Paper No.: 02

Paper Title: The Principles of the Food Processing & Preservation

Module No. : 30

Module Title: Preservation of Food using Bacteriocins

30.0 Introduction

Ever since the era of Louis Pasteur and Robert Koch, there hasbeen scientific recognition of an essential need to control detrimentalmicroorganisms in our environment. The discovery ofpenicillin by Alexander Fleming in 1929 opened the door for useof therapeutic antibiotics by the medical and veterinary communitiesto combat specific disease-causing organisms. Althoughtherapeutic antibiotics are prohibited for use in foods, the utilizationof antagonistic additives with preservative or antimicrobialproperties has since become a trademark approach in food safetyand preservation. In foods and beverages, addition of antimicrobialcompounds to processed products has become a traditionalweapon in the food preservation arsenal.Comprising a subgroup within the far larger body of commercialfood preservatives are the bacteriocins. Bacteriocins are producedby bacteria and possess antibiotic properties, but bacteriocinsare normally not termed antibiotics in order to avoid confusionand concern with therapeutic antibiotics that can potentiallyillicit allergic reactions in humans.

Bacteriocins differ from most therapeutic antibiotics in being proteinaceousand generally possessing a narrow specificity of actionagainst strains of the same or closely related species. Bacteriocins are ribosomally synthesized polypeptidespossessing bacteriocidal activity that are rapidly digested byproteases in the human digestive tract..Bacteriocins are a heterogenous group, characteristically selectedfor evaluation and use as specific antagonists against problematicbacteria; however, their effectiveness in foods can become limitedfor various reasons, and cost remains an issue impeding broaderuse of bacteriocins as food additives.

30.1 Ecology of Bacteriocins

On an evolutional basis, it appears that the ability to synthesizeone or more bacteriocins has been a highly advantageous characteristic.A clear opportunity for survival and proliferation of an organismcan be envisioned if it can eliminate a competing organ tense given the diversity of species and rapid growth of bacteria. Low-molecular-weight antibiotics (for example, tetracyclines),lytic agents, toxins, bacteriolytic enzymes, bacteriophage,and metabolic by-products, such as organic acids, hydrogenperoxide, and diacetyl, also function in a somewhat similar capacity,but nonetheless the capability to produce bacteriocins andproducer-cell immunity occurs abundantly in prokaryotes, botheubacteria and archaebacteria. Bacteriocins play a fundamentalrole in bacterial population dynamics even though the degree ofbacteriocin interactions is so complex at the ecological and evolutionarylevels in mixed populations (such as biofilms) that muchremains uncertain.

30.2 Classification of Bacteriocins

First discovered by Gratia in 1925, ñprincipe Vò was producedby 1 strain of E. coli against another culture of E. coli. The termñcolicineò was coined by Gratia and Fredericq (1946); ñbacteriocineòwas used by Jacob and others (1953) as a general term forhighly specific antibacterial proteins. The term colicin now impliesa bacteriocidal protein produced by varieties of E. coli andclosely related Enterobacteriaceae.

Bacteriocins (as colicins) were originally defined as bacteriocidalproteins characterized by lethal biosynthesis, a very narrowrange of activity, and adsorption to specific cell envelope receptors. Later, the recognized association ofbacteriocin biosynthesis with plasmids was added to the description.The definition has since been modified to incorporate theproperties of bacteriocins produced by gram-positive bacteria. Bacteriocins from gram-positive bacteriacommonly do not possess a specific

receptor for adsorption although exceptions exist, are mostfrequently of lower molecular weight than colicins, have a broaderrange of target bacteria with different modes of release and celltransport, and possess leader sequences cleaved during maturation.Today, bacteriocidal peptides or proteins produced by bacteriaare typically referred to as bacteriocins. Usually, to demonstratethe proteinaceous nature of a newly characterized bacteriocin,sensitivity to proteolytic enzymes such as trypsin, chymotrypsin,and pepsin is an expected demonstration. Evaluation for use as afood additive requires estimation of its heat resistance given thewidespread use of thermal processing in food production.

