1 produced by *Carnobacterium piscicola*. The structural gene is located on the bacterial chromosome while its expression depends on the export and immunity related genes localized on a 61 kb plasmid (Quadri et al. 1994).

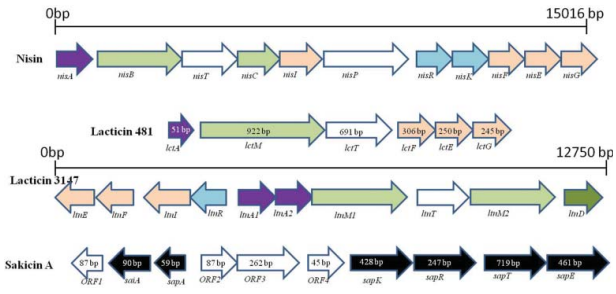

Figure 2. Organization of the operons and biosynthetic gene clusters of Lantibiotics- nisin, lacticin 3147 and class IIa bacteriocin sakicin A (adapted from Vos et al. (1995)). Letter A denotes structural genes, the structural genes are highlighted in blue; genes with similar proposed functions are highlighted in the same colour: light orange for immunity, white for transport/processing, light blue for regulatory proteins, light green for modifying proteins, and purple for unknown function. Gene designations are made according to Vos et al. (1995). Locus symbols used are: nis-nisin, lct- lacticin 481, ltn- lacticin 3147, sap- sakicin. Identical capital letters are used for genes encoding similar functions as deduced from functional analysis or sequence conservation. The presumed functions include modifying enzymes (B, C or M), oxidative decarboxylation (D), Immunity (I), leader peptidase (P), export (T), regulation (R, K), accessory self-protection (FG or EFG).

Production of more than one bacteriocins are also found in some strains like in the case of *Lactococcus lactis* encoding for both *LsbA* and *LsbB* bacteriocins which have an synergetic effect. The enterocin A and B are both expressed by their respective genes present in *Enterococcus faecium*. *Lactobacillus plantarum* are known to produce three different bacteriocins such as two-peptide containing bacteriocins like plantaricin E/F and J/K along with a single peptide bacteriocin plantaricin N (Diep et al. 1996). *Enterococcus faecium* also produces one two-peptide bacteriocin like enterocin L50 and two single peptide bacteriocins like enterocin P and enterocin Q (Cintas et al. 2000). It is also observed that in case of more than one bacteriocin produced by a single bacterium the peptides may belong to different classes like in the case of *Streptococcus mutans* UA140 they produce the lantibiotic mutacin I and the two peptide bacteriocin mutacin IV belonging to class II (Qi et al. 2001).

In class II bacteriocins like lactococcins A, B and M (Holo et al. 1991, Van Belkum et al. 1991, Stoddard et al. 1992, van Belkum, Kok, and Venema 1992, Venema, Venema, and Kok 1995a), plantaricin A (Diep et al. 1994, Diep and Nes 1995, Diep et al. 1996), pediocin PA-1 (Marugg et al. 1992, Motlagh et al. 1992, Bukhtiyarova, Yang, and Ray 1994, Venema et al. 1995) the organization of the genes responsible for the biosynthesis of bacteriocins share similarities among themselves with respect to the systematic sequential arrangement of the gene clusters encoding for the precursor peptide followed by immunity related genes and then the genes responsible for the ABC transporter and an accessory protein, which are responsible for the export of class II bacteriocins. Regulatory genes are also found in some bacteriocin clusters. These accessory proteins are not found in the lantibiotics gene cluster (Nes et al. 1996, Sablon, Contreras, and Vandamme 2000, Sahl and Bierbaum

1998, van Kraaij et al. 1999, Ennahar et al. 2000, McAuliffe et al. 2001) (see Fig. 2)

5. Genetics and regulation of bacteriocin biosynthesis

The initial product in the bacteriocin biosynthesis is a biologically inactive form of bacteriocin consisting of a prepeptide with a leader peptide in the N-terminal region attached to a propeptide containing a C-terminal region. The leader peptide is proposed to have various functions like they acts as a recognition site enzymes mediating prepeptide processing leading to maturation and transport, protection of the bacteriocin producer strain by maintaining the lantibiotics in an sedentary state until it is exported out of the cell, they are also known to modify the pro-peptide domain thus providing them with an appropriate conformation for the interaction of enzyme and substrate (van der Meer et al. 1994, Belkum, Worobo, and Stiles 1997, Sablon, Contreras, and Vandamme 2000, McAuliffe et al. 2001). In the lantibiotics the serine, threonine and the cysteine residues in the pro-peptide region is post-translationally modified in to Lan/MeLan. The various steps involved in the biosynthetic pathway of lantibiotics are the biosynthesis of prepeptide, post transactional modifications, splicing of the leader peptide by proteolytic enzymes and the transport of the processed pro-peptide outside the cytoplasmic membrane. The cleavage and the maturation of the leader peptide would take place before, after or during the transfer from the cytoplasm of the cell. Depending on the pattern of this process the genetic organizations of the lantiobiotics are categorized in to two groups I and II (Sahl and Bierbaum 1998, Guder, Wiedemann, and Sahl 2000, McAuliffe et al. 2001). The group I maturation pathway is found in nisin, subtilin, epidermin and Pep5 where the dehydration reaction is catalyzed by LanB enzyme where LanC mediates the thioester bond formation. Later the serine protease LanP, process the modified prepeptide and they are translocated through the ABC-transporter protein LanT. While in group II lantibiotics maturation pathway the modification of the propeptide is done by a single LanM enzyme like in the case of lacticin 481, mersacidin and cytolysin (van Kraaij et al. 1999, McAuliffe et al. 2001) and the processing takes place simultaneously during transport by LanT (P). In lactosin S the modification and processing are done by a single enzyme system LanM (Skaugen et al. 1997).

In the Class II bacteriocins the prepeptide is composed of a N-terminal leader region which is highly conserved and have a characteristic proteolytic processing site composed of a pair of glycine moiety except in the case of class IIc bacteriocins, which contains a specific sec-type signal sequence in the N-terminal region and are secreated by means of the general secretory pathway (Leer et al. 1995, Worobo et al. 1995). The class II bacteriocins do not go through elaborate post-translational modification instead the synthesized prepeptide is cleaved to remove the leader peptide followed by export mediated by ABC transporter along with the orchestrated action of the accessory protein (Nes et al. 1996, Ennahar et al. 2000).

A two component regulatory system is usually involved in the regulation of the biosynthesis of both lantibiotics and non-lantibiotics. They are composed of histidine protein kinase (HPK) which is membrane bound and a cytoplasmic response regulator (RR) (Stock, Ninfa, and Stock 1989, Parkinson 1993, Nes et al. 1996). The regulation of bacteriocin biosynthesis is made by the autophosphorylation of the histidine residue (which is a highly conserved residue) by HPK in the intracellular domain on sensing a threshold level of bacteriocin in the environment (Fig. 3). This is why bacteriocin is added externally to the fermentation medium to induce and enhance the production of bacteriocin in industrial production. The RR accepts the phosphorylated group through the conserved aspartic acid group present in them, which in turn causes some intramolecular changes triggering the response regulator mediated transcription of the structural gene, export genes and also the regulatory genes (Kuipers et al. 1998). In case of nisin and subilin the bacteriocin themselves acts as an external autoregulator in the signal transduction process (Kuipers et al. 1995, Guder, Wiedemann, and Sahl 2000). While in the case of the regulatory pathway in the class II bacteriocins (Fig. 3) the activation of the transcription is mediated by bacteriocin like peptide named induction factor (IF) which are heat stable, cationic hydrophobic peptide and do not have any antimicrobial activity. The IF is biosynthesized as a prepeptide with a pair of glycine leader sequence. The ABC transporter cleaves the leader peptide containing IF during the transport of mature bacteriocin from the cells and thus the IF is also released outside the cell along with the bacteriocin thereby triggering the transcription of the genes responsible for the production of bacteriocin production by their signaling activity (Nes et al. 1996, Ennahar et al. 2000).

