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REVIEW ARTICLE

Immobilization of Bacteriocins from Lactic Acid Bacteria and Possibilities for Application in Food Biopreservation

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Abstract: Bacteriocins are biologically active compounds produced by a large number of bacteria, including lactic acid bacteria (LAB), which exhibit antimicrobial activity against various saprophytic and pathogenic microorganisms. In recent decades, bacteriocins are increasingly becoming more important in different branches of the industry due to their broad antibacterial and antifungal spectrum - in the food industry for natural food preservation and expiry date extension; in the health sector for preparation of probiotic foods and beverages; in the clinical practice as alternatives of conventional antibiotics; in the agriculture as biocontrol agents of plant pathogens and alternatives of chemical pesticides for plant protection. The broad antimicrobial spectrum of bacteriocins has stimulated the research attention on their application mainly in the food industry as natural preservatives. Most scientific achievements concerning the application food biopreservation are related to bacteriocins produced by LAB. The lactic acid bacteria bacteriocins can be produced in the food substrate during its natural fermentation or can be added in the food products after obtaining by *in vitro* fermentations under optimal physical and chemical conditions. Moreover, the immobilization of LAB bacteriocins on different matrices of organic and inorganic origin has been proposed as an advanced approach in the natural food preservation for their specific antimicrobial activity, anti-biofilm properties and potential use as tools for pathogen detection.

Keywords: Bacteriocins, Lactic acid bacteria, Immobilization, Antimicrobial activity, Anti-biofilm properties.

1. INTRODUCTION

Lactic acid bacteria (LAB) are a large group of beneficial bacteria belonging to different taxonomic groups, but unified on the basis of their shared metabolic and physiological characteristics. Morphologically, they are Gram-positive, non-motile, non-spore forming cocci or rods, which produce lactic acid as the major metabolic end product of carbohydrate fermentation. Lactic acid bacteria also possess low proportions of guanine and cytosine (G+C) in their DNA molecule (<55%). Taxonomically, LAB belong to the bacterial genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* [1 - 4]. Lactic acid bacteria exhibit various beneficial properties as proteolytic activity, lactose and citrate fermentation, production of polysaccharides, high resistance to freezing and lyophilization, ability for adhesion and colonization of the digestive tract mucosa and production of substances with antimicrobial activity, which make them suitable for application as probiotics in human health [4].

Naturally LAB are associated with many different foods of animal and plant origin. They are found in milk and dairy products, meat, fish and sea products, fermented vegetables, wine, bread and baked products. They are generally recognized as safe (GRAS) bacteria widely applied as starter cultures for the production of a number of fermented milk and non-milk foods and beverages. The fermentation process and production of lactic acid contribute to improving of

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the organoleptic qualities, nutritional characteristics and textural profile of these food products. The rapid acidification caused by LAB through the production of lactic acid and other organic acids inhibits the growth of spoilage microorganisms, which leads to extension of the expiry date of fermented foods. Lactic acid bacteria are also known to produce other fermentation products and metabolites such as ethanol, aromatic compounds, exopolysaccharides, enzymes and bacteriocins associated to the food maturation, development of the sensory characteristics and biopreservation [1]. The health-promoting properties of some LAB strains and their beneficial effects on consumer's health, make them attractive for industrial application in the composition of functional foods and beverages [1, 5, 6].

The capacity to produce a broad spectrum of bacteriocins and bacteriocin-like substances (BLIS) was detected in all genera of LAB. These biologically active compounds are effective against competitive microorganisms, and thereby generate a selective advantage for their producers. Lactic acid bacteria bacteriocins are substances of great importance for application as natural food preservatives due to their antimicrobial effects on different microorganisms, including spoilage-related microflora and food-borne pathogens [7].

2. CLASSIFICATION OF LACTIC ACID BACTERIA BACTERIOCINS

Bacteriocins produced by LAB are small, ribosomally synthesized peptides that possess antimicrobial activity towards closely or distantly related microorganisms, whereas producer cells are immune to their own bacteriocin(s) [8]. In recent years, studies on the classification of bacteriocins synthesized by LAB are focused mainly on the biochemical properties and genetic characteristics of these biologically active compounds. The general proposals for the classification of bacteriocins produced by LAB are based on their chemical structure, molecular mass, mode of action and determination of susceptible strains.