Over the years, several publications have reviewed colicins,bacteriocins, bacteriocins from LAB, and applications of specificbacteriocins. Most of the bacteriocins from LAB are cationic, hydrophobic,or amphiphilic molecules composed of 20 to 60 amino acid residues. These bacteriocins are commonlyclassified into 3 groups that also include bacteriocins from othergram-positive bacteria. Examples of bacteriocins from these 3 classes are summarizedin Table 1.

Table 1—Examples of bacteriocins

Bacteriocins	Producer
Class I-type A lantibiotics	
nisin	*Lactococcus lactis*
lactocin S	*Lactobacillus sake*
epidermin	*Staphylococcus epidermidis*
gallidermin	*Staphylococcus gallinarum*
lacticin 481	*L. lactis*
Class I-type B lantibiotics	
mersacidin	*Bacillus subtilis*
cinnamycin	*Streptomyces cinnamoneus*
ancovenin	*Streptomyces* ssp.
duramycin	*S. cinnamoneus*
actagardin	*Actinoplanes* ssp.
Class IIa	
pediocin PA-1/AcH	*Pediococcus acidilactici*
sakacin A	*L. sake*
sakacin P	*L. sake*
leucocin A-UAL 187	*Leuconostoc gelidum*
mesentericin Y105	*Leuconostoc mesenteroides*
enterocin A	*Enterococcus faecium*
divercin V41	*Carnobacterium divergens*
lactococcin MMFII	*L. lactis*
Class IIb	
lactococcin G	*L. lactis*
lactococcin M	*L. lactis*
lactacin F	*Lactobacillus johnsonii*
plantaricin A	*Lactobacillus plantarum*
plantaricin S	*L. plantarum*
plantaricin EF	*L. plantarum*
plantaricin JK	*L. plantarum*
Class IIc	
acidocin B	*Lactobacillus acidophilus*
carnobacteriocin A	*Carnobacterium piscicola*
divergicin A	*C. divergens*
enterocin P	*E. faecium*
enterocin B	*E. faecium*
Class III	
helveticin J	*Lactobacillus heleveticus*
helveticin V-1829	*L. helveticus*

- Lantibiotics (from lanthionine-containing antibiotic) are small (<5 kDa) peptides containing the unusual amino acids lanthionine(Lan), methyllanthionine (MeLan), dehydroalanine, and dehydrobutyrine.These bacteriocins are grouped in class I. Class I isfurther subdivided into type A and type B lantibiotics according tochemical structures and antimicrobial activities.
    - TypeA lantibiotics are elongated peptides with a net positive chargethat exert their activity through the formation of pores in bacterialmembranes.
    - Type B lantibiotics are smaller globular peptides andhave a negative or no net charge; antimicrobial activity is relatedto the inhibition of specific enzymes.

- Small (<10 kDa), heat-stable, non-lanthionine-containing peptidesare contained in class II. The largest group of bacteriocins inthis classification system, these peptides are divided into 3 subgroups.
    - Class IIa includes pediocin-like peptides having an N-terminalconsensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys. Thissubgroup has attracted much of the attention due to their anti- Listeria activity.
    - Class IIb containsbacteriocins requiring 2 different peptides for activity
    - ClassIIc contains the remaining peptides of the class, including sec-dependentsecreted bacteriocins.

- The class III bacteriocins are not as well characterized. This group houses large (>30 kDa) heat-labile proteins that are of lesserinterest to food scientists.

- A 4th class consisting of complex bacteriocinsthat require carbohydrate or lipid moieties for activity hasalso been suggested by some scientists; however, bacteriocinsin this class have not been characterized adequately at thebiochemical level to the extent that the definition of this class requiresadditional descriptive information.