The immunity to the produced bacteriocins is conferred to the producer strains by means of immunity proteins. Like in the case of lantibiotics the immunity is mediated by LanI and LanFEG (belongs to ABC transport proteins). These two

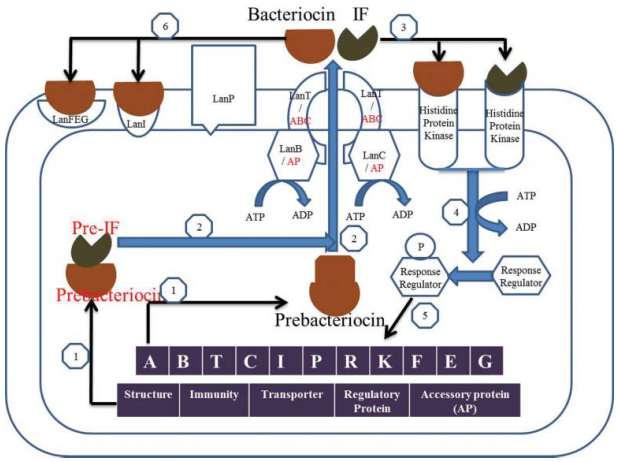

Figure 3. A Schematic representation of the biosynthetic pathway of lantibiotics and class II bacteriocins (marked in red, if not specified both lantibiotics and class II bacteriocins share the same pathway) (Adapted from Chen and Hoover (2003)): (1) Formation of prebacteriocin and prebacteriocin along with prepeptide of induction factor (IF); (2) The prebacteriocin is modified by LanB and LanC, translocated through a dedicated ABC-transporter LanT and processed by LanP, resulting in the release of mature bacteriocin; the prebacteriocin and pre-IF are processed and translocated by the ABC-transporter, resulting in the release of mature bacteriocin and IF; (3) Histidine protein kinase senses the presence of bacteriocin / IF and autophosphorylates; (4) The phosphoryl group (P) is transferred to the response regulator; (5) Response regulator activates transcription of the regulated genes; and (6) Producer immunity mediated by immunity proteins, LanI and dedicated ABC-transported proteins, LanFEG; Producer immunity.

immunity proteins are coded on to multiple open-reading frames (Reis et al. 1994, Siegers and Entian 1995, Peschel and Götz 1996, Saris et al. 1996, McAuliffe et al. 2001). The immunity proteins acts synergistically in protecting the producer bacterium from its own bacteriocins (Klein and Entian 1994). The LanI immunity protein prevents the formation of pores by the bacteriocins in the producer cells by attaching themselves to the outer surface of the cytoplasmic membrane and modulating the interaction of the bacteriocin with the producer cell's surface. The LanFEG exerts its protective effect by transferring the bacteriocin molecules attaching to the producer cell's surface on to the outside medium and keeps the concentration of the bacteriocins attached to the cell surface at check and maintains the critical level of binding.

In the producer cells of class II bacteriocins, the immunity is conferred by proteins found in the cytoplasm. Like in the case of carnobacteriocin B2 and mesentericin Y105 the respective immunity protein CbiB2 (Quadri et al. 1995) and MesI (Dayem et al. 1996) which are found in the cytoplasm protects the producer cells by binding to the cell membrane. These immunity proteins have the size ranging from 51 to 254 amino acid residues and are cationic in nature. These immunity proteins are found to confer total immunity against the bacteriocins secreated by the host (Nissen-Meyer et al. 1993, Venema et al. 1994, Nes and Holo 2000).

6. Mode of action of bacteriocin

The mode of action varies depending on the type of bacteriocins. The primary receptors of bacteriocins are the anionic lipid molecules present in the cytoplasmic membrane, the binding of the bacteriocin molecules causes pore formation in the membrane leading to the efflux of ions and other molecules out of the cells causing irrecoverable damage and death of the cell. In lantibiotics the stability of the pore formation is augmented by lipid II and the peptidoglycan precursor which acts as a docking molecule. In the class II bacteriocins the specificity is determined by receptor molecules present in the cell membrane (Venema, Venema, and Kok 1995a, Venema, Venema, and Kok 1995b) (Fig. 5). The pore formation by the class I bacteriocin would follow wedge like model, while the class II bacteriocin would follow barrelstave like model or carpet model, where the bacteriocins orient themselves parallel to the surface of the membrane and disrupt the cell membrane (Moll, Konings, and Driessen 1999). Nisin, which is the widely accepted bacteriocin in food preservation are proposed to be surface active molecules with cationic detergent like action. This bacteriocin gets adsorbed to the bacterial cell membrane and binds to the lipid II component present in the cell membrane and thus stabilizing the poration complex causing the degradation of sulfhydryl group (Bruno, Kaiser, and Montville 1992) leading to pore formation and cell disruption (Fig. 4-A). Also they are known to sequester the lipid II molecules and impair the repair mechanism of the bacterial cell by inhibiting the biosynthesis of cell wall. In type B lantibiotics like mersacidin they interact with lipid II molecules and causes inhibition of the cell wall biosynthesis (Fig. 5-B). The lacticin 3147 belonging to the class lantibiotic are composed of a two component system lac 1 and lac 2 (McAuliffe et al. 1998) and their synergistic action is required

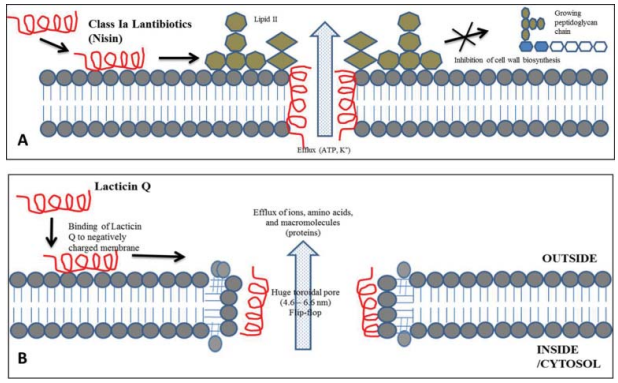

Figure 4. Schematic representation of the proposed mode of action of lantibiotics-nisin and lacticin Q. Type A lantibiotics (i.e., nisin) (Figure-A) initially interact with the cell membrane and binds to lipid II, thus stabilizing the complex and causing pore formation in the target site. They are also involved in sequestering the lipid II that causes inhibition of cell wall biosynthesis. The cationic lacticin Q bacteriocin (Figure-B) swiftly bind to the outer cell membrane and form huge toroid shaped pores modulated by lipid flip-flop (adapted from Rai et al. (2014)).

for their ion-specific pore formation activity in the membrane of the target cells (Fig. 5-C). The interaction of lacticin molecule with the cell membrane takes place by using the peptide A1 followed by its binding to the lipid II component in the cell wall. This causes a change in the conformational of A1 peptide which creates an affinity binding site for the second component A2 of the bacteriocin molecule effecting the formation of pores in the cell membrane (Islam et al. 2012). In subclass IIa group of bacteriocins like pediocin they bind to the IIAB, IIC, IID subunits which belongs to mannose phosphotransferase system