Lactic acid bacteria bacteriocins, as defined by Klaenhammer [9] are divided into four main classes: class I - lantibiotics, small membrane-active peptides (<5 kDa) containing the unusual amino acids lanthionine, b-methyl lanthionine, and dehydrated residues - nisin, lacticin 481, carnocin U149, lactocin S; class II - small heat-stable, non-lanthionine-containing membrane-active peptides (<10 kDa). The mature bacteriocins are predicted to form amphiphilic helices with varying amounts of hydrophobicity, b-sheet structure, and moderate (100°C) to high (121°C) heat stability - pediocin PA-1, lactococcin A, B, M, leucocin A, sakacin A, curvacin A, sakacin P, and lactacin F. Three subgroups are defined within the class II bacteriocins: subclass IIa - *Listeria*-active peptides with a consensus sequence in the N-terminal of -Tyr-Gly-Asn-Gly-Val-Xaa-Cys-; characterized by pediocin PA-1, sakacin A, sakacin P, leucocin A and curvacin A; subclass IIb - poration complexes consisting of two proteinaceous peptides for activity - lactococcin G, lactococcin M and lactacin F; subclass IIc - thiol-activated peptides requiring reduced cysteine residues for activity - lactococcin B; class III - large heat-labile proteins (>30 kDa) - helveticin J, helveticin V-1829, acidophilucin A, lactacins A and B; class IV - complex bacteriocins, composed of protein plus one or more chemical moieties (lipid, carbohydrate) required for activity- plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1.

According to other classifications [8, 10] LAB bacteriocins are divided into three main groups: lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose inactive prepeptides are subject to extensive post-translational modification; peptide bacteriocins or small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocin-like or *Listeria*-active bacteriocins (subclass IIa), two-peptide bacteriocins (subclass IIb) and circular bacteriocins (subclass IIc), and bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III). Extensive efforts have been made to resolve the relationship between the chemical structures and function for both class I and class II bacteriocins. The majority of bacteriocins, belonging to the class I and class II are active in the nanomolar range, causing membrane permeabilization, leading to the dissipation of membrane potential and the leakage of ions, ATP and other vital molecules from the target microorganisms.

Some authors as Cotter *et al*. [11] suggested a different classification of bacteriocins produced by LAB, in which they are divided into two main classes- lantibiotics (class I) and not containing lanthionine lantibiotics (class II), while high molecular weight thermolabile peptides, which are formally components of the above class III would be separately designated as "bacteriolysins". The authors also suggested that the above class IV should be extinguished.

3. CHARACTERISTICS OF THE MAIN LACTIC ACID BACTERIA BACTERIOCINS

3.1. Nisin

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*. Nisin is a 3488-Da hydrophobic peptide consisting of 34 amino acid residues, five thioether crossbridges and three dehydrated residues formed from serine or

threonine. It can form dimers or oligomers with molecular mass of 7000 - 14000 Da, which are believed to arise by intermolecular reactions between the dehydroresidues of two or more nisin molecules [9].

Nisin belongs to class I bacteriocins (lantibiotics) and possesses strong antimicrobial activity against a variety of Gram-positive bacteria *i.e*. *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium* sp., *Bacillus* spp. and some LAB [9, 12, 13]. Nisin has been the first substance considered a GRAS in the USA, which exhibits antimicrobial activity against a broad spectrum of food-spoilage microorganisms and pathogenic bacteria. Nowadays, nisin is the only one LAB bacteriocin officially approved and licensed for industrial use as a food additive and biopreservative in over 46 countries worldwide [9], known also as E 234 [4].

The investigations on the mode of action showed that nisin reveals its effect after adhesion to the microorganism surface and incorporation into bacterial cytoplasmic membrane, where the pore formation leads to a loss of intracellular ions and disruption of the pH gradient and proton motive force [9].

