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AMA : Antimicrobial activity

ATCC : American Type Culture Collection

AU : Arbitrary unit

CCD : Central composite design

CECT : Colección Española de Cultivos Tipo

cEPS : Capsular exopolysaccharide

DHBA : 2,3-dihydroxybenzoic acid

EE : Encapsulation efficiency

Ent35 : Enterocin CRL35

EPS : Exopolysaccharide

FAO : Food and Agriculture Organization

FFD : Fractional factorial design

fEPS : Free exopolysaccharide

GNB : Gram-negative bacteria

GPB : Gram-positive bacteria

H₂O₂ : Hydrogen peroxide

hVISA : Heterogenous vancomycin-intermediate *Staphylococcus aureus*

IPTG : Isopropyl β-D-thiogalactopyranoside

LAB : Lactic acid bacteria

LDH : Layered double hydroxides

MccV : Microcin V

MRSA : Methicillin-resistant *Staphylococcus aureus*

NDF : Nanofibers of 2,3-dihydroxybenzoic acid combined with nisin

NICE : Nisin controlled gene expression system

nis : Nisin

nis : Nisin gene

OD : Optical density

OFAT : One factor at a time

PA-1 : Pediocin PA-1

PC : Distearoylphosphatidylcholine

PCR : Polymerase chain reaction

PDB : Plackett-Burman design

PG : Distearoylphosphatidylglycerol

RSM : Response surface methodology

WHO : World Health Organization

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INTRODUCTION

In the course of history, lactic acid bacteria (LAB) have been incorporated, unconsciously, in the preparation of traditional and fermented foods and drinks (**Sanni et al., 2013; Salvucci et al., 2016**). Recently, works of Carl Wilhelm Scheele (1780) and Louis Pasteur (1875) have led to the conclusion that LAB were responsible for the processing of fermented foods like in cheese, yoghurt, sausage, wine, etc. (**Patel and Parikh, 2016**). Studies have also demonstrated the beneficial technological properties of this bacterial group such as proteolysis, lipolysis and development of flavors and aroma (**Karsloğlu et al., 2014; Esteban-Torres et al., 2015**). Lactic acid producing bacteria are also a priceless source for a number of valuable metabolites, in particular, organic acids, exopolysaccharides and biosurfactants (**Salvucci et al., 2016; Kalam, 2019**).

Bacteriocins are ribosomal antimicrobials produced by a broad number of bacteria and active against both Gram-positive and Gram-negative bacteria (**Walsh et al., 2015; Rasheed et al., 2020**). Bacteriocins from LAB, especially from probiotic strains, are of high interest because of their generally recognized as safe (GRAS) status (**Kaktcham et al., 2019**).

As well as their parent producers, bacteriocins have found their use in food and feed industries as biopreservatives and in medical and veterinary field as antimicrobial agents (**An et al., 2017**). However, several limitations restricted their use and production at large scale. Low production and high recovery costs are the most important constraints. Furthermore, the proteinaceous nature of these substances makes them vulnerable to alteration outside the cell (**Fahim et al., 2017; Telke et al., 2019**).

Presently, previous studies have been carried out in order to (i) improve the physicochemical properties of bacteriocins (ii) enhance their antimicrobial activity and spectrum (iii) optimize and increase their production. The advance studies of bacteriocin biology and the development of technologies were relevant to the purposes cited above (**Field et al., 2012; Dyae and Luti, 2019; Sidhu et al., 2019**).

This review contains two parts. The first part consists of an overview of LAB, their technological properties and antimicrobial potential along with bacteriocins, their classification and applications. The second part summarizes the recent developments and innovations in terms of bioengineering and nanotechnology approaches to improve bacteriocins' properties and activity, in addition to the production optimization techniques.

PART ONE

Chapitre 1 Lactic acid bacteria

1 Historical background

Throughout history, lactic acid bacteria (LAB) have been used in the production of several traditional fermented foods and beverages as well as in the preservation of foods and feeds and that in almost all societies (**Makarova et al., 2006; Sanni et al., 2013; Salvucci et al., 2016**). Though humans ignored the existence of living microorganisms, they have used some traditionally-prepared starters like sourdough as leavening agent in baking (**Liu et al., 2019**).

Lactic acid producing bacteria were first isolated from milk then they were recognized as natural microflora of raw milk (**Alnakip et al., 2016**). At the end of the 19th century, Doderlein revealed the presence of LAB in vagina. Later, the vaginal microbiota was matched to the growth inhibition of pathogens (**Garg et al., 2009; Foligné et al., 2010**). In the early 1900s, Ilya Ilyich Mechnikov stated the beneficial effects of LAB on health and longevity of Bulgarian farmers (**Duangjitcharoen et al., 2014; Süle et al., 2014**).

Nowadays, LAB are widely used in the production of a broad range of fermented foods, probiotics and metabolites (**Sanni et al., 2013; Esteban-Torres et al., 2015**).

2 General characteristics

LAB are a heterogeneous group of bacteria that produce lactic acid as the major end product of hexose sugars fermentation (**Makarova et al., 2006; Nikita and Hemangi, 2012; Alnakip et al., 2016**). Traditionally, they are described as Gram-positive, non-motile, non-spore-forming, spherical or rod-shaped bacteria (**Dinçer and Kivanç, 2018; Dangmanee, 2019**). LAB are anaerobic or aerotolerant, catalase negative (**Dinçer and Kivanç, 2018; Juwana et al., 2020**), they lack the heme group and thus they do not utilize the cytochrome system for terminal oxidation (**Pascual et al., 2006**). Like other prokaryotes, LAB contain a single circular chromosome (**Kleerebezem et al., 2002**). They are low G+C% bacteria (**Gómez et al., 2016**) and most of them harbor plasmids which encode for several functions such as, conjugal plasmid transfer, metabolic pathways and bacteriocin production (**Kleerebezem et al., 2002; Makarova et al., 2006**).

Two glucose metabolic pathways have been noticed in LAB. On the one hand, in homofermentative pathway, LAB catabolize glucose by glycolysis and produce mainly lactic acid. On the other hand, when glucose is catabolized by the phosphoketolase pathway in

heterofermentative organisms, it resulted in equimolar amounts of lactic acid, ethanol and CO₂ (Thornhill and Cogan, 1984; Makarova et al., 2006). LAB differ also in their metabolic profile of citrate (Thornhill and Cogan, 1984).

3 Diversity

In general, the classification of LAB is based on phenotypic (morphology) and biochemical properties (mode of glucose fermentation, configuration of the produced lactic acid, ability to grow at different temperatures, pH and high salt concentrations) (Nikita and Hemangi, 2012; Alnakip et al., 2016). Figure 1 shows a diagram used for LAB identification based on phenotypic and biochemical properties. However, most properties are strain-dependent (Salvucci et al., 2016), thus for a strain identification level, other techniques must be used. For instance, chemotaxonomic markers, cell wall compounds, PCR-based fingerprinting and protein fingerprinting (Alnakip et al., 2016; Juwana et al., 2020).

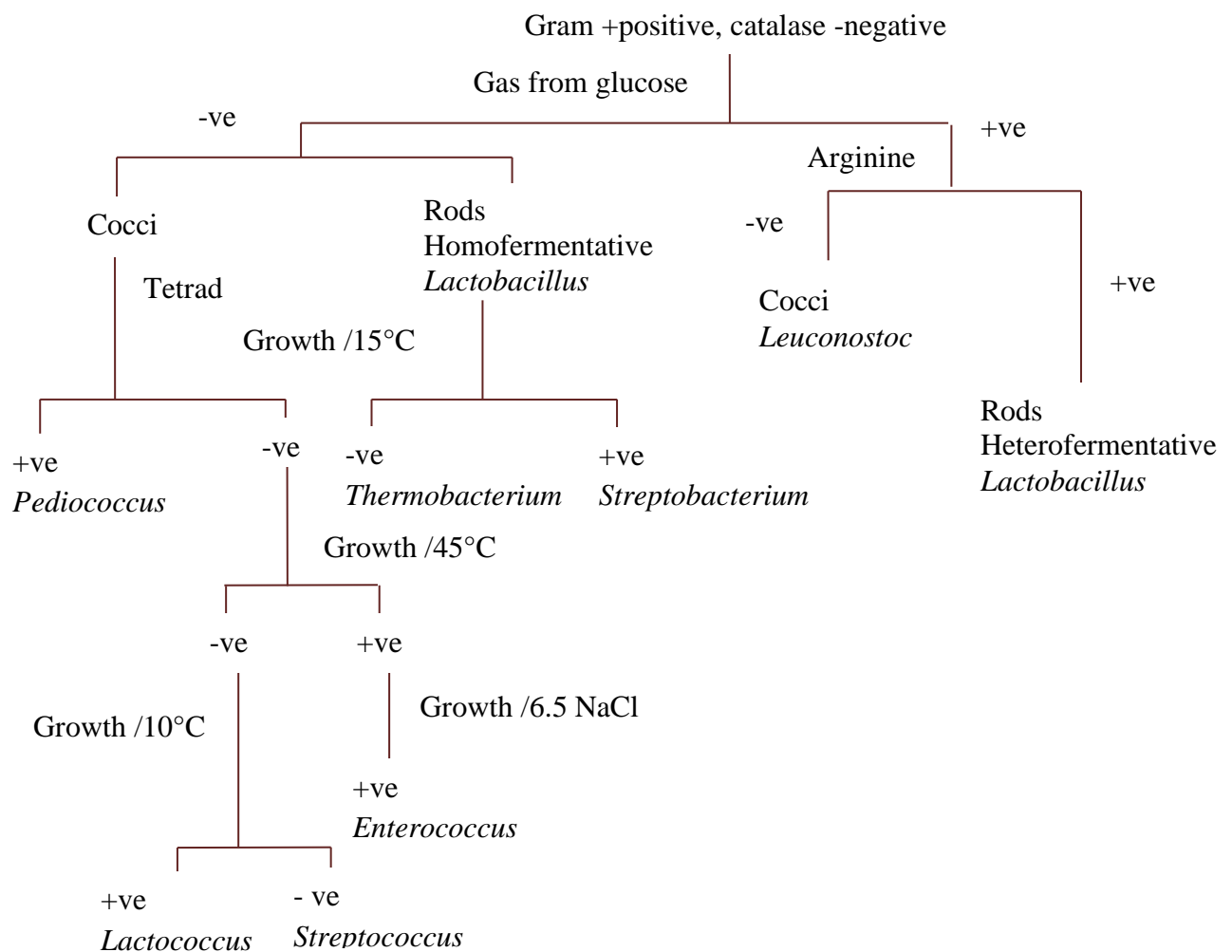


Figure 1 : Route for identification of LAB at genus level (Adnan et al., 2017).