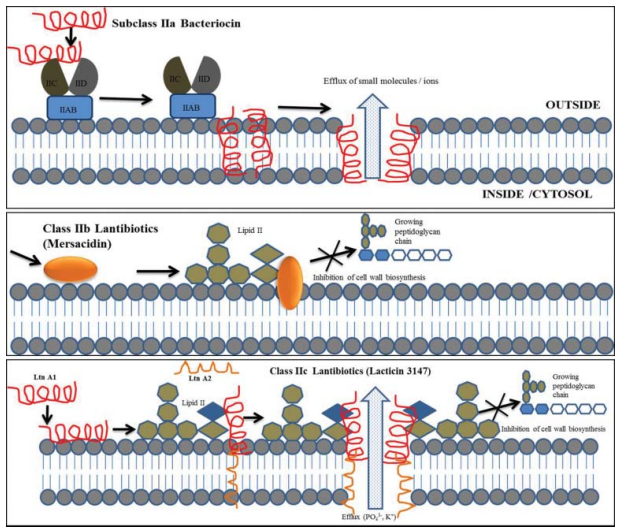

Figure 5. Schematic representation of the proposed mode of action of class IIa bacteriocins, class IIb and class IIc lantibiotics. In class IIa bacteriocins (figure- A), the mannose phosphotransferase systems (M-PTS) is represented by the subunits IIAB, IIC, and IID. The IIC and IID subunits are recognized by bacteriocins and the IIC subunit acts as a receptor for the bacteriocin. Later the bacteriocin impregnates themselves into the membrane and forms pores resulting in the efflux of metabolites and ions. In subslass IIb (figure- B) (i.e. mersacidin) lantibiotics interacts with lipid II and inhibits the cell wall biosynthesis. In subclass IIc (figure- C) which is a two-component lantibioitcs (i.e. lacticin 3147) interacts with the cell membrane through A1 peptide and later binds to lipid II. This triggers a change in the conformation of A1 peptide, which results in a high-affinity binding site for the A2 peptide, facilitating the pore formation. They also known to inhibit the biosynthesis of cell wall. (Adapted from Rai et al. (2014)).

(M-PTS) (Fig. 5-A), the bacteriocins could recognize IIC and IID subunits and the IIc subunits acts as a receptor. Later the bacteriocin impregnates itself in to the cell membrane, forming pores which causes the efflux of ions and molecules (Héchard and Sahl 2002). In case of cationic lacticin Q, (Fig. 4-B) the bacteriocin binds to the cell membrane and causes huge toroid shaped pores facilitated by flip-flop of lipids within the membrane (Yoneyama et al. 2011).

7. Selection criteria and safety of bacteriocins to be used in food preservation

There are various desirable qualities to look for in a bacteriocin to be used in food preservation application, they are:

i. Safety of the consumers and harmless to the consumers intestinal microflora,
ii. Wide antibacterial spectrum of the bacteriocin against the food spoilage organism,
iii. Resistance to enzymes present in food matrixes, and
iv. Thermal stability and activity at a wide range of pH and salt concentration, for inclusion in a wide range of food systems.

In order to establish the safety of the bacteriocins, various assays like cytoxicity assay in eukaryotic cell lines (Weyermann et al. 2005, Murinda, Rashid, and Roberts 2003) ability to induce apoptosis, growth inhibition, metabolic impairment, haemolytic activity, cross-resistance in-vitro and acute, subchronic, chronic toxicity, reproduction impairment and sensitization in animal models, has to be performed and ruled out (Vaucher Rde et al. 2011). However a certain degree of cytotoxicity was found in most of the bacteriocins, but it was found that the concentration at which they are cytotoxic are far higher than the minimum inhibitory concentration required for the food spoiling microbes (Lohans and Vederas 2012). Bacteriocins produced by LAB are generally accepted as safe however exceptions exist like the enterococcal cytolysin, which has a wide spread cytotoxic activity (Cox et al. 2005). Nisin has been used in biopreservation since 1950 (Table 3). Similarly bacteriocinogenic strains which are not genetically modified are being used as starter cultures for fermented food preparations.

For bacteriocins to be used as food preservatives it has to be declared by FDA either as food additives or as a GRAS substance. For bacteriocins to be declared as GRAS substance the particular bacteriocins must have a history of being used as food additive prior to 1958 otherwise clearance has to be sought from FDA describing the proposed use and its safety, brief details about the formulations like its physical and chemical properties and the conditions under which they are to be used. For bacteriocins to be declared as a food additive or GRAS ingredient the following details would have to be disclosed to the FDA (Hoover D, 1993).

i. The bacteriocin must be identified and characterized with respect to its structure, molecular formula and molecular weight. The probable breakdown products or the potential by-products formed during intake or during the manufacturing process has to be disclosed. The limits of impurity present in the final product and the purity of the bacteriocin formulations, their susceptibility to pH, temperature and the digestibility of the particular bacteriocin formulation have to be discussed in detail.
ii. The producer organism or source organism its characteristics, fermentation process, the substrates and material used for the growth and fermentation conditions have to be disclosed along with the separation, purification steps and final formulation.
iii. The specific methodology and analytical protocol for determining the bacteriocin activity, stability and potency is also to be disclosed in a precise, accurate and reproducible manner.
iv. The efficacy of the bacteriocin and the adequate quantity to be added to the food matrix for obtaining the expected efficacy along with the estimated daily intake of the same by the consumption of such preserved food products by the consumer from all such food sources must be provided.
v. The toxicological and the possible allergenic properties associated with the higher levels of bacteriocins in the food products have to be tested.

The usage of bacteriocin producing cultures for in situ production of bacteriocin are preferred in cases of fermented foods reducing the cost involved in the purification of the bacteriocins. The advantageous technological traits like high acid and flavor production by the LAB strains offers a good avenue for being used as sole starter cultures for fermentation which have a dual advantage of preservation and fermentation (Table 4). However if the strains are not suitable for use in fermentation they can be used as adjunct cultures along with the active main fermenting cultures provided they do not interfere with the activity of the fermenting bacterial strain. The bacteriocin producing strains are used for the biopreservation of the non-fermented foods if they do not confer any off flavor or undesirable odor and also if they do not affect the organoleptic quality of the food. According to food safety regulations for inclusion of bacteriocin producing starter cultures for the preservation of food, the producer culture must have GRAS status, and in cases

Table 3. Usage of nisin and their permitted concentration around the globe (Adapted from Cleveland et al. (2001)).

Country	Food matrix	Permitted concentration IU/g
US	Pasteurized processed cheese spreads	10,000
Russia	Dietetic processed cheese, canned vegetables	8000
UK	Cheese, canned foods, clotted cream	No limit
Australia	Cheese, processed cheese, canned tomatoes	No limit
EU	E234, may also labeled as "natural preservative"	varies according to product
Peru	Nisin is a permitted additive	No limit
Argentina	Processed cheese	500

Table 4. Usage of bacteriocinogenic starter cultures for insitu production of bacteriocins.

Bacteriocinogenic starter culture	Bacteriocin produced	Food system	Reference
Pediococcus acidilactici as starter culture	Pediocin P A-1	Dry sausage, Turkey summer sausage, Cheddar cheese, Frankfuters, Vacuum packed wieners, munster cheese	Berry et al. (1990), Foegeding et al. (1992)
Lactobacillus sake co-inoculated with starter culture	Bavaracin MN	Beef cubes, fresh minced meat	Winkoswki et al. (1993)
Leuconostoc gelidum UAL 187 as an adjunct culture co-inoculated with a sulfide producing *Lactobacillus sake*	Leucocin A	Vacuum packed beef	Leisner, Greer, and Stiles (1996)
Lactobacillus sake 2a inoculated to the sausage	Sakacin K	Brazilian sausage in modified atmosphere packing	Shilliner et al. (1991)
Lactobacillus plantarum WHE 92 sprayed on munster cheese surface at the beginning of ripening	Pediocin AcH	Munster cheese ripening	Ennahar et al. (1996)
Expression of pediocin operon in *Saccharomyces cerevisiae*	Pediocin	Preservation of wine and baked products	Schoeman et al. (1999)
Incorporation of pediocin producing *Pediococcus acidilactici* in milk	Pediocin 5	Prevention of Listeria growth in milk	Huang et al. (1994)

where the purified bacteriocins are being used, then as per U.S FDA (Food and Drug Administration 1993) guidelines for the safety assessment of new preservatives, the bacteriocins must be chemically identified and characterized. Its use and efficiency must be characterized along with the toxicological data, pharmacodynamics of the molecule after ingestion, along with the manufacturing process and standardized assay for the same must be reported for the grant of approval.