The use of nisin in food processing and biopreservation showed some disadvantages because of its structural instability, loss of activity by interaction with food and cell matrixes and development of tolerant and resistant *Listeria* strains, which leads to the need of adding of excessive nisin amounts in food products in order to guarantee an effective pathogen bacteria inhibition. However, these important problems can be solved by the application of some advanced formulation approaches such as immobilization [13 - 16].

3.2. Plantaricins

Lactobacillus plantarum is known to synthesize at least six different bacteriocins, primarily produced as precursors containing a double glycine moiety. Plantaricins are peptides that inhibit a wide range of LAB including their natural competitor *Lb. plantarum* and members of other bacterial genera as *Pediococcus*, *Carnobacteria*, *Clostiridia* and *Propionobacteria*.

The plantaricins JK and EF are bacteriocins that act as synergetic peptides. They consist of approximately 30 to 40 amino acid residues in length and show little sequence similarity to any other plantaricins. These bacteriocins act with strict specificity and any other combination, except JK or EF leads to complete loss of synergy. Plantaricin S is a two-peptide system isolated from *Lb. plantarum* spp. used to ferment green olives. These two peptides are of 26 and 27 amino residues in length. Plantaricin S is considered to control the fermentation process and preserve the olives [17].

Another two-peptide bacteriocin also synthesized by *Lb. plantarum* is plantaricin W (PlW) consisting of two protein molecules - Plwa (comprising 29 amino acid residues) and Plwb (comprising 32 amino acid residues). These lantibiotics possess low individual antimicrobial activity, but acting synergistically when put in a mixture (ratio components 1:1) [18].

3.3. Pediocins, Leucocins, Enterocins, Sakacins and Carnocins

Some other bacteriocins synthesized by LAB also exhibited potential for use as food biopreservatives. Since there are difficulties using nisin in some food applications, the use of other bacteriocins has been well examined.

Pediococci are widely associated with the fermentation of meat and vegetables. Two species - *Pediococcus acidilactici* and *Pediococcus pentosaceus* have been found to produce pediocins which are effective against most LAB and numerous Gram-positive pathogens. The best studied bacteriocins are pediocin A and pediocin PA-1 (AcH) produced by *P. acidilactici* [9]. Pediocin PA-1 shares sequence similarities with other important antilisterial bacteriocins (sakacin A and P, leucocin A, and carnocins BM1 and B2) synthesized by LAB normally associated with meat, poultry and fish. These pediocins are peptides active against a broad range of Gram-positive bacteria such as *L. monocytogenes,Pediococcus* sp., *Leuconostoc mesenteroides* and *Enterococcus faecalis* [9, 19]. The most promising results in meats were obtained using pediocin PA-1 which reduces immediately the number of target microorganisms, but is not yet an officially approved food additive in some countries (USA). Used alone or in a combination with diacetate, pediocin PA-1 was active against the food-borne pathogen *L. monocytogenes* and *Lactobacillus curvatus*. According to some studies, pediocin PA-1 was less effective than nisin against *Lb. curvatus* in the model meat system, and neither preservative was effective when used in a commercially manufactured meat product. Pediocin PA-1 and enterocin (synthesized by *Enterococcus faecium*) inhibited effectively the growth of *L. monocytogenes*, while leucocin A (produced by *Leuconostoc* sp.) was active mainly against the spoilage microorganisms. It was proven that leucocin A, enterocins, sakacins and the carnocins can prolong the shelf life of fresh meat [20]. Sakacin A is a bacteriocin produced by *Lactobacillus sakei*, which possesses a significant spectrum of activity against numerous LAB and various Gram-

positive pathogens (*L. monocytogenes*). It should be noted that *Lb. curvatus* and *Lb. sakei* are facultatively heterofermentative LAB usually used in the composition of starter cultures for fermented foods and beverages, but sometimes they are recognized as spoilage microorganisms in vacuum-packed cooked sausages [21, 22].

4. APPLICATION OF LACTIC ACID BACTERIA BACTERIOCINS IN FOOD BIOPRESERVATION

The strategies of application of LAB bacteriocins for natural preservation in food systems are based on their beneficial properties on human heath: a) they are GRAS substances like nisin; b) they are non-toxic on eukaryotic cells; c) they become inactivated by digestive proteases and have insignificant influence on the normal gut microflora; d) they are usually thermostable and pH-tolerant; e) they possess a wide antimicrobial spectrum against many food spoilage and pathogenic bacteria; f) they demonstrate a bactericidal mode of action by acting on bacterial cytoplasmic membrane that does not lead to cross resistance to conventional antibiotics [4, 23, 24].