Most lactic acid bacteria are listed among the generally recognized as safe (GRAS) microorganisms and the qualified presumption of safety (QPS) list allowing their utilization in food and drug industries (Alnaki et al., 2016; Salvucci et al., 2016). Nonetheless, some species of *Enterococcus* are excluded from those lists. It is worth noting that *Enterococcus* genus includes variable strains: pathogenic strains, food spoilage strains and safe strains. The latter are used as starters and probiotics (Quintela –Baluja et al., 2013; Li et al., 2020).

LAB proportion among the bacterial count in animal milk can reach 30% and it changes in number and diversity according to the animal species, season, feed habit (Alnaki et al., 2016; Revathy et al., 2019). Table 1 shows this diversity of LAB in two milks from two different regions.

Table 1: Diversity of lactic acid bacteria isolated from milk.

LAB genera	Origin	Reference
<i>Enterococcus</i> (51.22%)	Raw cow's milk	Alnaki et al., 2016
<i>Aerococcus</i> (26.82%)	(Elsharika province, Egypt)	
<i>Lactobacillus</i> (7.32%)		
<i>Lactococcus</i> , <i>Leuconostoc</i> , <i>Pediococcus</i> (14.64%)		
<i>Aerococcus</i> (30%)	Cow and buffalo milk	Revathy et al., 2019
<i>Pediococcus</i> (26%)	(Tamil Nadu, India)	
<i>Lactobacillus</i> (8%)		
<i>Streptococcus</i> , <i>Enterococcus</i> , <i>Tetragenococcus</i> , <i>Streptobacterium</i> , <i>Thermobacterium</i> (36%)		

4 Habitat

LAB are found both in association with natural hosts and free in natural environment. They are considered as autochthonous inhabitants (de Almeida Junior et al., 2015; Gómez et al., 2016). Nevertheless, they can come from exogenous sources (Quintela –Baluja et al., 2013).

4.1 LAB associated with natural hosts

Lactic acid producing bacteria are tightly associated to both humans and higher animals. They are indigenous inhabitants of mucosal surfaces, e.g., oral cavities, gastrointestinal tracts and vaginal tracts (de Almeida Junior et al., 2015; Juwana et al., 2020; Li et al., 2020).

Interestingly, vaginal microbiome which is dominated by lactobacilli maintains the balance of the vaginal ecosystem (**Bouridane et al., 2016**). Similarly, the presence of LAB prevents the growth and colonization of pathogenic microorganisms on or inside the mammals (**Roos, 2020**).

4.2 LAB free in natural environment

LAB are widely distributed in natural environment. Several ecological niches offer good conditions for their development, for example soils, waters, plants, waste products (**Nikita and Hemangi, 2012; Salvucci et al., 2016**) and foods (**Gómez et al., 2016**). Some LAB species were spread out in these niches through gastrointestinal tracts (faecal contamination) and genital tracts (**Quintela –Baluja et al., 2013; Juwana et al., 2020**).

5 Nutritional requirements

LAB are heterotrophic bacteria with complex nutritional requirements and limited biosynthetic capabilities. They require carbohydrates, peptides, amino acids and growth factors such as vitamins (**Nikita and Hemangi, 2012; Juwana et al., 2020**). They are programmed genetically for the use of a number of sugars as carbon source and for the generation of energy. They also possess genes encoding for protein-degradation, uptake and catabolism of peptides and pathways for amino acids biosynthesis (**Kleerebezem et al., 2002**).

These microorganisms are often found in various fermented foods in which salt concentration is up to 20% (**Esteban-Torres et al., 2015; Salvucci et al., 2016**). To tolerate such concentrations, some LAB uptake or synthesize a number of solutes (**de Almeida Junior et al., 2015**).

6 Usage and benefits

6.1 Technological properties

LAB are of great value in food industry especially fermented food because of their technological and nutritional properties. Interestingly, most of them are characterized by acidifying abilities, proteolytic activities, lipolytic activities and production of exopolysaccharides. Hence, they are widely used as starters (**Sanni et al., 2013; Gómez et al., 2016; Salvucci et al., 2016**).

6.1.1 Lipolytic activity

Lactic acid producing bacteria possess lipolytic potential due to their esterases and lipases. These enzymes are involved in the development of aroma and flavor of fermented foods

(Esteban-Torres et al., 2015; Dinçer and Kivanç, 2018). Moreover, Karsloğlu and co-workers (2014) reported that LAB have the ability to modify the lipid content during meat fermentation and both assayed commercial starters (*Lactobacillus sakei* and *Lb. pentosaceus*) showed lower thiobarbituric acid and free fatty acid amounts compared to the control groups (Karsloğlu et al., 2014).

6.1.2 Proteolytic activity

Proteolytic activity is essential for LAB to grow on protein-rich environments (Kleerebezem et al., 2002; de Almeida Junior et al., 2015). LAB possess a variety of proteinases and peptidases (aminopeptidases, dipeptidases, carboxypeptidases) degrading proteins and peptides respectively (de Almeida Junior et al., 2015). This activity is important in food industry since it has a role in flavor development, texture and it enhances the nutritional quality of the product (Pescuma et al., 2010; Salvucci et al., 2016).

6.1.3 Aromatic activity

All metabolic activities of LAB lead to the production of a number of aroma and taste compounds (Kalam, 2019; Silva et al., 2019). Liu and collaborators (2019) identified a number of volatile compounds, e.g., alcohols, aldehydes, ketones, acids and esters in some fermented sourdoughs samples (Liu et al., 2019).

6.1.4 Production of exopolysaccharides

Some LAB are known for their ability to produce exopolysaccharides (EPSs). These biopolymers *in situ*-produced affect rheological and physical properties of end products even when synthesized in small amounts (Mende et al., 2012; Dertli et al., 2016). For that reason, LAB are widely used in fermented dairy products to modify the viscosity, firmness, elasticity or syneresis (Gentès et al., 2011; Mende et al., 2012). LAB can produce EPSs, either free EPS (fEPS) if it is excreted into the medium or capsular EPS (cEPS) if it remained attached to the cell walls, it is also called ropy EPS (Mende et al., 2012; Khanal and Lucey, 2017). EPSs can exert their technological properties differently according to their characteristics: monosaccharide composition, charge, molecular mass, linkage and branching type and their interactions with milk compounds (Gentès et al., 2011; Mende et al., 2012).

6.2 Probiotics and antimicrobial properties of LAB

Nowadays, the antimicrobial potential of LAB is well known and some strains are even considered as probiotics (Dong et al., 2019; Li et al., 2020). LAB exert their antagonistic effect

through a number of antimicrobial metabolites, e.g., organic acids, fatty acids, hydrogen peroxide, diacetyl, bacteriocins (**Dong et al., 2019; Juwana et al., 2020**), biosurfactants (**Gómez et al., 2016**) and exopolysaccharides (**Dertli et al., 2016**).

6.2.1 Probiotic

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms which, when administered in adequate amounts confer a health benefit to the host (**Gómez et al., 2016; Silva et al., 2019**). From this definition, it can be concluded that probiotic bacteria should be present and remain viable at a level that allowed them to exert their requested effects (**Pescuma et al., 2010; Ouled-Haddar et al., 2014**). Therefore, LAB used as probiotics must require some criteria. For instance, they must survive the transit across digestive system, resist to low pH, bile salts, gastric juice, and pancreatic juice and have the ability to adhere the epithelial cells (**Sanni et al., 2013; Gómez et al., 2016**). Additionally, other probiotics, e.g., vaginal probiotics must meet further characteristics such as, adhesion to the vaginal epithelial cells and resistance to spermicides (**Bouridane et al., 2016**). Probiotics have positive effects in both prevention and treatment of several diseases. **Table 2** summarizes some of these effects.

6.2.2 Antimicrobial properties

6.2.2.1 Production of organic acids

As a result of carbohydrates fermentation, LAB produce different organic acids like lactic, acetic and propionic acids which acidify the environment and thus can inhibit the growth and metabolic activities of other microorganisms including food borne pathogens (**Sanni et al., 2013; Salvucci et al., 2016; Roos, 2020**). More interestingly, vaginal LAB maintain a low vaginal pH by the production of such organic acids which protect this ecosystem from potential pathogens. The predominant species of *Lactobacillus* produced mainly lactic acid (**Pascual et al., 2006; Bouridane et al., 2016**).

6.2.2.2 Production of hydrogen peroxide

Hydrogen peroxide (H_2O_2) is an antimicrobial metabolite produced by several species of LAB (**Kalam, 2019; Silva et al., 2019**). Its antagonistic effect has been demonstrated in both vagina and intestinal mucous (**Pascual et al., 2006; Bouridane et al., 2016; Silva et al., 2019**). H_2O_2 is produced by LAB possessing flavoproteins and affect the growth of microorganisms which lack or produce small amounts of catalase or peroxidase enzymes (**Pascual et al., 2006**).

Table 2 : Some benefic effects of lactic acid bacteria probiotics

Probiotic effect	References
Stimulation and enhancement of the immune system	Bosch et al., 2011 ; Dangmanee, 2019 ; Li et al., 2020
Management of inflammatory bowel diseases	Ouled-Haddar et al., 2014 ; Süle et al., 2014 ; Li et al., 2020
Anticancer	Süle et al., 2014 ; Li et al., 2020
Antidiabetic	Li et al., 2020
Stabilization and balance of the intestinal microbiota	Dangmanee, 2019 ; Li et al., 2020
Prevention and treatment of intestinal infections	Bosch et al., 2011 ; Ouled-Haddar et al., 2014
Restoration of the intestinal flora after antibiotic-treatment	Süle et al., 2014 ; Li et al., 2020
Reduction of serum cholesterol	Süle et al., 2014 ; Dangmanee, 2019
Alleviation of lactose intolerance	Süle et al., 2014 ; Dangmanee, 2019
Prevention of urogenital tract infections	Bosch et al., 2011 ; Bouridane et al., 2016
Reduction of allergy and respiratory infections	Bosch et al., 2011
Prevention of caries	Bosch et al., 2011
Improvement of feed efficiency and growth rate of animals	Li et al., 2020

6.2.2.3 Production of bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by some LAB. They possess bactericidal or bacteriostatic activity against a wide range of pathogenic bacteria (Salvucci et al., 2016; Kalam, 2019) and yeast (Eissa et al., 2018; Tumbraski et al., 2019).

6.2.2.4 Production of biosurfactants

LAB produce biosurfactants which are amphiphilic metabolites with very interesting properties: antibacterial, antifungal, antiviral, antiadhesive and antibiofilm properties (Gómez et al., 2016; Ghasemi et al., 2019) that confer a protection for the intestinal and vaginal tracts (Dong et al., 2019). They have variable chemical structures according to the producer strain.