Listeria monocytogenes has been reported to be the most notorious food contaminant of dairy and meat products and has caused several listeriosis outbreak (Hachler et al. 2013, Makino et al. 2005). This pathogenic microbe is known to grow in a diverse physico-chemical environmental condition ranging from a temperature of 0–50°C and pH as low as 4.5 (Farber and Peterkin 1991). Due to this reason the bacteriocin producing strains are mined for anti-listerial activity targeting a wide market in the preservation of a range of food systems. Bacteriocin producing bioprotective cultures targeted towards *Listeria monocytogenes* are commercially available in various trade names such as Bactoferm F-Lc for use in fermented sausages marketed by Chr. Hansen, Denmark which is a mixture of *P. acidilactici* and *L. curvatus*, producing pediocin and sakacin A respectively. HOLDBAC™ are protective cultures marketed by Danisco (Copenhagen, Denmark) for the prevention of spoilage and pathogenic contamination of sea foods, poultry, meat, and dairy products. They are a mixture of bacterial strains of *Propionibacterium freudenreichii* subsp. shermanii, *Lactobacillus rhamnosus, L. sakei, L. paracasei, and L. plantarum* which produces bacteriocins as well as other antimicrobials thus preventing the growth of *Listeria*, yeasts, and molds.

For bacteriocinogenic cultures even though the organisms along with the food matrix are considered as GRAS status because of their presence in fermented foods and have been consumed by humans for hundreds of years, it still requires sanction from regulatory authorities like FDA for its intended use in other purposes like the preservation of non-fermented food, or when they are used in ingredients added to foods, since the naturally occuring bacteriocin producing strains which is an integral constituent of fermented food are now being used for a new purpose and hence its safety has to be tested and proved before being widely used. (Hoover D, 1993)

The class II bacteriocins which are linear and do not undergo post translational modifications are very sensitive to intestinal proteases thus eliminating the risk of negatively impacting the natural microflora present in the gut. The Class I bacteriocins which undergo rigorous post translational modifications are more resistant to proteases, this is particularly advantageous in the case where when they are used for preservation of food systems like meat which have natural proteases. It is also very unlikely that the bacteriocins which are active against food pathogens and spoiling organisms would have an effect on the beneficial microflora owing to its high specificity. It is difficult to get bacteriocins targetting a wide spectrum of antibacterial activity because some bacteriocins which inhibit gram postivive bacteria have hardly any activity on gram negative bacteria and vice versa. For example nisin, pediocin PA-1 failed to kill *Salmonella* unless it is combined with additional hurdles like high hydrostatic pressures or other kind of treatement to compromise the outer membrane integrity (Kalchayanand, Hanlin, & Ray, 1992, Chalón, Acuña, Morero, Minahk, & Bellomio, 2012)

The enterocin AS-48 is an exception which have a broad spectum of antimicrobial activity (Sanchez-Hidalgo et al. 2011). The spectrum of activity of other bacteriocins can be enhanced by employing physical or chemical hurdles that alter the permeability of cell wall of the bacteria, such improvement in antibacterial spectra was found with nisin (Chalón et al. 2012). Increased diffusion and homogenous distribution in food matrixes are an important criteria for the bacteiocins to be used in food systems (Rollema et al. 1995). As bacteriocins are acting on the biological membranes they are expected to have higher affinity to the lipid rich phases of food.

8. Factors affecting the usage of bacteriocins as biopreservatives in food systems

The food system is a complex environment with various factors influencing the efficiency of bacteriocins. The in situ production of bacteriocins in the food system is affected by reduced growth and/or production of bacteriocins by the bacteriocinogenic strains due to unfavourable pH, a_w, temperature fluctuations during processing and storage like freezing, thawing, homogenization which impair the viability of the bacteriocin producing bacterial cells and inhibition by other food components like spices, additives, salt concentration, food structure, buffering capacity etc. Infection by bacteriophages and antagonism of other bacterial fermenters co-inoculated would negatively impact bacteriocin production and biopreservation (Martí, Horn, and Dodd 2003) by exhibiting competition for nutrition in the food system, and by the production of

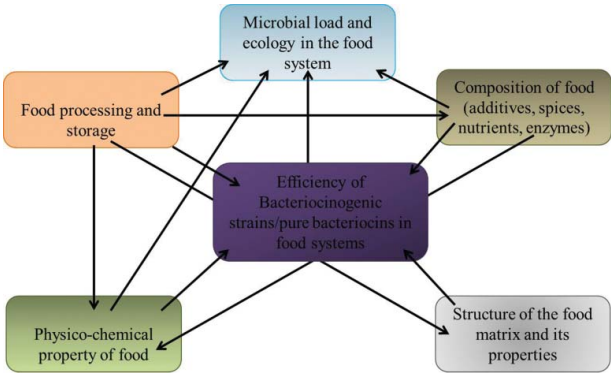

Figure 6. Factors influencing the efficiency of bacteriocins produced in-situ by bacteriocinogenic strains or added to the food systems.

antibacterial agents by the co-cultures. Apart from them the emergence of pathogenic strains resistant to the produced bacteriocins, and genetic instability of the producer strains would also reduces the efficacy of the biopreservation by bacteriocinogenic cultures. Thus these factors are interrelated to each other and effect the biopreservation techique. And hence selection of the strains which are more suitable for the food system is very essential for efficient biopreservation (Galvez et al. 2007).

The efficacy of pure bacteriocins in the food systems are affected by the permeability of the bacteriocins in the food matrix, and the pH of the food matrix which influences the soluability of the bacteriocin, distribution in the food matrix, inactivation by the enzymes present in the food systems like meat products, interaction with food additives and food components like complex formation with glutathione (Rose et al. 1999) such as in meat products greatly reduces its efficacy (See Fig. 6)

There are variations which may occur in the sensitivity of the targetted sensitive food spoiler microbe due to mutations in the producer strains or due to the resistance developed by the targetted food borne pathogens which may intensify the problem. There are reports that strains of *L. monocytogenes* and *Staphylococcus aureus* becoming resistant to nisin (Martinez, Bravo, and Rodriguez 2005), plantaricin C19 (Rekhif, Atrih, and Lefebvre 1994), sakacin A (Dykes and Hastings 1998). The other factors which make them less effective against the otherwise sensitive microbes are spore formation and biofilm formation by the pathogenic microbes which are impermeable to the bacteriocins (Kumar and Anand 1998). These shortcommings can be overcome by using a complementary hurdle technology, physico-chemical treatments along with the bacteriocin biopreservation and this have been proven to be effective as shown in Table 5.