In recent decades, different advanced formats for application of LAB bacteriocins in food biopreservation have been developed: a) by inoculation of food with LAB - starter or protective cultures for *in situ* production of bacteriocins; b) use of previously fermented food by fermentative of a bacteriocin-producing strains as an ingredient in food processing; c) by addition of semi- or purified LAB bacteriocins [4]. *In situ* production is readily cost-effective provided that the bacteriocin producers are technologically suitable. Nisin-producing dairy starters have been designed to specifically inhibit *S. aureus* in acid-coagulated cheeses and *Clostridium tyrobutyricum* in semi-hard cheeses. Protective cultures, which do not contribute to the organoleptic properties of the food, have been mainly applied to enhance the hygienic quality of raw meat and fish products. The use of bacteriocins as ingredients or additives requires some new strategies for large scale production in suitable low-cost food-grade media. For example, lacticin 3147 and the enterocin AS-48 have been produced in whey-based media suitable as a dairy ingredient. The use of whey as a substrate is an attractive option because it also contributes to recycling a by-side product of the dairy industry [25].

Regardless of the way of application in food, the main goal of LAB bacteriocins is to inhibit the spoilage microorganisms and food-borne pathogenic bacteria during the food processing and storage. Moreover, the application of LAB bacteriocins in food biopreservation is important due to some other useful features: a) decreases the risk of food poisonings; b) decreases the risk of contamination with food-borne pathogens through the food chain; c) extends the shelf life of food products; d) reduces the intensity of physical treatments and protects the food during temperature abuse conditions; e) decreases economic losses caused by the food spoilage; f) reduces the adding of chemical preservatives and thus to avoid their harmful effects on human health; g) provides better preservation of the food nutrients, vitamins and improves the organoleptic properties of food; h) provides alternative preservation barriers for "novel" foods less acidic, with a lower salt content, and with a higher water content [24]. All of these beneficial properties make LAB bacteriocins suitable for industrial use and adequate to satisfy the increasing consumers' demands for fresh-tasting, safe, naturally preserved and minimally-processed food products with improved organoleptic and nutritional characteristics.

The immobilization of LAB bacteriocins as a modern technique can also find a wide application for development of bioactive food packaging. As mentioned above, some LAB bacteriocins demonstrate additive or synergistic effects when used in a combination with other bacteriocins and may be an attractive approach to avoid development of resistant strains of microorganisms. The increase of antimicrobial effects in foods can be reached also by their combination with some chemical preservatives and natural phenolic compounds. In this respect, thymol and carvacrol can facilitate the disruption of the outer membrane of Gram-negative pathogens enhancing the antimicrobial activity of bacteriocins [26]. The combination of LAB bacteriocins and physical treatments (high pressure processing or pulsed electric fields) also offers good opportunities for more effective preservation, providing an additional barrier to the resistant forms such as bacterial endospores. However, the effectiveness of LAB bacteriocins is closely related to the environmental conditions like pH, temperature, chemical composition as well as the food microflora [24, 25].

5. IMMOBILIZATION OF LACTIC ACID BACTERIA BACTERIOCINS AS AN ADVANCED APPROACH IN FOOD BIOPRESERVATION

It is clear that the use of bacteriocins as biopreservatives is limited by their loss of activity, therefore immobilization could overcome this problem by confining these antimicrobials in food grade materials [13, 14, 16], liposomes [27] and nanocarriers. For example, the incorporation of bacteriocins into the polymeric packaging leads to food active packaging solutions which are considered a strategy to assure not only a protective barrier but also a biopreservative function. In this respect, the commercial bacteriocins nisaplin and lacticin 3147 were adsorbed to

polyethylene/polyamide pouches. The best performances have been achieved by nisin and the resulting active packaging was useful for shelf life extension of sliced cheese and ham stored for three months, both at room temperature and under refrigeration [28]. In the field of packaging, glutaraldehyde up to 20% may be considered a suitable crosslinking agent for a polyvinyl alcohol (PVA) composite film and nisin. In this way the active packaging exhibits a synergistic effect against the tested bacterial strains of *S. aureus* and *Escherichia coli*. [29].