Table 2 – Properties of some class I and class IIa bacteriocins

Bacteriocins	MW* (Da)	Properties
Class I		
lacticin 3147A	2847	Heat stable at 100 °C for 10 min at pH 5 or 90 °C for 10 min at pH 7.
lacticin 3147B	3322	Sensitive to trypsin, α-chymotrypsin, proteinase K, and pronase E, resistant to pepsin.
nisin	3488	Heat stable at 121 °C for prolonged heating at pH 2. Become less heat stable at pH 5-7. Sensitive to α-chymotrypsin, resistant to trypsin, elastase, carboxypeptidase A, pepsin, and erepsin.
plantaricin C	3500	Stable at room and low temperatures, heat stable at 100 °C for 60 min or 121 °C for 10 min. Most stable at acid and neutral pHs. Sensitive to pronase, trypsin, and α-chymotrypsin, resistant to pepsin, proteinase K, α-amylase, and lipase.
Class IIa		
bavaricin A	3500-4000	Heat stable at 100 °C for 60 min. Stable at pH 2.0 to 9.7. Sensitive to pepsin, trypsin, pronase E, proteinase K and chymotrypsin A_4, resistant to catalase.
lactococcin MMFII	4143	Heat stable at 70 °C for 30 min. Stable at pH 5 to 8. Sensitive to proteinase K, trypsin and papain, resistant to glucoamylase, lipase, α-amylase and lysozyme.
pediocin PA-1	4624	Stable at pH 4 to 6, becomes less stable as pH increases. Heat stable at 80 °C for 60 min or 100 °C for 10 min. Sensitive to trypsin, papain, ficin, α-chymotrypsin, protease IV, XIV, and XXIV, and proteinase K, resistant to phospholipase C, catalase, lysozyme, DNAses, RNAses, and lipase.
piscicolin 126	4416	Stable at pH 2 after 2-mo storage at 4 °C. Heat stable at 100 °C for 120 min at pH 2 to 3. Becomes less heat stable as pH increases. Sensitive to α-chymotrypsin, β-chymotrypsin, protease type I, XIV, XXIII, and trypsin, resistant to catalase, lipase, and lysozyme.

*MW = molecular weight

30.3 Mode of action

The antibiotic activity of bacteriocin from Gram positive bacteria is based on interactionwith bacterial membrane. Some of bacteriocins elaborated amphiphilic property generalizedmembrane disruption by pore formation. Lactic acid bacteria produce several types of poreforming peptides. Most of bacteriocins produced from lactic acid bacteria are bactericidalpeptides which act primarily by creating pores in the membrane of their target cells.Although the formation of pore is a general feature. The size, stability and conductivity of these pores differ considerably from bacteriocins to bacteriocins. The formation of porationcomplexes, causing an ionic imbalance and leakage of inorganic phosphate. Thesemechanisms rely upon stabilizing interactions between membrane phospholipids and thecationic residues of the peptides allowing the insertion of hydrophobic regions into theouter leaflet of the membrane. One associated with the membrane surface a number of theordered bacteriocins could potentially aggregate.The bacteriocin complex can in principle completely span the membrane thereby forming atransient pore. In which there is dissipation of protonmotive force (PMF), which involves the partial or total dissipation of either or both thetransmembrane potential and a pH gradient.Anyway most bacteriocin interacts with anionic lipids that are abundantly present in themembrane of Gram Positive Bacteria. Theses anionic lipids may enhance the conductivityand stability of antibiotic pores by as docking molecule or may acts as receptors in class IIbacteriocins.

30.4 Medical significance

Bacteriocins are of interest in medicine because they are made by non-pathogenic bacteria that normally colonize the human body. Loss of these harmless bacteria following antibiotic use may allow opportunistic pathogenic bacteria to invade the human body Bacteriocins have also been suggested as a cancer treatment. They have shown distinct promise as a diagnostic agent for some cancers, but their status as a form of therapy remains experimental and outside the main thread of cancer research. Partly this is due to questions about their mechanism of action and the presumption that anti-bacterial agents have no obvious connection to killing mammalian tumor cells. Some of these questions have been addressed, at least in part.Bacteriocins were tested as AIDS drugs around 1990, but did not progress beyond in-vitro tests on cell lines.Bacteriocins can target individual bacterial species, or provide broad-spectrum killing of many microbes. As with today's antibiotics, bacteria can evolve to resist bacteriocins. However, they can be bioengineered to regain their effectiveness. Further, they could be produced in the body by intentionally introduced beneficial bacteria, as some probiotics do.