9. Strategic usage and application of bacteriocins in food preservation

Various strategies has been employed for the incorporation of bacteriocins in to food matrixes and also for enhancing the antibacterial effects of the bacteriocins in food systems like usage of bacteriocins directly to the food system (Pawar et al. 2000), spray treatments (Cutter and Siragusa 1994), incorporation of bacteriocins in to packaging films (Appendini and Hotchkiss 2002), vacuum impregnation of bacteriocins (Andrés-Bello et al. 2015) and usage of various physico-chemical treatments along with bacteriocin (see Fig. 7) like modified atmosphere packing (Fang and Lin 1994), vacuum packing (Winkowski, Crandall, and Montville 1993, Vignolo et al. 1996), usage of various hurdle technologies along with bacteriocins like in combination with heat treatment (Budu-Amoako et al. 1999), chelating agents (Szabo and Cahill 1998), carbon dioxide treatment (Nilsson et al. 2000), high hydrostatic pressure (Masschalck, Van Houdt, and Michiels 2001, Morgan et al. 2000, Kalchayanand et al. 1998), pulsed electric field (PEF) (Pol et al. 2000, Calderón-Miranda, Barbosa-Cánovas, and Swanson 1999a, Calderón-Miranda, Barbosa-Cánovas, and Swanson 1999b) antimicrobial like potassium sorbate (0.3%) (Buncic et al. 1995), sodium diacetate (0.5%) (Schlyter et al. 1993), carvacol (0.3 mmol) (Periago and Moezelaar 2001), monolaurin (0.25 mg/l) (Mansour and Millière 2001), lactoperoxidase system (Boussouel et al. 2000). The hurdle technology of using various physicochemical treatments along with the bacteriocin is gaining greater attention since the additional hurdles employed in the preservation strategy have helped to overcome the various inadequacies of bacteriocin alone treatment, like enhanced activity spectra against gram negative pathogens in combination with hurdle technology. The hurdles in the form of chelating agents like EDTA can bind the magnesium ions from the lipopolysaccharide layer causing disruption of the outer membrane of the gram negative bacteria and also permitting easy access of the nisin (Abee 1995). Also the inactivation efficiency of heat treatment is found to be enhanced when treated along with nisin thereby reducing the holding time of thermal treatment resulting in enhanced quality of food. The usage of bacteriocin nisin along with the lactoperoxidase system has shown to have a synergistic effect on inhibiting the growth of *L. monocytogenes* in skim milk (Zapico et al. 1998). The usage of various cocktails of bacteriocins has been demonstrated to enhance the antibacterial activity and improved preservative effects in food systems (Hanlin et al. 1993, Mulet-Powell et al. 1998). The usage of high hydrostatic pressure (HP) (Black, Setlow, et al. 2007, Black, Wei, et al. 2007, Hicks et al. 2009, Roberts and Hoover 1996) and pulsed electric field (PEF) treatment has been widely accepted non-thermal based food preservation technology. It is found that the usage of bacteriocins in synergy with these treatment techniques enhanced the bacterial inactivation efficiency. This is because the bacterial pathogen undergoes sub-lethal damage to the pathogenic cell, which makes them more permeable to the bacteriocins and to entrust their effect (Kalchayanand et al. 1994). (see Table 5).

Introduction of bacteriocins on to packaging films for the control of food borne diseases and spoilage of food has kindled great interest among the food industry. The antimicrobial films exerts their beneficial effect of controlling the food spoilage microbe by two methods, one is by direct contact of the packaging material with the food and the other is by the gradual release of the bacteriocins from the packaging film in to the food surface and diffusion in to the food matrix which are more advantageous than spraying and dipping in bacteriocin preparation because the bacteriocins may be inactivated because of the components present in the food matrix or may get eventually diluted within the food matrix (Appendini and

Table 5. Strategic usage of bacteriocins for improved efficacy in various food systems.

Bacteriocin/ Bacteriocinogenic strains employed	Food system	Strategy employed	Study outcomes	Target organism	References
Nisin	Brined white cheese	Heat treatment (63°C for 5 min)	Synergistic effect of heat treatment and nisin completely eliminated the pathogen	*L. innocua*	(Al-Holy et al. 2012)
	Whole and low -fat milk	Extract of ginseng by-product (1.0–2.0%)	Enhanced the inactivation of pathogen by synergistic action	*L. monocytogenes*	(Kim et al. 2012)
	Rainbow trout fillets	Essential oil (from Zataria multiflora) 0.2% to 0.4%	Increased the shelf life by inhibiting the growth of pathogen	LAB & Psychrophilic bacteria	(Zakipour Rahimabadi, Rigi, and Rahnama 2013)
	Sausages	Modified atmosphere storage	Nisin inhibited bacterial growth while the modified atmosphere along with nisin inhibited yeast and mould	LAB, mesophilic and psychrophilic bacteria, yeast and mold	(Khajehali et al. 2012)
	Soymilk	Garlic shoot juice 3–6%	Synergistic effect inhibited pathogen and increased shelf life	*L. monocytogenes*	(Hsieh et al. 2011)
	Sturgeon caviar and lobster	Moderate heat (60°C for 5 min) treatments/chemical preservatives	Nisin, brine and heat combination showed 1 to 3 log reduction of pathogen and reduced the drained weight loss of lobster	*L. monocytogenes L. innocua/L. monocytogenes*	(Budu-Amoako et al. 1999)
Nisin + pediocin	Raw meat	Gamma radiation	Synergistic effect completely eliminated the pathogen	*L. monocytogenes*	(Mohamed, Elnawawi, and Yousef 2011)
	Fresh cut mung bean sprouts, cabbage, broccoli	Phytic acid (0.02%), citric acid (10 Mm), sodium lactate (2%), potassium sorbate (0.02%)	Washing with preservative solution reduced the pathogen load while the bacteriocin treatment inhibited pathogen growth	Cocktail of five strains of *L. monocytogenes*	(Bari et al. 2005)
Pediocin (ALTA 2341^{TM})	Frankfurters	Thermal pasteurization and vacuum packaging	Heat treatment at 81° C for less than 60s after vacuum packing along with bacteriocin prevented the growth of pathogen for up to 12 weeks at 4–10° C	Five strain mixture of *L. monocytogenes*	(Chen et al. 2004a)
	Frankfurters	Vacuum packaging and post-packaging irradiation 2.3 kGy	Synergistic effect of irradiation and pediocin enhanced the shelf life by inhibiting pathogen	*L. monocytogenes*	(Chen et al. 2004b)
	Bologna sausages	Sodium diacetate (2.5%), sodium lactate (4.8%), and heat treatment	The synergistic effect of bacteriocin and preservative agent reduced the decimal reduction time of the pathogen	*L. monocytogenes*	(Grosulescu, Juneja, and Ravishankar 2011)
Nisin+ enterocin AS-48	Rice pudding	High Hydrostatic Pressure (HHP), cinnamon (0.2% v/wt) and clove oil (0.25% v/wt)	Time and the intensity of the HHP treatment is reduced by the synergistic action of bacteriocin and natural oil, for the efficient inactivation of pathogen	*S. aureus*	(Pérez Pulido et al. 2012)
Nisin	Goat milk cheese	High hydrostatic pressure (500 MPa)	Synergistic effect of nisin and HHP significantly reduced indigenous microbiota and *Bacillus subtilis* spore germination	Inactivation of indigenous microbiota	(Capellas et al. 2000)
Lacticin 3147	Milk and whey	Increased pressure (150 to 275 Mpa)	Concentrated lacticin from culture supernatant and HHP in combination caused more than 6 log reduction of pathogens and HHP greater than 400 MPa doubled the bacteriocin activity	*S. aureus* and *Listeria innocua*	(Morgan et al. 2000)
Pediocin AcH	Peptone solution	High hydrostatic pressure (345 Mpa)	The addition of bacteriocin enhanced the inactivation of pathogens by HHP treatment to 8 log units.	*Listeria monocytogenes, S. aureus, S. typhimurium, Lactobacillus sake, E. coli O157:H7, Serratia liquefaciens, Leuconostoc mesenteroides, and P. fluorescens*	(Kalchayanand et al. 1998)
Nisin	Experimental matrix (simulated milk ultrafiltrate media)	Pulsed electric field	Nisin is inactivated by pulsed electric field but in the presence of bacterial cells they showed an enhanced pathogen inactivation	*E. coli*	(Terebiznik et al. 2000)

(*Continued on next page*)

Table 5. (*Continued*)