Therefore, there are lipopeptides, glycopeptides, glycolipids, polysaccharides, lipopolysaccharides, lipoproteins, etc. (Ghasemi et al., 2019; Matei et al., 2019).

6.2.2.5 Production of other antimicrobial compounds

LAB synthesize other antimicrobial metabolites such as 3-hydroxypropionaldehyde, also known as reuterin produced by *Lb. reuteri* (Kalam, 2019; Roos, 2020). Diacetyl (Kalam, 2019; Juwana et al., 2020) and exopolysaccharides (Dertli et al., 2016) are also characterized by their antimicrobial effect.

6.2.2.6 Inhibition of biofilm formation

Biofilms are considered as potential reservoirs of pathogenic microorganisms. However, several LAB strains have the ability to inhibit the biofilm formation and pathogens colonization by producing antimicrobial substances (Gómez et al., 2016; Shi et al., 2019). Bajpai and collaborators (2016) have noticed an antagonistic effect of the cell free supernatant of *Pediococcus pentosaceus* on the biofilms formed by *Staphylococcus aureus* and *Escherichia coli* O157:H7 (Bajpai et al., 2016).

Chapitre 2 Bacteriocins

1 Definition and general characteristics

1.1 Definition

Bacteriocins are ribosomally-synthesized antimicrobial metabolites produced by numerous Gram-negative and Gram-positive bacteria (An et al., 2017; Rasheed et al., 2020). These compounds enhance the growth and the survive of the producer strains in their ecological niche by inhibiting other competitive and invader bacterial species (Martin-Visscher et al., 2008) either close (related species) or distant (other genera) (Walsh et al., 2015; Wayah and Philip, 2018). In general, they are cationic and amphipathic (Sharma et al., 2010; Salvucci et al., 2016) proteinaceous substances that can be found as peptides, proteins or protein complexes (Mirkovic et al., 2016) often with low molecular weights (Wayah and Philip, 2018; Pei et al., 2020).

1.2 Genetics

Bacteriocins are encoded by gene clusters which are located differently from a bacteriocin to another either on the bacterial chromosome (Mirkovic et al., 2016) or carried by plasmid (Piard et al., 1993; Stern et al., 2005). These gene clusters encode for the putative bacteriocin and for other proteins required for its synthesis, post-translational modification, transport and immunity (Birri et al., 2012; Scholz et al., 2014; Borrero et al., 2018). It is worth noting that immunity determinants of *Pediococcus acidilactici* PAC1.0 were reported to be located chromosomally, whereas the presumed-bacteriocin (pediocin PA-1) gene cluster was detected on the plasmid pSRQ11 (Marugg et al., 1992).

1.3 Composition

Bacteriocins of LAB are usually small with sizes up to 60 amino acids, although others contain more than 60 amino acids (Nissen-Meyer et al., 1992; Moll et al., 1996). Their three-dimensional structures are diverse. An and colleagues (2017) reported that bacteriocin M1-UVs300 possesses β -sheet (content of 52.43%), α -helix (16.17%), β -turn (15.27%) and random coil (16.12%) (An et al., 2017). Leucocin A features to contain triple-stranded β -sheets in N-terminal and an α -helix in C-terminal (Sit et al., 2012).

1.4 Biosynthesis

It has been reported that up to 90% of bacteria produce at least one bacteriocin (Walsh et al., 2015) whereas other strains can produce more than one. *Enterococcus faecium* NKR-5-3B, isolated by Himeno and collaborators (2015) produces five different bacteriocins (Himeno et al., 2015). Generally, the production is initiated during the exponential phase with a highest production reached by the early stationary phase (Gilbreth and Somkuti, 2005; Rasheed et al., 2020).

Bacteriocins are synthesized as precursor peptides. The N-terminal extension of the prepeptides determines the type of excretion of the bacteriocin. On the one hand, the double-glycine type leader peptides are cleaved simultaneously with export by the adenosine triphosphate-binding cassette transporters (ABC transporters). Most of lantibiotics and non-lantibiotics follow this pathway. On the other hand, the sec-dependent signal peptides are cleaved concomitantly with the transport by the general secretory pathway (GSP) or sec-dependent pathway (Maldonado et al., 2003; Gilbreth and Somkuti, 2005; Borrero et al., 2011).

Bacteriocin biosynthesis is a high-energy-consuming process and thus it is well regulated. The regulatory mechanism required various molecules (Maldonado et al., 2003). For example, salivaricin MMAYE1 production was found to be regulated by a quorum sensing mechanism that include a histidine protein kinase (HPK), a response regulator (RR) and an inducing peptide (IP) (Wayah and Philip, 2018). Lantibiotics production is also monitored by a quorum sensing system which involves a histidine protein kinase (Lan K), a response regulator (Lan R), and a peptide pheromone (Birri et al., 2012).

1.5 Mode of action

Bacteriocins possess bactericidal or bacteriostatic effects on sensitive cells (An et al., 2017; Kaktcham et al., 2019) as well as different mechanisms of action. The most known mode is cell wall/membrane permeabilization (Gilbreth and Somkuti, 2005; Zhang et al., 2020), which is similar to that of ionophore antibiotics (Stern et al., 2006). Firstly, they must bind to their specific cell receptors (Grosu-Tudor et al., 2014; Pei et al., 2020), then, the installation of bacteriocins across the wall/membrane affects the phospholipid bilayer integrity by pore-formation that causes the leakage of intracellular electrolytes, nucleic acids and proteins, and eventually the death of cells (Pei et al., 2020; Qiao et al., 2020; Zhang et al., 2020). There are two pore types: the “barrel-stave-like” and the “wedge-like” pores, both consist of the

oligomerization of amphiphilic bacteriocins across the phospholipid bilayer membrane (Moll et al., 1999).

Other mechanisms including the interruption of cell wall/membrane synthesis (Pei et al., 2020; Zhang et al., 2020) and DNA replication (Mossallam et al., 2014; Galvan et al., 2020) were reported. Moreover, bacteriocins played an important role in the synchronization of population behavior and are used as signaling peptides (Kubašová et al., 2020). More interestingly, bacteriocins might also interfere and inhibit the formation of biofilms (Pei et al., 2020). Chopra and collaborators (2015) reported that sonorencin, a bacteriocin produced by *Bacillus sonorensis* MT93, inhibit the formation of *St. aureus* biofilm (Chopra et al., 2015). Bacteriocin FT256, produced by *Lb. paraplantarum* FT256, was found to interfere with the formation of *Listeria monocytogenes* biofilm (Winkelströter et al., 2015).

1.6 Immunity

Bacteriocins are highly specific antimicrobial peptides that are active on other bacterial strains rather than their own producer (Scholz et al., 2014; Qiao et al., 2020). Bacteriocin producers possess distinct immunity system (Mirkovic et al., 2016; Kubašová et al., 2020) granted by specific immunity proteins (Birri et al., 2012; Mu et al., 2014; Walsh et al., 2015).

2 Classification of LAB bacteriocins

Bacteriocins are very various and are distinguished by their molecular weights, structures, biochemical characteristics and mode of action (Kubašová et al., 2020). Klaemhanner (in 1993) classified bacteriocins from LAB into four major classes. Class I includes lantibiotics, class II includes small, heat-stable, non-lanthionine-containing peptides, class III bacteriocins are large and heat-labile proteins and class IV includes complex proteins (Gilbreth and Somkuti, 2005; Stern et al., 2006). Recently, authors have de-established the fourth class and thus the classification was restricted to only the first three classes (Song et al., 2014a; An et al., 2017; Kubašová et al., 2020).

2.1 Class I

Class I bacteriocins are named lantibiotics. They are small (<5KDa), heat-stable and post-translationally modified peptides (Birri et al., 2012; Jiang et al., 2016). To earn its biologically active form, the prepeptide is subjected to some modifications. These latter consist of dehydration of serine and threonine which form dehydroalanine and dehydrobutyrine, respectively, that interact with the close cysteine residues to form a thioether linkage leading to

the two unusual amino acids, lanthionine and methyllanthionine, respectively (**Castiglione et al., 2007; Field et al., 2010; Birri et al., 2012**).

According to their structure and mode of action, lantibiotics can be classified into flexible type A lantibiotics and rigid and globular type B lantibiotics. Type A is pore-forming lantibiotics which interact with lipid II. Type B lantibiotics act by inhibiting enzymes functions (**Castiglione et al., 2007; Mirkovic et al., 2016**). Other classification categorizes them on two subclasses according to the enzymes involved in their synthesis, modification and exportation (**Birri et al., 2012; Mirkovic et al., 2016**).

2.2 Class II

Class II bacteriocins are small (<10KDa), heat-stable, non-lanthionine-containing (**Castellano et al., 2003; Grosu-Tudor et al., 2014; Kubašová et al., 2020**) and membrane active peptides (**Jiang et al., 2016; Sun et al., 2018**). They are divided into four subclasses: subclass IIa- the pediocin-like bacteriocins; subclass IIb- the two-peptide bacteriocins; subclass IIc- the circular bacteriocins; and subclass IId- the non-pediocin-like linear one-peptide bacteriocins (**Song et al., 2014a; Jiang et al., 2016**).

2.2.1 Subclass II a

Subclass IIa consists of pediocin-like bacteriocins. They are called so because they shared the N-terminal consensus sequence of YGNGVxC with pediocin PA-1 (**Fimland et al., 2000; Tymoszevska et al., 2017**). Furthermore, they possess the highest antimicrobial activity against *L. monocytogenes* (**Castellano et al., 2003; Gilbreth and Somkuti, 2005; Grosu-Tudor et al., 2014**). Pediocin PA-1 is the prototype of this subclass, produced by *P. acidilactici* PAC 1.0 (**Gonzalez and Kunka, 1987**).

2.2.2 Subclass II b

Subclass IIb includes two-peptide bacteriocins whose activity depends on the complementary action of the two peptides (**Castellano et al., 2003; Maldonado et al., 2003**). Both peptides may have almost identical amino acid sequences like in plantaricin A or distinct ones such as those of plantaricin S (**Jimenez-Dial et al., 1995**). Lactococcin G, the representative of subclass IIb bacteriocins, kills sensitive cells by formation of potassium selective pores that lead after several cytosolic phenomena to cell death (**Nissen-Meyer et al., 1992; Moll et al., 1996**).