30.5 Production

There are many ways to demonstrate bacteriocin production, depending on the sensitivity and labor intensiveness desired. To demonstrate their production, technicians stab inoculate multiple strains on separate multiple nutrient agar Petri dishes, incubate at 30 °C for 24 h., overlay each plate with one of the strains (in soft agar), incubate again at 30 °C for 24 h. After this process, the presence of bacteriocins can be inferred if there are zones of growth inhibition around stabs. This is the simplest and least sensitive way. It will often mistake phage for bacteriocins. Some methods prompt production with UV radiation,Mitomycin C, or heat shock. UV radiation and Mitomycin C are used because the DNA damage they produce stimulates theSOS response. Cross streaking may be substituted for lawns. Similarly, production in broth may be followed by dripping the broth on a nascent bacterial lawn, or even filtering it. Precipitation (ammonium sulfate) and some purification (e.g. column orHPLC) may help exclude lysogenic and lytic phage from the assay.

30.6 Application of bacteriocin in food preservation & other food applications

The principle physical,chemical, enzymatic and microbiological reactions responsible for food deterioration arewell known. Various preservation techniques to avoid different forms of spoilage and foodpoisoning, including reduction in temp, water activity and pH as well as addition of preservatives such as, antimycotic, inorganic and organic compounds are known to slow orprevent growth of microorganisms. Nisin, the bacteriocin produced byLactococcus lactis subsp. Lactishas been applied as foodpreservatives in several countries. It has been used to control some food borne pathogens,especially some species of the generaAeromonas, Bacillus, Clostridium, Enterococcus,Listeria, Micrococcus, and Staphylococcus. The potential application of bacteriocins is consumer friendly. Bio-preservatives either the form of protective culture or as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agents, lactic acid bacteria and their by products have been shown to be more effective and flexible in several applications. In addition of that, functional properties in lactic acid bacteria improve preservatives effect and add flavor and taste.

Consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are drawn to natural and ñfresherò foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally processed foods with long shelf life and convenience, has stimulated research interest in finding natural but effective preservatives.

Bacteriocins, produced by LAB, may be considered natural preservatives or bio-preservatives that fulfill these requirements. Bio-preservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life. Three approaches are commonly used in the application of bacteriocins for bio-preservation of foods:

- Inoculation of food with LAB that produce bacteriocin in the products. The ability of the LAB to grow and produce bacteriocin in the products is crucial for its successful use.
- Addition of purified or semi-purified bacteriocins as food preservatives.
- Use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing

30.7 Hurdle technology to enhance food safety

The major functional limitations for the application of bacteriocinsin foods are their relatively narrow activity spectra and moderateantibacterial effects. Moreover, they are generally not activeagainst gram-negative bacteria. To overcome these limitations,more and more researchers use the concept of hurdle technologyto improve shelflife and enhance food safety (Table 3). It iswell documented that gram-negative bacteria become sensitive tobacteriocins if the permeability barrier properties of their outermembrane are impaired. For example, chelating agents, such asEDTA, can bind magnesium irons from the lipopolysaccharidelayer and disrupt the outer membrane of gram-negative bacteria,thus allowing nisin to gain access to the cytoplasmic membrane.