Bacteriocin/ Bacteriocinogenic strains employed	Food system	Strategy employed	Study outcomes	Target organism	References
Nisin	Liquid whole egg and skim milk	Pulsed electric field	Exposure of the pathogen to nisin followed by pulsed electric field treatment exhibited an enhanced pathogen inactivation	*L. innocua*	(Calderón-Miranda, Barbosa-Cánovas, and Swanson 1999a, Calderón-Miranda, Barbosa-Cánovas, and Swanson 1999b)
Enterocin AS-48	Commercial sauces	Mild heat (50 −60° C) and 2-nitro-1-propanol (25 Mm)	The combination of bacteriocin and 2-nitro-1-propanol reduced the effective concentration of the antimicrobial required, while heat treatment inhibited the proliferation of the pathogen.	*S. aureus*	(Burgos et al. 2012)
	Ready-to-eat salad	Essential oils, fatty acids, Nisaplin (nisin)	The anti-listerial activity of the bacteriocin is enhanced by essential oils extracted from herbs, lactic acid and *p*-hydroxybenzoic methylester acid	*L. monocytogenes*	(Molinos et al. 2009)
Sakacin C2	Low fat and whole milk	Lecithin, tween-80, ε-polylysine	Tween 80 and milk fat decreased the activity of bacteriocin but lecithin and ε-polylysine enhanced the activity of sakacin C2	*E. coli ATCC 25922*	(Gao, Li, and Liu 2013)
Gassericins A and T	Custard cream	Glycine	Glycine (0.5% wt/wt) prevented the growth of gram positive pathogens and also enhanced the activity of the bacteriocins against gram negative pathogens by reducing the concentration of bacteriocin required for inhibition.	*Achromobacter denitrificans* AK1113, *B. cereus* AK1124, *L. lactis* subsp. *lactis AK1155*, *Pseudomonas fluorescens* AK1195	(Arakawa et al. 2009)
Pediocin (ALTA^{TM} 2351)	Sliced ham	Incorporation of pediocin into cellulose based film as packing material.	Pediocin incorporation on to the films up to 25% reduced the *Salmonella* count and 50% incorporation of the bacteriocin inhibited *L. innocua*	*L. innocua*	(Santiago-Silva et al. 2009)
Nisin	Vacuum packed cold smoked salmon	Nisin coated plastic films	Enhanced the efficiency of nisin by maintaining its antibacterial activity for an extended period of storage.	*L. monocytogenes*	(Neetoo et al. 2008)
Lactocin 705	Fresh soft cheeses	Linear low density polyethylene (LLDPE) non-edible packing film is coated with bacteriocin.	The adsorption of the bacteriocin on to a multilayer film reduced the minimum inhibitory concentration required for the antibacterial activity and also maintained its antimicrobial activity after conformational reorganization of the adsorbed bacteriocin.	*L. monocytogenes*	(Massani et al. 2013)
Live *Enterococcus casseliflavus 416K1* strain producing Enterocin 416K1	Seasoned cheese	Coating of Polyethylene terephthalate (PET) films with live cultures of Enterococcus casseliflavus 416K1	Coating enhanced the survival of the bacteriocinogenic strains for a long time in the food matrix, which can respond to the accidental rise in temperature of the preserved food by outgrowing the pathogen, and producing antimicrobial bacteriocins.	*L. monocytogenes*	(Iseppi et al. 2011)
Live LAB strains and nisin	Smoked salmon	Impregnated into alginate films along with nisin enhanced shelf life.	Enhanced diffusion of bacteriocin like substance produced by the LAB strains impregnated on to the alginate films, while the combinatorial effect of the LAB and added nisin enhanced the bacteriostatic effect for over a period of 28 days under refrigerated conditions.	*L. monocytogenes*	(Concha-Meyer et al. 2011)
Live L. plantarum strain	Experimental matrix	Incorporated into bioactive cellulosebased films	Enhanced the bacteriocin production by the entrapped LAB strain and a significant antimicrobial activity was observed.	*L. monocytogenes*	(Sánchez-González, Saavedra, and Chiralt 2013)
Live lactic acid bacteria (LAB) and a nisin solution	Gilthead sea bream fillets	Vacuum impregnation was carried out at 4°C	Vacuum impregnation extended the shelf life by reducing the initial load of pathogen and delaying its growth. It also enhanced the distribution of the bacteriocin on to the food matrix.	*L. monocytogenes*	(Andrés-Bello et al. 2015)

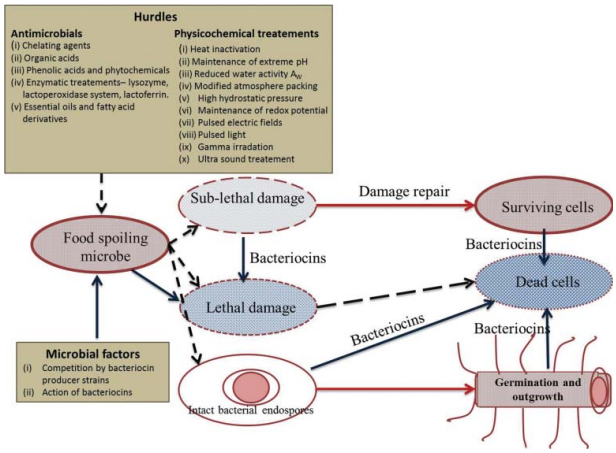

Figure 7. Schematic representation of the application of bacteriocins as part of hurdle technology for increased bacterial inactivation (Adapted from Galvez et al. (2007).

Hotchkiss 2002). There are three methods of preparing bacteriocin incorporated packaging films: (i) incorporation of bacteriocins directly in to the polymeric matrix of the packaging film, for example the incorporation of nisin in to films made up of soy protein and corn zein or biodegradable protein films (Padgett, Han, and Dawson 1998), this method had the disadvantage that some components which are used in the making of such packaging film may interfere with the efficiency of the incorporated bacteriocin (Coma et al. 2001). (ii) Coating or the adsorption of the bacteriocins on to the packaging film like polyethylene, ethylene vinyl acetate, polyamide, polyester, polypropylene, polyvinyl chloride and polyacrylic films (Appendini and Hotchkiss 2002, MING et al. 1997) (iii) Incorporation or immobilization of the bacteriocins in polymeric inserts like cellulose based, polyethylene and polyamide matrixes. These inserts are kept in contact with the food matrix to be preserved. It was also found that these insert materials maintained its bioactivity and stability up to 3 months under refrigerated and non-refrigerated conditions (Scannell et al. 2000). Thus bacteriocins incorporated packaging materials holds the promise of an economically viable options in the light of large scale food spoilage and loss to the food industries and also as an alternative natural food preservation strategy.

10. Production and purification of bacteriocins

In case of food preservation by in-situ production of bacteriocins, the task can be achieved by carefully selecting the producer strains which is suitable for the particular type of food matrix intended to be preserved. The bacterial strains with highest activity in the particular food matrix that do not confer any negative characteristics like off flavor and other organoleptic properties, interaction with food components and other preservatives used in the food matrix should be ruled out before selecting an appropriate producer strains. They must be used after prior testing in intended storage conditions in the actual food.

Bacteriocins from LAB can be used as a crude preparation from the bacteriocin containing LAB fermentate which may contain other antibacterial compounds other than bacteriocins. In case of purified bacteriocins the preparation contains only pure bacteriocins, which is the only antibacterial substance present in the purified preparations, which are very active at a very low concentration (Chen and Hoover 2003). Usage of pure bacteriocins in food preservation is limited by the development of producer strains which are industrially viable and also the cost involved in the purification of bacteriocins. Production of bacteriocins in industrial scale is only viable by process optimization of bacteriocin production by high yielding producer strains or genetically engineered strains. It was found that the production of bacteriocins is highest at the end of exponential and early stationary phase (Daba et al. 1993, Naidu 2000, Hurst and Kruse 1972, Callewaert and De Vuyst 2000) which means that critical process parameters has to be optimized for each producer strains in order to produce industrially economical amount of bacteriocins. The industrial scale production of bacteriocins involves various steps which include:

1. Screening of high yielding strains from large number of candidate bacteriocin producers,
2. Optimization of medium components and physicochemical parameter for the increased production of bacteriocins, and
3. Purification of the bacteriocins to homogeneity, identification, characterization and safety assessment of bacteriocins.