2.2.3 Subclass II c

Subclass IIc involves circular bacteriocins which have a head-to-tail backbone formed by a covalent ligation between their N- and C-termini (Mu et al., 2014; Borrero et al., 2018). They are first synthesized as a linear precursor that undergo later some post-translational modifications starting by the removing of its leader peptide followed by linking of the N- and C-termini to form the mature bacteriocin (Himeno et al., 2015; Borrero et al., 2018). The final three-dimensional structure consists of several α -helices encircling a hydrophobic core (Scholz et al., 2014; Himeno et al., 2015). This particular nature makes them robust bacteriocins (Martin-Visscher et al., 2008; Mu et al., 2014). Enterocin AS-48 from *Streptococcus faecalis* S-48, was the first bacteriocin discovered in this subgroup (Galvez et al., 1986). Several other subclass IIc bacteriocins were described later, e.g., carnocyclin A, produced by *Carnobacterium maltanomiticum* (Martin-Visscher et al., 2008) and amylocyclin from *B. amyloliquefaciens* FZB42 (Scholz et al., 2014).

2.2.4 Subclass II d

Subclass IId regroups all bacteriocins that do not belong to the other class II bacteriocins, i.e., linear one-peptide non-pediocin-like (Song et al., 2014a; Jiang et al., 2016). Numerous subclass IId bacteriocins are known: leucocin B, produced by *Leuconostoc mesenteroides* TA33a with limited activity spectrum to *Leuconostoc* and *Weissella* genera (Wan et al., 2015); and garvicin Q, with a broad antimicrobial spectrum, including, among others, *L. monocytogenes* (Tymoszevska et al., 2017).

2.3 Class III

Class III bacteriocins are non lantibiotics, heat-labile with high molecular weight (>30KDa) proteins (Castellano et al., 2003; Jiang et al., 2016; Kubašová et al., 2020). Several class III bacteriocins have been described such as: enterolysin A, from *Enterococcus faecalis* LMG2333, possesses a broad inhibitory spectrum against LAB strains (Nilsen et al., 2003) and helveticin M, produced by *Lb. crispatus* K313 which has a bacteriostatic mode of action against Gram-positive and Gram-negative bacteria (Sun et al., 2018).

2.4 Class IV

Class IV bacteriocins are large and thermostable protein complexes that required other essential moieties of lipids or carbohydrates (Gautam and Sharma, 2009; Sankar et al., 2012). The best-known representatives of class IV bacteriocins were: leuconocin S from *Leuconostoc*

paramesenteroides (Lewus et al., 1992) and pediocin SJ-1, produced by *P. acidilactici* SJ-1 (Schved et al., 1993).

2.5 Other classification

Some authors preferred to classify bacteriocins into two groups. Class I consists of lanthionine-based bacteriocins and class II includes non-modified or slightly modified bacteriocins (Himeno et al., 2015; Walsh et al., 2015; Pei et al. 2020).

3 Application and usage

Similarly to most LAB species, bacteriocins are now known by their GRAS status (Kaktcham et al., 2019; Rasheed et al., 2020). Consequently, they are used as natural preservatives in food and feed industries as well as alternative antimicrobial agents in pharmaceutical industry (An et al., 2017).

3.1 Food industry

Bacteriocins know an increase use in food industry as alternatives of chemical preservatives (Wayah and Philip, 2018; Kaya and Simsek, 2019) and to overcome resistance among existing antibiotics (Grosu-Tudor et al., 2014; Rasheed et al., 2020). They are used to control several food spoilage microorganisms and food-borne pathogens such as *L. monocytogenes*, *E.coli* and *St. aureus* (Birri et al., 2012; Kaya and Simsek, 2019; Qiao et al., 2020). Their proteinaceous nature and physicochemical characteristics gave them such potential (O'Shea et al., 2010; Zhang et al., 2020). However, only few bacteriocins are certified for commercialization such as nisin and pediocin PA-1 (Fagundes et al., 2016; Kaktcham et al., 2019).

In addition to their biopreservation potential, bacteriocins are also used in cheese making to speed the ripening process (Gilbreth and Somkuti, 2005; Mirkovic et al., 2016).

3.2 Therapeutic use

As a result of their origin, nature and potent activities, bacteriocins are widely used in human medicine. Consequently to their antimicrobial potential, they offer a suitable alternative for antibiotics, especially with drug and multi-drug resistant pathogens (Field et al., 2010; Kubašová et al., 2020). Bacteriocins have antagonistic effect against several important human pathogens (Birri et al., 2012). For example, some studies have shown that bacteriocins may be used to control vaginosis caused by *Gadnerella vaginalis* (Aroutcheva et al., 2001), to inhibit

the growth of *Probiobacterium acnes* (Kang et al., 2009), to control respiratory tract infections caused by *St. aureus* (de Kwaadsteniet et al., 2009) etc.

Furthermore, many bacteriocins have shown their potential use in contraception by exhibiting a spermicidal activity such as nisin (Reddy et al., 2004) and lacticin 3147 (Silkin et al., 2008).

3.3 Animal feed and veterinary medicine

Bacteriocins present potentialities in veterinary medicine in treatment of bacterial infections (Borrero et al., 2018). Wang and colleagues (2011) reported that albusin B stimulated the growth of broiler chicken by increasing the intestinal absorption and decreasing the number of pathogenic bacteria (Wang et al., 2011). Similarly, a bacteriocin-based treatment before poultry slaughter reduced chicken colonization by *Campylobacter jejuni* (Stern et al., 2005).

Nisin-based commercial products are now available, e.g., Wipe Out®, anti-mastitis wipes, and Mast Out®, an intra-mammary infusion (Field et al., 2012).

PART TWO

Chapitre 1 Bioengineering of bacteriocins from lactic acid bacteria

1 Nisin and its natural variants

Nisin is a 34-amino acids polycyclic lantibiotic produced by *Lc. lactis* (Zhou et al., 2016; Galvan et al., 2020). It possesses ten natural variants (O’Sullivan et al., 2020) which are slightly different in their peptide sequences (Figure 2.A, Appendix 1). For example, nisin Z differs from nisin A by only one amino acid at position 27 (Field et al., 2015) while twelve amino acids were found different in nisin U (Wirawan et al., 2006). More interestingly, it was revealed that nisin O cluster has four copies of its structural gene *nsoA1234* (Hatzioanou et al., 2017), which encode for three identical peptides (nisO123) and another which is highly divergent peptide from nisin A (nisO4) (Figure 2.B) (O’Sullivan et al., 2020).

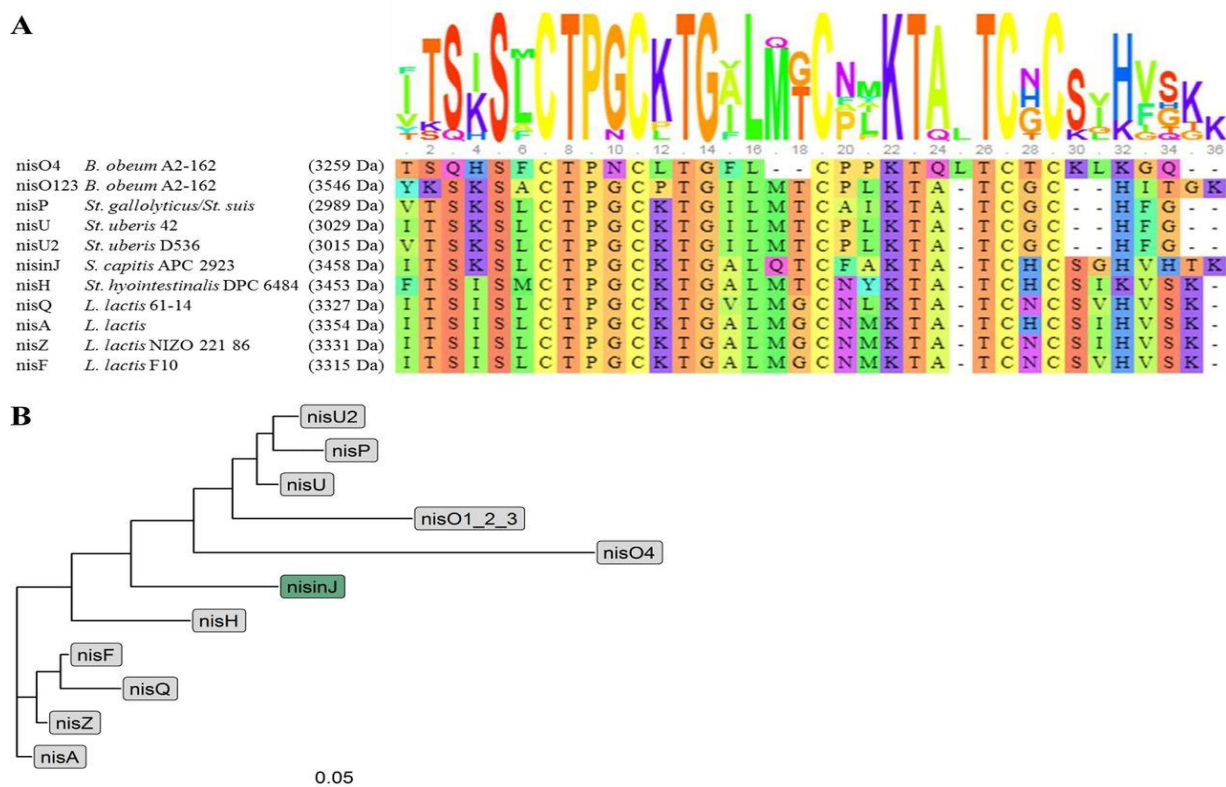


Figure 2 : Nisin natural variants. (A) The multiple-sequence alignment of all natural nisin variants aligned with strain origin obtained by Muscle Program. The total height of the sequence logo at each position reflects the degree of conservation at that position in the alignment, while the height of each letter in that position is proportional to the observed frequency of the corresponding amino acid at that position. (B) Phylogenetic relatedness in primary structures of all known natural nisin variants. The branch length represents phylogenetic distance (scale: 0.05) (O’Sullivan et al., 2020).

Hence, these variants show some diversities in their antimicrobial activity (AMA), spectrum and/or physicochemical properties (**Table 3**).

Table 3 : Physicochemical properties, antimicrobial activity and spectrum of some nisin natural variants.

Bacteriocin	Producer strain	Properties	References
Nisin A	<i>Lc. lactis</i> strains	Potent activity against GPB	Zhang et al., 2020
		Slight effect on GNB; Decreased stability and low AMA in the pH range of 5.0-7.0	Qiao et al., 2020
		Easily oxidized at a low pH or by freeze drying	Yoneyama et al., 2018
Nisin Z	<i>Lc. lactis</i> NIZO 22186	Similar AMA to nisin A; Higher rate of diffusion; Less soluble at low pH	Field et al., 2015
Nisin F	<i>Lc. lactis</i> F10	More active against several drug-resistant strains than nisin A, Z and Q	Piper et al., 2010
Nisin Q	<i>Lc. lactis</i> 61-14	Higher stability under oxidative conditions; Longer time-inhibition against GPB	Yoneyama et al., 2018

AMA: antimicrobial activity; GNB: Gram-negative bacteria; GPB: Gram-positive bacteria.