Entrapment of bacteriocins into liposomes might address the stability issue which is a big hurdle for their direct application in food. In addition, the encapsulation increases the nisin resistance to low- or high-pH and moderate heat treatments, maintaining its antimicrobial efficacy for controlling spoilage and pathogenic microorganisms in food [30]. Several types of liposomes can be defined according to structure and size of the vesicles while the encapsulation of bacteriocins is mainly achieved by thin-film hydration method [27]. Since bacteriocins can be confined to the aqueous phase and the liposome membranes, they might explain a complementary antimicrobial effect due to the release of the encapsulated nisin and desorption of the membrane immobilized nisin [31]. Liposomes obtained through the neutral phoshatidylcholine (PC) show a more entrapment efficiency than liposomes resulting from the anionic phosphatidylglycerol [32], therefore the recovery of PC from lecithin of soy, sunflower and egg yolk can guarantee an easy and safe process with low costs [33].

Biofilm formation is a serious problem for food processing device and packaging which needs specific materials with anti-adhesion and antimicrobial properties even if it is difficult finding these characteristics together. Immobilization of nisin on multi-walled carbon nanotubes (MWNTs) has been carried out to address this issue. The MWNT–nisin deposit film, obtained by using poly(ethylene glycol) (PEG1000) as a linker covalently attached to MWNTs and nisin, showed a 100-fold higher anti-biofilm property than the MWNT deposit film alone and a very good stability against leaching [34]. In this respect, the results confirmed that the antimicrobial activity was due to the immobilized nisin and not to the free one, which has been leached. In particular, the MWNT–nisin system displayed an antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *Bacillus subtilis* 7-fold higher in comparison to multi-walled carbon nanotubes without nisin. The antimicrobial activity was maintained for over 60 days at room temperature, therefore the MWNT–nisin system might be considered a stable, effective and relatively inexpensive antimicrobial material. The antibacterial activity of the surfaces can contribute to avoiding biofilm formation, which is a challenge during water purification processes. For this reason, the covalent attachment of antimicrobial peptides (AMPs) was carried out on reverse osmosis membrane surfaces by filtration and subsequent irradiation with UV light. The antimicrobial capacity of the immobilized system was tested on *Ps. aeruginosa* which viability on modified surfaces decreased up to 55% [35].

Bacteriocins are antimicrobial peptides displaying activity by binding to the surface of target cells, therefore may be used as potential detection agents or biosensors, for obtaining of immediate analytical results.

In this respect other AMPs as magainin-1, polymyxins B and E, cecropin A and melittin were confined as biological component of biosensors for the detection of *E. coli* O157:H7, *Salmonella typhimurium* and *Coxiella burnetii* [36]. Leucocin-A, a Class-IIa bacteriocin, and its C-terminal fragment immobilized on gold supports displayed a binding capacity for Gram-positive pathogens such as *L. monocytogenes*, *S. aureus* and *Enterococcus hirae* while the Gram-negative bacteria were not captured [37, 38]. Contrary to this, plantaricin 17C (Pln-17C) immobilized on the surface of silanized glass, demonstrated antilisterial activity and a binding capacity for a variety of Gram-negative bacteria, including *E. coli*. To date, this short antimicrobial peptide may be used alone or in combination with other AMPs in biosensors for pathogen detection or due its antimicrobial properties is useful for creating surface-coating materials [39]. Another important issue is to assess the microbiological quality of drinking water reducing the analysis time and for this reason a portable biosensor system with colicin S4 bacteriocin for fast and sensitive detection of *E. coli*. has been designed [40].

CONCLUSION

The recent studies on the immobilized LAB bacteriocins reported many advantages in comparison to the free ones. Therefore, these results support the scientific efforts focusing on LAB bacteriocins immobilization as an advanced approach in natural food preservation and improvement of food quality and safety.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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