There have been reports of Induction Factors (IF) which are bacteriocin like low molecular weight, cationic peptides of 19–26 amino acids residues in length inducing the production of bacteriocins (Cintas et al. 2001, Riley and Wertz 2002a, Balciunas et al. 2013, Nes et al. 2007). Two models has been proposed by Nes et al on the regulation of the production of bacteriocins by these induction factors, one is the quorum sensing model which states that IF is produced and accumulated by the producer strains at lower concentration during the growth phase. Later the induction of the bacteriocin genes occurs when the IF concentration reaches the threshold level for auto-induction. Thus this model predicts the induction of the bacteriocin based on the cell density of the cultures (Cintas et al. 2001, Nes et al. 1996). The second model suggest that the level of IF reaches its threshold level for facilitating the induction of bacteriocins by some external intervention like modifications in the environmental conditions, changes in the physico-chemical parameters of growth conditions or change in the nutrient levels (Cintas et al. 2001, Nes et al. 1996). Besides these the recent studies points towards the regulation of bacteriocin production through a three component system which includes an inducing peptide, the transmembrane histidine kinase (receptor) and a regulator of response. Apart from them the auto-induction of bacteriocin production, such as in the case of lantibiotics like nisin and plantaricin has been reported for the improved production of bacteriocins (Nes et al. 2007, Balciunas et al. 2013).

Most of the bacteriocins are produced by Lactic Acid Bacteria (LAB) which are fastidious organisms which require often rich nutrient conditions for their growth, each strain has a specific requirement of vitamins, amino acids, minerals and carbon source for their growth and bacteriocin production (Bouguettoucha, Balannec, and Amrane 2011). These demands for complex media composition including peptides that are in

the molecular weight range of the bacteriocins makes the purification of bacteriocins a complex task (Carolissen-Mackay, Arendse, and Hastings 1997). The production of bacteriocins using the agro-industrial waste has been reported to reduce the production cost (Bali, Panesar, and Bera 2016).

Since the bacteriocins are disparate in nature a single method of purification protocol for all categories of bacteriocins is not possible. Since the bacteriocins are secreted in small quantities in the large volume of culture broth, the initial step in purification is concentration of the supernatant containing the bacteriocins, followed by further purification protocols utilizing the charge and hydrophobic properties of the intended bacteriocin class. Ammonium sulphate mediated precipitation for the initial concentration of the bacteriocins is the most preferred method for all the class of bacteriocins, other methods like the usage of amberlite resins, ultrafiltration and lyophilisation are also used. The next following step uses the specific property of the bacteriocins like their charge and/or hydrophobicity to purify them to homogeneity (Parada et al. 2007). Various schemes like the usage of ion exchange chromatography, hydrophobic interaction chromatography, and reverse phase chromatograph are commonly employed to achieve this task. The final polishing step employs gel filtration chromatography followed by RP-HPLC (Reverse Phase High Performance Liquid Chromatography) to purify the bacteriocins to homogeneity. The purified protein is then assayed by running in native gel electrophoresis and determining the activity of the band formed by the purified protein against the indicator pathogens (Bhunia, Johnson, and Ray 1987). Further the purified bacteriocins are characterized by protein sequencing and mass spectroscopic techniques.

Alternative purification schemes have been employed in the recent decades to increase the final yield of the product without much loss in the purification steps. One such method is the utilization of the cationic nature of the bacteriocins which have a tendency to get adsorbed to the cell surface at a particular pH 6.0 and desorption at acidic pH 2.0 as suggested by Yang, Johnson, and Ray (1992). By this method they could recover about 90% of bacteriocins like leuconocin Lcm1, pediocin, nisin, and pediocin AcH after a single final purification step. Other strategies like the usage of chilled ethanol at 4°C with the cell free supernatant minimized the purification steps which could selectively precipitate and concentrate lactococcin B (Venema et al. 1997), sakacin C2 (Gao et al. 2010) and pediocin SA131 (Lee et al. 2010) and further within two steps of polishing the bacteriocins was be purified to homogeneity. Another strategy of using chemically defined simple medium which is devoid of contaminating proteins and smaller peptides has been successfully utilized by Vera Pingitore et al. (2009). By this method a single step ultrafiltration of the culture supernatant yielded a high concentration of purified salivaricin 1328 from *Lactobacillus salivarius 1328*. Further the development of new strategies could help in the reduction of the overall production cost.

The characterization of the purified bacteriocins is very essential for determining its safety, elucidating the mechanism of action, its novelty and other wealth of information regarding the suitability of the bacteriocins to be used in food matrices like active pH range, hydrophobicity, interaction effects and inactivation by enzymes present in the food matrix etc. by using in-silico methods and comparing them with the database of previously characterized bacteriocins like BACTIBASE, BAGEL etc. (Galvez et al. 2007). The steps in characterization includes the elucidation of the amino acid sequence of the protein by sequencing and designing of specific primers for the bacteriocin gene after codon optimization for the particular genus and amplification and sequencing of the complete structural gene of the bacteriocin. From this the complete amino acid sequence of the bacteriocin molecule could be deciphered. Identification and characterization of many novel bacteriocins like plantaricin ASM1 (Hata, Tanaka, and Ohmomo 2010), lactocyclicin Q (Sawa et al. 2009), lactococcin Q (Zendo et al. 2006), and other novel bacteriocins have been characterized by this method (Sawa et al. 2013).

11. Genetically engineered bacteriocins, heterologous production and biosafety

The increase in demand for the production of bacteriocins which are least affected by the thermal processing of the food, enzymes present in the food matrix and also the enhanced solubility and distribution in the food system is very essential for the success of bacteriocins as a food preservatives. These have lead to the genetic modification of the naturally available bacteriocins to confer beneficial physico-chemical properties enhancing its activity in the food matrix.

The genetic modification of nisin A to nisin Z which has amino acid substitution in His31/Asn27 position has enhanced its diffusion in the food matrix by seven fold compared to its natural counterpart (Cotter, Hill, and Ross 2005a, Mulders et al. 1991). Linear peptides like class II bacteriocins are the easy targets for genetic manipulation because they undergo lesser post translational modification and can be heterologously expressed in other nonfastidious host which can act as a good starter cultures for the fermentation of the food or protective cultures which does not affect the flavour and consistency of the food matrix unlike the natural producers. Among the class I bacteriocins, nisin is the most widely studied bacteriocin for genetic manipulations, which includes mutations in the thiol bridge, changes made to the unusual amino acid composition of the peptide, mutations modifying the hinge region of the peptide and changes to the overall charge of the peptide (Cotter, Hill, and Ross 2005a). Mutations involving the unusual amino acids like dehydrobutyrine (Dhb) in the variants Dhb14S and A12L conferred significant resistance to trypsin but compromised its antimicrobial activity. Increased hydrophobicity of the subtilisin variant by modifying its N-terminal region is achieved by genetic modification which enhanced its activity by more than three fold than the natural subtilisin (Liu and Hansen 1992).

Mutations in the hinge region of nisin Z (N20K and M21K) enhanced the solubility and antibacterial activity against gram negative food borne pathogens like *Shigella, Pseudomonas* and *Salmonella* species (Chen et al. 1998) and other mutation in the same region has enhanced the solubility of the bacteriocin even at pH 8 and enhanced its thermal stability at neutral pH (Yuan et al. 2004). Mutations in the hinge region of nisin have generated a vast repertoire of nisin variants like nisin V and T which

exihibited a broad range of antagnostic activity against gram positive pathogens like *Listeria monocytogenes, Staphylococcus aureus* which were antibiotic resistant clinical pathogens and those isolated from contaminated food (Field et al. 2008). Apart from these, mutations made in the hinge region of S29 have generateded a novel nisin N20P, M21V and K22S with antagnostic activity against both gram positive and gram negative pathogens (Field et al. 2012) and enhanced diffusion capacity (Rouse et al. 2012). Modification in Microcin J25 was done to make it susceptible to enzymatic digestion by intestinal chymotrypsin ensuring the safety of the baceriocin for human consumption (Pomares et al. 2009).