Although bacteriocins are gene-encoded and ribosomally-synthesized (**Mirkovic et al., 2016; Kaya and Simsek, 2019**), that made them adequate candidates contrary to other antimicrobial metabolites- for further improvement through bioengineering-based techniques (**Field et al., 2015**).

2 Improvement of the antimicrobial activity and spectrum

Bacteriocins possess diverse antimicrobial spectra which can either be restricted, covering only bacteria that are closely related to the producer strain or extended, inhibiting a wide range of distantly related bacteria and other microbes (**Walsh et al., 2015; Wayah and Philip, 2018**). Several bioengineering-based strategies have been applied on bacteriocins to enhance their antimicrobial activity and broaden their spectrum.

2.1 Nisin mutant variants

Nisin has been the most engineered bacteriocin. Several mutagenesis assays have been achieved since its approved use in 1969 (Field et al., 2010). Field and co-workers (2008), using the site-directed and site-saturation mutagenesis, successfully generated three nisin derivatives with improved inhibitory activity against clinical and food-borne Gram-positive pathogens. The study revealed that M21V has superior specific activity against *L. monocytogenes* 10403S and EGDe and *St. aureus* ST528, and that K22T has enhanced specific activity against *St. aureus* ST528 and *Streptococcus agalactiae* ATCC13813 and that N20P possesses superior specific activity against only *St. aureus* ST528 (Field et al., 2008).

In 2012, Field and collaborators focused their study on S29. Oligonucleotides with NNK triplet rather than AGT were used to substitute the serine 29 with all standard amino acids. Then, the mutant genes were cloned in *E. coli* Top10 and expressed in *Lc. lactis* NZ9800. Four variants (S29G, S29A, S29D and S29E) displayed improved activity against at least five target cells. Interestingly, S29A had superior activity than nisin A against all tested strains including methicillin-resistant *St. aureus* (MRSA), heterogenous vancomycin-intermediate *St. aureus* (hVISA), *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium UK1 and *Cronobacter sakazakii* DPC6440 (Field et al., 2012).

2.2 Pediocin PA-1 mutant variants

Pediocin PA-1 is a cationic class IIa bacteriocin composed of 44 amino acid residues. Its N-terminal domain is highly conserved, hydrophilic and cationic, while the C-terminal region is less conserved, hydrophobic or amphiphilic (Song et al., 2014b).

Song and co-workers (2014) generated eight pediocin PA-1 mutants (Figure 3) based on previous alignment of some pediocin-like bacteriocins. The mutants were altered with cationic residues at different positions within their N-termini by site-directed mutagenesis, cloned and expressed in *E. coli* BL21. Three mutated variants of PA-1 (OK, S13K and OKS13K) had higher affinity for target cells (*Micrococcus luteus* and *St. aureus*) than PA-1. Although, mutant OK had an equal inhibitory activity as the wild type, S13k was 1.5-fold more potent whereas OKS13K's potency increased by 2-fold (Song et al., 2014b).

PA-1:	K YYNGVTCGKHSCSVDWGKATTCI INNGAMAWATGGHQGNHKC
0K:	K.
2K:	.K.
G10K:K.
S13K:K.
0K+S13K:	K.K.
2K+S13K:	.K.K.
0K+2K+S13K:	K.K.K.
0K+2K+G10K:	K.K.K.

Figure 3 : The eight constructed pediocin PA-1 mutants (Song et al., 2014b).

Sun and colleagues (2015) were interested in the C-terminal of Pediocin PA-1. Ten out of fourteen mutants showed improved activity against *L. monocytogenes*. The highest increase was obtained with the mutant G29A. This study highlighted also the role of the region 29G to 32A along with the hairpin-like structure in the inhibiting-mechanism of PA-1 (Figure 4) (Sun et al., 2015).

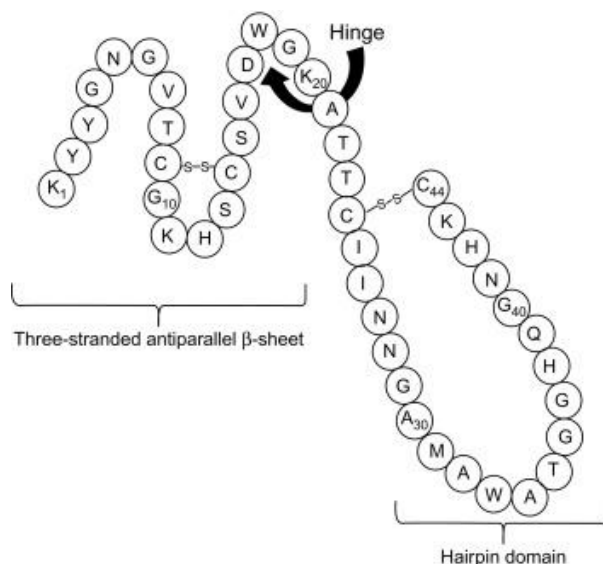


Figure 4 : Primary structure of pediocin PA-1 (Espitia et al., 2016).

2.3 Hybrid bacteriocins

Enterocin CRL35 (Ent35) is a class IIa bacteriocin with a strong antilisterial activity and no effect on Gram-negative bacteria. Differently, the inhibiting-effect of microcin V (MccV) is limited to Gram-negative bacteria. A chimerical gene was constructed with genes encoding enterocin CRL35 and microcin V namely *munA* and *cvaC*, respectively. Both genes were fused by asymmetrical PCR and then cloned and expressed in *E. coli* DH5 α . Interestingly, the hybrid peptide Ent35-MccV exhibited a broader antimicrobial spectrum including clinical isolates on

which both wild-type bacteriocins are inactive. Moreover, Ent35-MccV was more potent than microcin V against some Gram-negative bacteria (Table 4) (Acuña et al., 2012).

Table 4 : Inhibitory spectrum of Ent35-MccV and parental bacteriocins. Adapted from Acuña et al., (2012).

Strain	Inhibitory activity ^a		
	Ent35-MccV	Ent35	MccV
Gram-positive			
<i>L. innocua</i> 7	+++	+++	-
<i>L. monocytogenes</i> FBUNT1	+++	+++	-
<i>L. monocytogenes</i> FBUNT2	+++	+++	-
<i>Ec. faecalis</i> FBUNT1	-	+++	-
<i>St. aureus</i> FBUNT1	+	-	-
<i>St. aureus</i> FBUNT2	+++	-	-
<i>St. epidermidis</i> FBUNT1	+	-	-
Gram-negative			
<i>E. coli</i> MC4100	+++	-	+++
<i>E. coli</i> O157:H7	+++	-	++
<i>E. coli</i> (UPEC215)	+++	-	++
<i>E. coli</i> (UPEC217)	+++	-	++
<i>Eenterobacter cloacae</i>	+	-	+
<i>Serratia marscecens</i>	+	-	-
<i>Klebsiella pneumoniae</i>	+	-	-

^a Symbols represent relative activity by diffusion in agar, evaluated by measuring the average diameter (da) of inhibition zones. -: no inhibition; +: da < 5 mm; ++: 5 mm < da < 10 mm; +++: da > 10 mm.

Ent35: enterocin 35; MccV: microcin V.

Various studies featured that the combination of nisin with some chemicals resulted in an enhanced antimicrobial activity against Gram-negative bacteria (Zhou et al., 2016; Galvan et al., 2020). Other works highlighted the potent inhibitory effect of a number of antimicrobial peptides towards Gram-negative bacteria (Zhou et al., 2016).

Zhou and collaborators (2016), designed some hybrid peptides by combination of nisin with ten anti-Gram-negative peptides. Four groups (**Figure 5**) were formed by combining an anti-Gram-negative peptide tail with the ABC rings of nisin + hinge region (group1), full length nisin (group2), part of the C-terminal sequence-deleted nisin (group3) and the ABCDE rings with a Ser-Gly linker (group 4). The hybrid genes were cloned and expressed in *Lc. lactis* NZ9000. As a result, only the hybrid peptide GNT16 which contains a full length nisin and a tail from apidaecin1b displayed superior activity (2-fold) than nisin against *E. coli* CECT101. It is worth noting that the inhibitory effect of GNT16 against the nisin-sensitive strain *Lc. lactis* MG1363 showed a 32-fold decreased (**Zhou et al., 2016**).

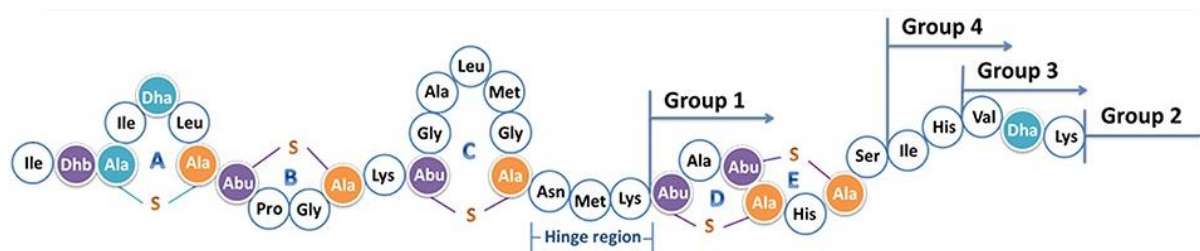


Figure 5 : Primary structure of nisin showing the positions of the tails. Adapted from **Zhou et al., (2016)**.

2.4 Synthetic bacteriocins

The head-to-tail backbone of circular bacteriocins affords several advantages (**Mu et al., 2014**), but it becomes challenging with chemical synthesis. In order to chemically synthesize enterocin AS-48, a class IIc bacteriocin, Hemu and colleagues (2016) adopted a two-step approach. Firstly, the linear peptide precursors were prepared by microwave stepwise synthesis. Contrariwise, the synthetic precursor peptides started with the residue after Asn 17 (i.e., V18) and the C-termini were elongated with a dipeptide HV (i.e., N17-HV). Secondly, the cyclization was mediated by butelase 1, a highly potent Asp/Asn (Asx)-specific ligase. The previous modifications are crucial for the butelase-mediated ligation (**Figure 6**). Importantly, synthetic AS-48 bacteriocin exhibited strong antimicrobial activity against a number of drug resistant strains such as MRSA DR15686 and carbapenem-resistant *E. coli* DR23975 and the food-borne *L. monocytogenes* (**Hemu et al., 2016**).