Table 3 – Hurdle Technology to enhance food safety

Bacteriocins	Inactivation effects
In combination with heat	
nisin	Nisin (1000 IU/g) enhances inactivation of *Listeria monocytogenes* in lobster by mild heat (60 or 65 °C).
nisin	Nisin (500 to 2500 IU/ml) enhances inactivation of *Salmonella* Enteritidis by mild heat (55 °C).
nisin, pediocin AcH	Both bacteriocins reduced the viability of gram-negative and gram-positive bacterial cells surviving sublethal stresses.
In combination with chelating agents	
nisin	When used with EDTA, citrate, or lactate, nisin (2000 IU/ml) is effective against gram-negative bacteria (*Salmonella* Typhimurium and *E. coli* O157:H7). in combination with modified atmosphere packaging (MAP)
nisin	When used with MAP and low temperature, nisin at a level of 400 IU/ml increases the lag phase of *L. monocytogenes,* and at 1250 IU/ml prevents its growth.
nisin	Combined use of MAP (100% CO_2, 80% CO_2 + 20% air) and nisin (1000 or 10000 IU/ml) inhibits growth of *L. monocytogenes* and *Pseudomonas fragi.*
In combination with antimicrobials	
nisin	The combined use of potassium sorbate (0.3%) and nisin (400 IU/ml) inhibited the growth of *L. monocytogenes.*
pediocin AcH	Synergistic effects between sodium diacetate (0.3 and 0.5%) and pediocin (5000 AU/ml) against *L. monocytogenes.*
nisin	Synergist effect between sucrose fatty acid esters and nisin on inhibition of gram-positive bacteria.
nisin	Carbon dioxide and nisin act synergistically against *L. monocytogenes.*
nisin	When combined with carvacrol (0.3 mmol /l), nisin (6 IU/ml) is more effective in reducing the counts of *Bacillus cereus* than when it is applied alone.
nisin	Nisin (100 IU/ml) and monolaurin (0.25 mg/l) act synergistically against *Bacillus* sp. vegetative cells in milk.
In combination with lactoperoxidase system	
nisin	A synergistic and lasting bactericidal effect on *L. monocytogenes* between nisin (100 or 200 IU/ml) and lactoperoxidase system.
nisin	Synergistic effect of nisin (10 or 100 IU/ml) and the lactoperoxidase system on inactivation of *L. monocytogenes* in skim milk.
In combination with other bacteriocins	
pediocin AcH	When used with nisin, lacticin 481, or lactacin F, pediocin AcH produced synergistic effects.
leucocin F10	In combination with nisin, leucocin F10 provides greater activity against *L. monocytogenes*.
curvaticin	Simultaneous or sequential additions of nisin (50 IU/ml) and curvaticin 13 (160 AU/ml) induces a greater inhibitory effect against *L. monocytogenes* than the use of a single bacteriocin.

30.8 Bacteriocins in packaging film

Incorporation of bacteriocins into packaging films to control food spoilage and pathogenic organisms has been an area of active research for the last decade. Antimicrobial packaging filmprevents microbial growth on food surface by direct contact of the package with the surface of foods, such as meats and cheese. For this reason, for it to work, the antimicrobial packaging filmmust contact the surface of the food so that bacteriocins can diffuse to the surface. The gradual release of bacteriocins from a packaging film to the food surface may have an advantage overdipping and spraying foods with bacteriocins. In the latter processes, antimicrobial activity may be lost or reduced due to inactivation of the bacteriocins by food components or dilution belowactive concentration due to migration into the foods. Two methods have been commonly used to prepare packaging films with bacteriocins. One is to incorporate bacteriocins directly into polymers. Examples include incorporation of nisin into biodegradable protein films. Two packaging film-forming methods, heat-press and casting, were used to incorporate nisin into films made from soy protein and corn zein in this study. Both cast and heat-press films formed excellent films and inhibited the growth of L. plantarum. Compared to the heat-press films, the cast filmsexhibited larger inhibitory zones when the same levels of nisin were incorporated. Incorporation of EDTA into the films increased the inhibitory effect of nisin against E. coli.

30.9 Conclusion

Bacteriocins represent one of the best-studied microbial defense systems. Althoughwe are still in the earliest stages of exploring their evolutionary relationships andecological roles, it is clear from their abundance and diversity that they are themicrobial weapons of choice. Sorting out why they are such a successful familyof toxins will require a substantial commitment to future research. It is expected that a better and deeper understanding of the molecular basis of the antimicrobial activity of bacteriocins will definitively result in safer food in the near future. Following a knowledge-based approach, new bio-preservation strategies as well as unique biotechnological applications of these natural antimicrobials are envisaged.