Bacteriocins with disulphide bridge at C-terminus domain of the peptide enhanced its antimicrobial activity even at elevated temperatures (Fimland et al. 2000). Pediocins which belong to the class II bacteriocins are chemically very unstable at room temperature or even at low temperature storage conditions, but this drawback is complemented by substituting the methionine residue in the bacteriocin to hydrophobic residue, which rendered the peptide higher stability and also maintained its antibacterial activity for a very long period of storage (Crameri et al. 1998). The usage of chimeric bacteriocins containing the counter parts from each type of bacteriocins enhanced its spectrum of activity as in the case of ent35-colV which is a chimeric bacteriocin produced by the fusion of enterocin CRL35 from *Enterococcus* and microcin V produced by *E.coli* (Acuna et al. 2012).

The genetic manupulation of bacteriocins or the producer strains has to pass through strict safety regulations and norms laid down by national regulatory agencies like FDA for approval to be used in human consumption. However self cloning approach within the LAB (accredited with GRAS status) like modification of the native plasmid by gene knock out, site directed mutagenesis, splicing by overlap extension (Cotter, Ross, and Hill 2013) and also transfer of DNA between the same species or very closely related species, which may otherwise occur by natural physiological process, etc. would not come under the scanner of Contained Use legislation of mutants as per the Council Directive 98/81/EC (1988). The FDA regulations require the following considerations for the usage of genetically engineered bacteriocins or engineered bacteriocin producing strains:

i. The genetic material to be introduced in to the organisms must be characterized and must be ensured of its safety. The genetic material or DNA from the organisms previously used in food systems are generally preferred. The overall genetic material used in such artificial constructs must be characterized and ensured that no additional genetic material. Also the donor organism and the intermediate hosts must be well characterized.
ii. The identity and origin of the host organism must have to be well characterized and proven to be safe. The presence of virulence factors, toxins etc., is aldo required to be tested.
iii. The vectors used in such genetic constructs must have been derived from organisms recognized as safe to be used in food. The selectable marker present in such vectors must not be encoding resistance to antibiotics used in therapeautic interventions. Also the stability of the vector/plasmid must be ensured along with the minimal chances of the transfer of such vector to other organisms.
iv. The pathogenicity, allergenicity and the toxigenicity of such engineered organisms must be ruled out. It has to be verified by in-vitro and animal studies. Absence of unintentional pleiotrophic effects has to be confirmed with respect to chemical, structural and organoleptic property of the food matrix compared to non-engineered counterparts.
v. The nutritional composition of the food containing the engineered microbial starter cultures has to be evaluated and the exposure levels of such bacteriocins produced by the starter cultures in the consumer population has to be evaluated with respect to the each product groups containing such engineered organisms.

Overall the evaluation regulations for the genetically engineered organisms are likely to vary case by case, where each organisms would be analyzed independently depending on the intended use or the product.

Bacteriocinogenic LAB starter cultures are main targets for genetic manupulation for enhancing the production of bacteriocins by modifying or complementing the LAB strains with genes responcible for the regulation and synthesis of desirable bacteriocins (Chen and Hoover 2003). Heterologous expression of the bacteriocins in the host which are not natural producers of bacteriocins are gaining attention in the recent days because of the following reasons:

1. LAB which are the natural producers of bacteriocins are very fastidious organism which require special nutrient conditions for their growth and bacteriocin production and hence the expression of bacteriocin in quick growing host which requires only minimal media requirements will reduce the production and purification cost to a great extent.
2. Some of the producer strains of bacteriocins may have some negative effects on the food system when used as starter culture for fermentation, owing its off flavour formation and changes in the organoleptic properties of the food and hence heterologous expression of the bacteriocin genes in them is an viable option.
3. Not all producer strains could establish themselves in the various food matrices and produce enough amount of bacteriocins to confer its protective effect on the food to be preserved.
4. Pathogenic nature of the producer strains and antimicrobial resistance of the strain makes them unfit to be used in food system.

The heterologous expression and production of the bacteriocin in an unrelated host system depends on various factors like the plasmid stability, ability to maintain appropriate copy number of the transferred genes in their plasmid, transcription and translational regulation of the host system, post translational modification system of the host and resistance of the host towards the produced bacteriocins. In such cases the transfer of bacteriocin resistance genes of the original producer strains along with the bacteriocin gene is essential for the successful

heterologous expression of bacteriocins (Rodríguez and Laviña 2003).

A vast repertoire of host systems are being used for the successful expression of bacteriocins like the *E. coli* and yeast system. Enterocin P (Gutierrez et al. 2005), enterolysin A (Nigutova et al. 2008), pediocin PA-1 (Liu, Han, and Zhou 2011) and nisin (Karakas-Sen and Narbad 2012) are successfully expressed in *E. coli* expression system. Alternatively many starter culture of LAB has been successfully employed for the heterologous expression of bacteriocins from otherwise pathogenic or problamatic producer strains. Enterocin A are produced by *Enterococcus* species which have strong antilisterial activity, but the *Enterococcus* species are known to cause infections in humans and they are also known to harbour antibiotic resistance genes by horizontal gene transfer, thus making them less suitable for being used as starter cultures in food systems. Thus a plasmid construct pENT02 encoding the genes for enterocin A, secretion, transport and host immunity were transferred to *Lactococcus lactis MG1614* strain, which is used as starter culture in cheese production. This heterologous expression system after transformation was termed *L. lactis*$_{ENT+}$ and was found to effectively combat the growth of *Listeria monocytogenes* without compromising the quality of the cheese (Liu et al. 2008). Further another construct of sec-dependent enterocin P genes in the plasmid pLEB590 which is transferred to *L. lactis MG1614* produced about 3.9 fold higher amount of enterocin P than its natural counterpart (Liu et al. 2011).

Heterologous expression of two different unrelated bacteriocins in a single host was also attempted to strategically evade the bacteriocin resistance phenomenon and also to expand the spectrum of antibacterial activity by the transformed strain, a nisin producer strain of *Lactococcus lactis* ESI515 transformed using the plasmid construct pF12160 consisting of genes encoding for pediocin PA-1 was able to produce both pediocin PA-1 and nisin simultaneously in a single system and was sucessfully used as insitu-bacteriocin producing starter cultures in cheese making (Reviriego et al. 2005).

12. Conclusion and future directions

Although some bacteriocins like nisin and lacticin are commercially being used in specific food systems, the limitations of the bacteriocins and other influencing factors of the variable food matrixes has restricted its wide spread usage. These limitations of the bacteriocins are overcome by engineering the bacteriocins and making it more robust with respect to thermal and pH stability, improved diffusivity, and other desired characteristics. Also, the production and purification cost impedes the widespread popularity of the bacteriocins. These can be overcome by using bacteriocin producing strains for insitu production of bacteriocins, and also by heterologous expression of the bacteriocins in bacterial host which have a minimal nutritional requirement. This would directly reduce the production cost to a great extent. With the advent of novel technologies for incorporating bacteriocins and bacteriocin producing strains in to the food matrix and packing material, which is promising and achievable at the industrial scale, the demand for bacteriocin in food preservation is expected to escalate in the forth coming years. Thus the emergence of new horizons for the application of bacteriocins as a natural food preservative is evident as an alternative to the conventional harsh physical and chemical preservatives.

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Conflict of interest statement

The authors declare that they have no conflict of interest disclosed in this work.

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