2.5 Analogues of lactacin 481

Lactacin 481 is a type B lantibiotic which inhibit the peptidoglycan biosynthesis of its target cells. Knerr and collaborators (2012) synthesized four analogues of lactacin 481. Firstly, linear

core peptide analogues were produced by solid phase synthesis where the following mutations: N15R+F21H, N15R+F21Pal, N15R+F21H+W23Nal and N15R+F21Pal+W23Nal (Pal=3-(4'-pyridyl) alanine; Nal=3-(2-naphthyl) alanine) were introduced. Secondly, the leader-LctM fusion enzyme (LctMCE-GS₁₅) was incubated with these core peptides to obtain the active analogue peptides. As a result, N15R+F21Pal and N15R+F21H exhibited the higher growth inhibitory activity with IC₅₀=201nM and IC₅₀=428nM, respectively, compared to the parent lactacin 481 (IC₅₀=785nM). Hence, the introduction of non-proteinogenic amino acids resulted in enhanced antimicrobial activity due to an improved affinity to the target (**Knerr et al., 2012**).

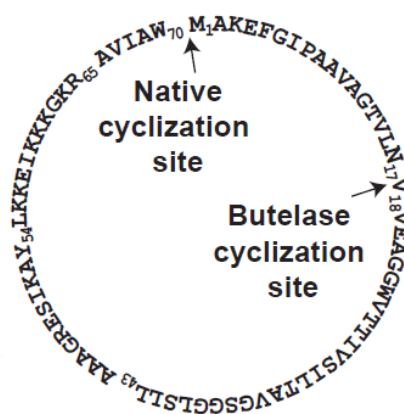


Figure 6 : Primary structure of enterocin AS-48 showing the native and the butelase cyclization sites. Adapted from **Hemu et al., (2016)**.

3 Improvement of the physicochemical properties

Besides tendency to enhance the antimicrobial activity and spectrum of bacteriocins, scientists have showed interest in developing the physicochemical characteristics of bacteriocins. A number of bioengineered strategies were conducted in order to increase the resistance of bacteriocins to proteolytic enzymes (**Birri et al., 2012; Wayah and philip, 2018**), improve their solubility and enhance their pH and temperature ranges' stability (**Rouse et al., 2012**).

3.1 Reduction of the temperature sensitivity

Some pediocin-like bacteriocins are characterized with an additional disulfide bridge in their C-terminal which gives them more potency towards target cells. Sakacin P unlike Pediocin PA-1 possesses only one disulfide bridge located in its N-terminal half. Fimland and co-workers (2000), using site-directed mutagenesis, produced some variants with an additional disulfide bridge in the C-terminal region (**Figure 7**). By that means two variants sak[N24C+44C] and

sak[G23T+N24C+44C] displayed a reduced temperature sensitivity than that of the wild type and had the same efficiency at 20 and 37°C as PA-1; temperatures at which sakacin P had low activity (Fimland et al., 2000).

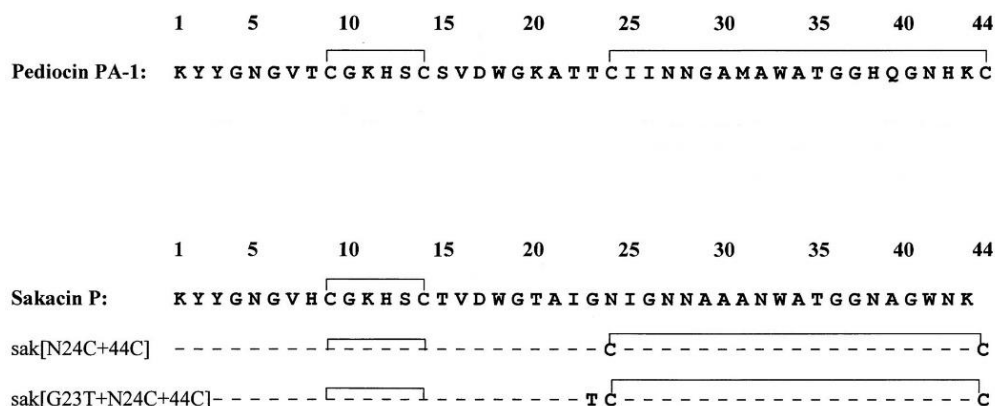


Figure 7 : Primary structure of pediocin PA-1, sakacin P and sakacin P variants. Adapted from Fimland et al., (2000).

3.2 Improvement of the solubility

The usage of bacteriocins in food-technologies requires their ability to diffuse through used matrices. Rouse and colleagues (2012) have generated some nisin variants by saturation mutagenesis of *nisA* gene mutated in the hinge region. The mutants were tested for antimicrobial activity with broth-based assays, agar- and carrageenan-based assays and carrageenan-containing chocolate milk product assessment. Variants SVA and NAK exhibited enhanced specific activity against *L. monocytogenes* UCC35 in carrageenan-containing chocolate milk. This was explained by an improved diffusion through the matrix (Rouse et al., 2012).

3.3 Improvement of the proteolytic resistance

Bacteriocins are proteinaceous antimicrobials and thus they are vulnerable to proteolysis by gut proteases (Waya and Philip, 2018; Kaya and Simsek, 2019). This may limit their use in some medical applications (O'Shea et al., 2010).

Salivaricin P is a class IIb bacteriocin. Its two peptides, Sln1 and Sln2, are sensitive to trypsin. O'Shea and collaborators (2010), using microwave-assisted solid phase peptide synthesis, designed a series of variants of the two peptides (Table 5). Amino acids which are sensitive to trypsin were substituted by other residues or followed by proline residues. Most of variants

acquired a trypsin-resistant phenotype but exhibited less antimicrobial activity than the parent bacteriocin against *L. innocua* DPC3572 (O'Shea et al., 2010).

Table 5 : Amino acid sequence, proteolysis phenotype and MIC₅₀ of salivaricin P components and their variants. Adapted from O'Shea et al., (2010).

Peptide	Amino acid sequence ^a	Proteolysis phenotype	MIC ₅₀ ^b (nM)
Sln1	K R GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	S	50
Sln1-1	R GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	S	80
Sln1-2	H GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	R	200
Sln1-3	KPH GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	R	100
Sln1-4	HRP GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	R	130
Sln1-5	KPR PNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	R	80
Sln1-6	K R GPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGLVGGALTCL	S	120
Sln2	K NGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH	S	50
Sln2-1	K NGYGGSGN H WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH	R	200
Sln2-2	KPNGYGGSGN H WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH	R	200
Sln2-3	H NGYGGSGN H WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH	R	120

^a Bold letters indicate inserted residues.

^b MIC₅₀ of the bacteriocin variant combined with the complementary wild-type peptide against *Listeria innocua* DPC3572.

3.4 Improvement of the oxidation resistance

Kaur and co-workers (2004) utilized a solid-phase peptide synthesis approach to synthesize a mutant of Pediocin PA-1 by substituting the methionine 31 by norleucine. The mutant acquired protection from oxidation, but had up to two-fold less activity than that of the parent PA-1 (Table 6) (Kaur et al., 2004).

Table 6 : Inhibitory activity of pediocin PA-1 and its variant M13Nle as a function of temperature. Adapted from Kaur et al., (2004).

	MIC (nM)			
	<i>L. innocua</i> ATCC33091		<i>C. divergens</i> LV13	
	25°C	37°C	25°C	37°C
PedPA-1	1.4	5.3	3.3	29.5
Ped[M31Nle]	2.7	7.7	4.6	20.9

The indicator strains used in the bacteriocin assay were *Listeria innocua* ATCC 33091 and *Carnobacteriocin divergens* LV1.

Chapitre 2 Bacteriocins and nanotechnology

1 Nanotechnology as an emerging tool for bacteriocin engineering

Nanotechnology is defined as the handling and manipulation of functional and structural materials in order to reshape them in a nanoscale size (Fahim et al., 2016; Lee et al., 2020). Given their diameters, usually varying from 1 to 100 nm, nanoparticles displayed impressive physicochemical and biological properties approving their use for different applications (Sidhu et al., 2019; Lee et al., 2020). Integration of nanotechnology in bacteriocin production contributed to the amelioration of their physicochemical properties, antimicrobial activity and applications in food industry and clinical field (Fahim et al., 2016; Sidhu et al., 2019).

Several nanotechnological approaches have been used according to their properties. **Table 7** summarises the most important advantages of these approaches.

Table 7 : Nanotechnological approaches and their advantages.

Nanotechnological approach	Advantages	References
Lipid-based nanoparticles E.g.: liposome	Non-toxic, biodegradable, applicable for encapsulating both hydrophilic and hydrophobic substances, biocompatible	Fahim et al., 2016
Carbohydrates-based nanoparticles E.g.: chitosan, alginate	Non-toxic, biodegradable, biocompatible, antimicrobial activity, drug delivery ability	
Metal-based nanoparticles E.g.: gold, silver	Large surface area, antimicrobial activity, highly stable	
Polymeric nanofibers E.g.: poly(ethyleneoxide), poly(D, L-lactide)	Large surface area, high physical stability, powerful encapsulation ability	
Layered double hydroxides E.g., MgAl-CO ₃ LDH	Large surface area, biocompatible, high anion exchange capacity, chemical stability, drug delivery ability	Fahim et al., 2017

2 Improvement of antimicrobial activity and spectrum

The combination of the antimicrobial property of some metallic nanoparticles with bacteriocins showed promising interest.

Mossallam and colleagues (2014) produced *Lb. acidophilus* CH1 bacteriocin-gold nanoparticle conjugates and tested their efficacy against the intestinal parasite *Enterocytozoon bieneusi* in immunosuppressed mice. These nanoconjugates exhibited the highest faecal spore load with 93.65% at the end of therapy and sustained active up to one week after the cessation of therapy with 94.26% efficiency. It is worth noting that the viability rate among spores encountered from stool of bacteriocin-gold nanoparticles group was 92.4%, however, they showed the least infectivity rate with 16.6% (Mossallam et al., 2014).

Sidhu and Nehra (2020) designed two groups of bacteriocin-capped silver nanoparticles (Bac-CSNPs), using two bacteriocins (Bac4463 and Ba22), both possessing different antimicrobial spectrum (Table 8). The nanoconjugates were tested for their AMA against six bacterial strains. Both bacteriocin nanoconjugates exhibited enhanced activity compared to that displayed by the parent bacteriocins against all the tested targets. Indeed, they both were active against strains on which wild bacteriocins are inactive i.e., *Shigella flexneri* and *E. coli* for Bac4463 and *L. monocytogenes* and *E. coli* for Bac22 (Table 8). Scanning electron microscopy revealed pore formation and cell deformation in *B. cereus* treated by Bac4463-CSNPs, whereas cell deformation and cell elongation were observed in *S. flexneri* exposed to Bac22-CSNPs (Sidhu and Nehra, 2020).

Table 8 : Antimicrobial activity of bacteriocin-capped silver nanoparticles and bacteriocins alone. Adapted from Sidhu and Nehra, 2020.

Bacterial strain	Mean zone of inhibition (mm)			
	Bac4463	Bac4463-CSNPs	Bac22	Bac22-CNSPs
<i>St. aureus</i>	24.6	30.3	14.6	21
<i>L. monocytogenes</i>	20	26.3	-	12.3
<i>P. aeruginosa</i>	14.6	19.3	18.3	26.6
<i>B. cereus</i>	20.6	27.6	12.6	21.3
<i>S. flexneri</i>	-	9.3	23	34.6
<i>E. coli</i>	-	11.3	-	13.3

P. aeruginosa: *Pseudomonas aeruginosa*; Bac4463-CSNPs: Bac4463-capped silver nanoparticles; Bac22-CSNPs: Bac22-capped silver nanoparticles.

Likewise, Gomaa (2019) had tested the efficiency of the bacteriocin produced by the probiotic strain *Lb. paracasei* conjugated with silver nanoparticles. Interestingly, the nanoconjugates displayed potent activity against multidrug resistant (MDR) bacteria isolated from wound swabs of patients (*St. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumonia*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Proteus mirabilis*). Measurements of protein and DNA concentration in the cell free supernatant proved the bactericidal effect of bacteriocin-silver nanoparticles conjugates (Gomaa, 2019).

3 Acquisition of anti-biofilm activity

Nanofibers of 2,3-dihydroxybenzoic acid (DHBA), a non-toxic siderophore, have showed their ability to inhibit the growth of a few number of Gram-positive and Gram-negative bacteria. Ahire and Dicks (2015) tested the effect of these nanofibers combined with nisin (NDF) on MRSA Xen31's biofilm formation. NDF treatment resulted in an 88%-decrease in biofilm formation after one day of exposure, a decrease in the number of viable cells in biofilm from \log_{10} 7.2 to \log_{10} 4.3 after 24h of exposure and an increase of planktonic cells from OD_{595nm} 0.03 to 0.02 after 24h of exposure. Moreover, NDF was potent in inhibiting the formation of *St. aureus* Xen31's biofilm when exposed to increased iron concentrations (Ahire and Dicks, 2015).

4 Improvement of stability

Layered double hydroxides (LDHs), anionic clay materials, are advantageous carriers by reason of their ability to intercalate neutral molecules and/or to exchange ions between the hydroxide layers. Fahim and colleagues (2017) designed three nanocomposites of LDH nanoparticles and avicin A, a pediocin-like bacteriocin: avicin A-MgAl-CO₃ LDH, avicin A-ZnAl-CO₃ LDH and avicin A-MgAl-NO₃ LDH. Therefore, avicin A-ZnAl-CO₃ LDH nanocomposites were the only to display a stability and retain around 6.14% of their initial antimicrobial activity after storage at room temperature for 24 days. Free avicin A showed decreased activity roughly lost after storage at room temperature and 4°C after 6 and 15 days of incubation, respectively. Nevertheless, no improvement in the antimicrobial activity was showed against the target cells tested (Fahim et al., 2017).

Although some food products undergone intense physicochemical treatments during their manufacturing, added food biopreservatives like nisin lost their activity. Temperature and pH are very crucial for the stability and activity of proteinaceous compounds. Taylor and collaborators (2007) have produced nisin encapsulated with liposomes nanoparticles prepared from distearoylphosphatidylcholine (PC) and distearoylphosphatidylglycerol (PG). The

encapsulation efficiency (EE) was measured for temperatures (25-60°C) and pH range of (5.5-11.0) as function of calcein entrapment. As a result, PC, PC/PG 8:2 and PC/PG 6:4 (mole ratio) liposome nanoparticles retained more than 60% EE after exposure to elevate temperatures and more than 70% EE after exposure to the pH range (**Taylor et al., 2007**).

Chapitre 3 Optimization of bacteriocin production

The production of bacteriocins is growth-associated and thus, it is influenced by various bacterial growth conditions such as pH, temperature, medium composition, and other factors like bacterial strain, phage infection and the neighbouring microorganisms (**An et al., 2017**). Moreover, the production level is usually very low and the purification process decreases more the yield (**Suganthi and Mohanasrinivasan, 2015; Jiang et al., 2016**). These issues may interfere with the study of bacteriocins and limit their production on large scale and usage (**Yildirim et al., 2007; Suganthi and Mohanasrinivasan, 2015**). For this reason, scientists showed more interest for the optimization of bacteriocin production.

1 Optimization of growth parameters

Bacteriocin production optimization involves well-determinate nutrients sources and physicochemical conditions (**Maldonado et al., 2003; Guerra et al., 2005**).

1.1 Culture medium

Commonly, bacteriocin production is achieved on conventional media such as de Man, Rogosa and Sharpe (MRS), Trypton Glucose Extract (TGE) and Trypticase Soy Broth (TSB) (**Kaktcham et al., 2019; Telke et al., 2019**). However, the composition of these media can highly interfere with the bacteriocin production (**Guerra et al., 2005**). The optimization processes tend to found the most effective nutrient sources. For instance, yeast extract showed its high suitability as nitrogen source (**Lee et al., 2012; Dyae and Luti, 2019**), skim milk was found to be a rich carbon source but it should be combined to trypton for an enhance production (**Telke et al., 2019**).

In addition to the carbon and nitrogen sources, the production of bacteriocins involves other ingredients. For example, the presence of NaCl was found to be crucial in the process (**Lee et al., 2012; Iyapparaj et al., 2013**) as well as minerals like MgSO₄ (**Lee et al., 2012**), NH₄Cr, CH₃COONa and K₂PO₄ (**Dyae and Luti, 2019**) and tween 80 (**Iyapparaj et al., 2013; Dyae and Luti, 2019**).

Although bacteriocin production is dependent to cell growth, it requires autonomous cultivation parameters e.g., temperature, pH and aeration (**Guerra et al., 2005**). In most studies, optimal growth conditions influence positively the bacteriocin production (**Telke et al., 2019**). However, it has been shown that non-optimum temperature and pH may trigger bacteriocin production (**Aasen et al., 2000**). Initial pH was also found to be an effective factor (**Lee et al.,**

2012). Additionally, some culture media can contribute in the regulation of pH (Sharma et al., 2010). Moreover, controlled aeration also showed its advantageous role in enhancing the garvicin KS production level (Telke et al., 2019).

1.2 Optimization techniques

Traditionally, optimization processes involve the one-factor-at-time (OFAT) method which consists of modifying one parameter at a time while keeping others settled. It is laborious, time-consuming and may lead to unsuitable conclusions (Suganthi and Mohanasrinivasan, 2015; Dyaee and Luti, 2019). Newly, statistical techniques are mostly used such as Plackett-Burman design (PBD) and response surface methodology (RSM). In the former, interactions between the factors are considered negligible (Embaby et al., 2014) while the latter respects both individual and interaction effects of several factors to improve the optimal process conditions (Suganthi and Mohanasrinivasan, 2015; Dyaee and Luti, 2019).

Lee and colleagues (2012) carried out a OFAT experiment to determine the optimum medium components and cultivation conditions for the production of the bacteriocin DF01 from *Lb. brevis* DF01. Glucose, yeast extract, MgSO₄, temperature and initial pH were the key determinants. Later, a fractional factorial design (FFD) revealed that glucose, yeast extract and initial pH were the most significant factors with positive effects. Finally, through a central composite design (CCD), a 14.56g/l concentration of yeast extract and 28.95g/l concentration of glucose at initial pH of 6.8 was found to be the optimum formula which increased the productivity by 4-fold (1280 AU/ml) (Lee et al., 2012). In Dyaee and Luti's trial (2019), glucose and yeast extract were also the most efficient factors for the production of the bacteriocin NH40 from *Lb. plantarum* NH40 with an increase of 8-fold (634.74 U/ml) (Dyaee and Luti, 2019).

The sequential statistical approach fulfilled by Embaby and collaborators (2014) resulted in 1.6-fold enhancement in bacteriocin YAS1 production. Firstly, nine factors (incubation time, pH, temperature, agitation speed, inoculum size, glycerol, glucose, starch, and yeast extract) were tested by PBD. Incubation time, agitation speed and yeast extract displayed positive impact while starch and glycerol negatively influenced the production. Secondly, RSM approach, through fifteen experimental trials, showed that an incubation of 62 hours under agitation speed of 207rpm and 0.48% (w/v) yeast extract was the determinant combination for an enhanced production of the bacteriocin from *Bacillus* sp. YAS1 strain (470AU/mL) (Embaby et al., 2014).

The production of the bacteriocin KC692718 from *P. pentosaceus* KC692718 was optimized up to 20-fold increased yield (25,600.34 AU/ml). At the first stage, a OFAT experiment yielded 2.5-fold and 4.7-fold increase with sucrose (2.4%) as carbon source and soyatone (1.03%) as nitrogen source, respectively. At the second stage, a CCD with thirty experiments, resulted in a 20-fold increased production with sucrose (2.4%), soyatone (1.03%), pH (5.5) and temperature (34.5°C) (**Suganthi and Mohanasrinivasan, 2015**).

Pandey and Malik (2019) carried out an optimization of the bacteriocin production from *Lb. gasserii* NBL 18 by only one RSM approach. A central composite rotatable design (CCRD) was adopted to test the four independent parameters: inoculation level (1-3%), incubation temperature (37-42°C), pH (4.0-8.0) and incubation time (6-24h) through thirty experiments. The empirical model of 37°C temperature, 8 pH, inoculation level of 3% and incubation time of 24 hours yielded in a maximum bacteriocin production of 2.56×10^4 AU/ml (**Pandey and Malik, 2019**).

2 Heterologous expression

Heterologous expression provides another tool to produce a bacteriocin by other microorganisms rather than the original strain. As a result of that, non-producing strains could acquire this novel phenotype, and thus, several limitations and difficulties related to the natural production could be solved (**Jimenez et al., 2013; Wayah and Philip, 2018**). The approach is based on the expression of the structural, transporter and sometimes even the immunity genes in a host organism (**Borrero et al., 2011; Jimenez et al., 2013**).

2.1 Factors that influence the production

Several factors are involved in the heterologous production of bacteriocins. The host strain, the expression vector (**Borrero et al., 2011; Jimenez et al., 2015**) and the promoter (**Borrero et al., 2011**) used to direct gene expression are the most critical parameters.

2.1.1 Effect of the host organism

Among the most used host organisms there are LAB (**Jimenez et al., 2015**), *E. coli* (**Mesa-Pereira, 2017**) and yeast such a *Kluyveromyces lactis* (**Jimenez et al., 2013**) and *Pichia pastoris* (**Jimenez et al., 2013; Hu et al., 2014**). **Table 9** shows that the production yield of enterocin A differs significantly from a producer host to another.

Table 9 : Heterologous expression of enterocin A.

Host organism	Production yield	References
<i>Lc. lactis</i>	Up to 18.5-fold	Borrero et al., 2011
<i>P. pastoris</i>	3-fold	Hu et al., 2014
<i>Lb. sakei</i>	2.7-fold	Jimenez et al., 2015
<i>Lb. casei</i>	4.9-fold	

2.1.2 Effect of gene dose

Leer and collaborators (1995) cloned the acidocin B-encoding gene in the high-copy-number *Lactobacillus* vector pLPE24M and in the low-copy-number vector pGKV21 (about 80 and 20 copies per bacterium, respectively). The expression in *Lb. plantarum* transformants displayed higher production yields with increase acidocin B-encoding gene dose (Leer et al., 1995).

2.1.3 Effect of induction system

The optimization depends also on the induction system. For example, the Nisin-Controlled gene Expression system (NICE system) resulted in a 7.8- to 18.5-fold increase in the production of enterocin A (Borrero et al., 2011). Isopropyl β -D-thiogalactopyranoside (IPTG) was found to be effective for divercin V41 production (Yildirim et al., 2007) and bactofencin A (Mesa-Pereira et al., 2017). However, lactose induction was significantly better than IPTG's for carnobacteriocin production optimization (Jasniewski et al., 2008).

2.1.4 Effect of secretion system

The fusion of a signal peptide to a putative bacteriocin gene may result in an increase of the production yield (Jimenez et al., 2013). Jasniewski and colleagues (2008) improved the production of carnobacteriocin with the chimeric gene of a thermostable thioredoxin fused to the bacteriocin gene (Jasniewski et al., 2008). Borrero and collaborators (2011) used signal peptides of the protein Usp45, the bacteriocin enterocin P and hiracin JM79 to enhance the production of enterocin A (Borrero et al., 2011).

2.1.5 Effect of production strategy

The production strategy has also proved its influence on the bacteriocin production. For example, batch and fed-batch production of divercin V41 has increased by 1.6- and 4.1-fold, respectively, compared to the shake flask cultivation (Yildirim et al., 2007).

2.2 Constructed expression systems

Ingham and collaborators (2005) have developed a versatile bacteriocin expression system adapted for *E. coli* which consists of a vector, named pSuV1, constructed by inserting a series of synthetic oligonucleotides downstream of the T7 promoter of the expression vector pTYB1 (Figure 8). The vector successfully exhibited the expression of active bacteriocins BacR1, pediocin PA-1, enterocin P and divercin V41 (Ingham et al., 2005).

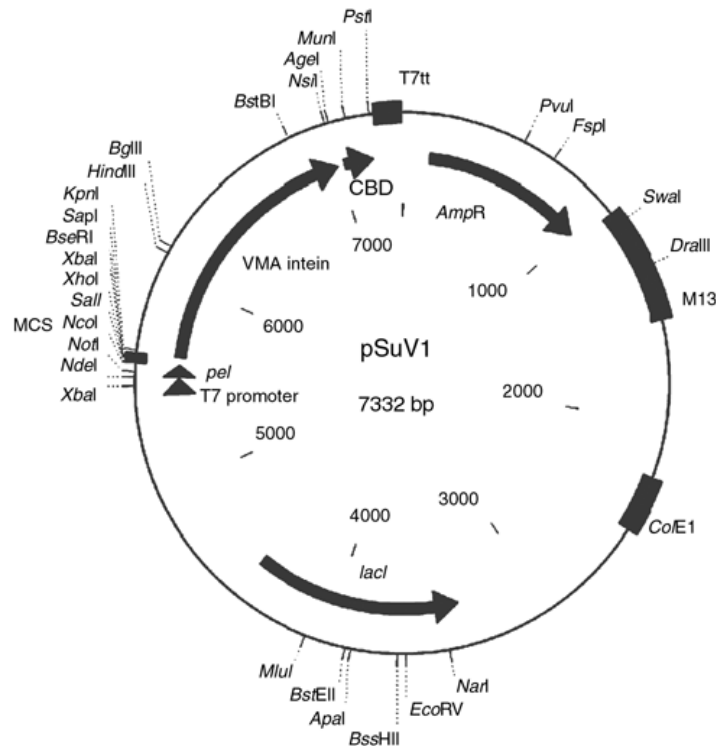


Figure 8 : Map of bacteriocin expression vector pSuV1. (T7promoter) IPTG-inducible T7 RNA polymerase promoter, (*pel*) peptate lyase section system, (VMA intein) the fusion partner open reading frames of the intein, (CBD) chitin-binding domain, (*AmpR*) ampicillin resistance gene, (*ColE1*) origin of replication, (*lacI*) lactose operon (Ingham et al., 2005).

3 Use of conjugation

Conjugation presents another tool for exogenous production. Piard and colleagues (1993) accomplished a 2-fold higher production of lacticin 481, a class I bacteriocin produced by *Lc. lactis subsp. lactis* CNRZ 481. The plasmid-borne lacticin 481 structural gene (*lct*) was transferred into the plasmid free strain *Lc. lactis subsp. lactis* IL1441. The resulted transconjugants were phenotypically positive for the lacticin 481 production. Moreover, the transconjugants possessed superior copy number of the structural gene than the wild strain. Plasmid analysis revealed that the derivative plasmid (120-130 KB) might be a dimer of the native plasmid (69Kb) (Piard et al., 1993).

CONCLUSION

This research review's purpose was to view the recent progress in the improvement of bacteriocin properties and production. Genetic characterization of bacteriocins has been of great importance for scientists to understand the gene-peptide primary structure relationship. Along with this, bioengineered bacteriocins were developed based on the data provided earlier. Specific amino acid modifications have been adopted when was thought to be determinant for the stability, solubility or antimicrobial activity of bacteriocins of interest. Additionally, synthetic and hybrid bacteriocins have also been shown to display improved properties. Moreover, numerous nanotechnologies approaches have been evolved and tailored to food and therapeutic applications of bacteriocins.

Production optimization has been performed through three lines of research (i) media composition (ii) cultivation conditions and (iii) producer organisms. It is clear from the reviewed research that statistical-based methods were potent than classical ones in the prediction of best formula for higher production. Furthermore, heterologous expression has allowed the expression of bacteriocins in other organisms rather than the parent producer, especially when the latter is prohibited. Therefore, several expression schemes have exhibited enhanced production yields.

It is important to conduct more studies on:

- ❖ Development of novel heterologous-expression systems that are amenable for a large-scale production.
- ❖ Investigation of the applicability of existing nanotechnological approaches to design nanoparticles with bacteriocin combinations.
- ❖ Sever toxicological studies of the modified bacteriocins and their potential use in the food and biomedical industries.

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Appendix

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Theme: Bacteriocins of lactic acid bacteria: recent advances and production optimization

Abstract

Due to increasing demands for natural antimicrobial agents, bacteriocins emerged as the proper alternative for chemical preservatives and some hurdle technologies. However, only few bacteriocins are available in the market because of several constraints including low yield, high recovery cost and instability during the food-processing stages. This review highlights the recent methods employed to improve the properties and antimicrobial activity of bacteriocins as well as the optimization of their production. Bioengineering and nanotechnology approaches displayed potent tools for improving the physicochemical properties and enhancing the antimicrobial activity and spectrum. Bacteriocin production optimization trials exhibited higher efficiency with statistical-based methods rather than classical ones. Heterologous expression is also potent to multiply the bacteriocin production by other organisms rather than the original producer strain.

Key words: Lactic acid bacteria, bacteriocin, production optimization, heterologous expression, bioengineering, nanotechnology

Résumé

Face à la demande croissante d'agents antimicrobiens naturels, les bactériocines sont apparues comme une alternative intéressante des conservateurs chimiques et de quelques techniques de la technologie des barrières. Cependant, peu de bactériocines sont disponibles sur le marché en raison de plusieurs contraintes, notamment le faible rendement, le coût élevé de purification et l'instabilité lors du processus de fabrication des aliments. Cette synthèse met en évidence les méthodes récentes adoptées pour améliorer les propriétés et les activités antimicrobiennes des bactériocines ainsi que l'optimisation de leur production. Les approches de bioingénierie et de nanotechnologie présentent des outils puissants pour améliorer les propriétés physicochimiques et amender l'activité et le spectre antimicrobiens. Les essais d'optimisation de la production de bactériocine sont plus efficaces avec les méthodes statistiques plutôt qu'avec les méthodes classiques. L'expression hétérologue est également opérationnelle pour augmenter la production par d'autres organismes différents de la souche productrice d'origine.

Mots clés : bactéries lactiques, bactériocine, optimisation de la production, expression hétérologue, bioingénierie, nanotechnologie

ملخص

في ظل الطلب المتزايد للمضادات الجرثومية الطبيعية، ظهرت البكتيريوسينات كبديل أمثل للمواد الحافظة الكيميائية ولبعض تقنيات تكنولوجيا العوائق. بالرغم من ذلك لا يتوفر منها إلا عدد قليل جدا في السوق بسبب عدة قيود من بينها المردود الضعيف، تكلفة الاستخلاص المرتفعة وعدم استقرار البكتيريوسينات خلال مراحل صنع الأغذية. تبرز هذه المراجعة أحدث الطرق المستخدمة في تحسين خصائص البكتيريوسينات ونشاطها المضاد للجراثيم وكذلك تحسين الإنتاج. تقدم نهج الهندسة الحيوية وتقنية النانو وسائل فعالة لتحسين الخصائص الفيزيوكيميائية وتعزيز النشاط المضاد للجراثيم ونطاق الفعالية. أبرزت تجارب تحسين الإنتاج بالطرق الإحصائية نجاعة أكبر من الطرق التقليدية. التعبير المغاير فعال أيضا لمضاعفة إنتاج البكتيريوسين باستخدام كائن مغاير للسلسلة الأصلية.

الكلمات المفتاحية: البكتيريا اللبنية، بكتيريوسين (مضاد جرثومي)، تحسين الإنتاج، تعبير مغاير، الهندسة الحيوية، تقنية النانو

