BIO-BASED ANTIMICROBIAL AGENTS TO IMPROVE AGRICULTURAL AND FOOD SAFETY

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Bio-Based Antimicrobial Agents to Improve Agricultural and Food Safety

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CONTENTS

FOREWORD

The use of chemical preservatives and pesticides in agriculture and food has long been a worldwide concern since it has been shown over and over again to result in food safety issues detrimental to human health and the environment. Thus, innovative modern food safety and preservation tools, especially natural bio-safe ways, are being investigated to reduce food contamination and spoilage and extend the shelf-life of food. Recently, the discovery and application of new bio-based antimicrobial agents have been the focus of many researchers, healthcare professionals, farmers, and agricultural companies to satisfy the consumers' demand for agricultural quality and food safety. This book brings together updated, innovative technologies of antimicrobial agents originally from microorganisms and plants, as well as their application in agriculture and food. Some of the technologies presented in the book are still emerging and will be of great benefit to researchers, healthcare professionals, farmers, and agricultural companies interested in staying on the forefront of innovative bio-based antimicrobial agents to improve agriculture and food safety. It also presents some practical guided approaches that could assist in the improvement of microbial strains and food quality.

Y. Martin Lo
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PREFACE

Food safety includes many approaches, starting from crop plantation, animal feeding, and food processing to storage, which involve microbial contamination control, quality assurance, and preservation. However, the microbiological risks in food are still one of the main sources of foodborne illnesses. In addition, the greatest losses in the food industry are attributed to microbial contamination, which dramatically affects the shelf life of food. Meanwhile, many plant pathogens influence the production of crops. Specifically, pathogenic fungi as one of the major production constraints, not only reduce crop production but also produce mycotoxins.

Nowadays, lasting exposure to chemical preservatives and pesticide residues in plants and food results in serious health impacts that are becoming global concerns. Therefore, modern safety and preservation tools, especially natural bio-safe ways, are being investigated to reduce food contamination and spoilage and to extend the shelf-life of food. This public perception has generated heightened interest in "biopreservation and biocontrol" in terms of the use of biologically producing compounds that possess a broad spectrum of antimicrobial activities as natural preservatives. Recently, the discovery and application of new bio-based antimicrobial agents have been focused on by many researchers, healthcare professionals, farmers, and agricultural companies to satisfy the consumers' demand for agricultural quality and food safety.

The book titled "**Bio-Based Antimicrobial Agents to Improve Agricultural and Food Safety**" aims to bring together the most recent progress in the development and application of novel antimicrobial agents, such as lipopeptides (surfactin, fengycin, bacillomyci, and brevibacillin) from *Bacillus,* bacteriocins from lactic acid bacteria and others. Biopreservation and biocontrol by the new antimicrobial agent are introduced, and new biopreservation tools to improve agricultural production and food safety are discussed in this volume. This book is mainly fruitful for biotechnologists, microbiologists, food scientists, food industrial companies, and also any reader interested in recent progress in the field of new preservatives and biopreservation methods. Our volume contains eight chapters prepared by outstanding authors from China, the USA, and the UK.

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CHAPTER 1

Surfactin Biosynthesis and its Potential Applications in Agriculture and Food System

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Abstract: Surfactin is a biosurfactant of the lipopeptide-type that has excellent physicochemical properties and biological activity. However, surfactin's high cost and low productivity of the wild strains restrict its large-scale manufacturing and application. Hence, numerous engineered bacteria have been utilized to boost surfactin biosynthesis. The current review includes information on the structure, physicochemical properties, and antibacterial mechanism of surfactin. This article also summarizes the regulatory network of surfactin biosynthesis, the molecular modification strategies, and the major function of surfactin, as well as its applications in agriculture, livestock, aquaculture and the food field. Finally, future prospects for surfactin research are discussed.

Keywords: Application, Biosynthesis, Genetic engineering strategies, Productivity of improvement, Surfactin.

INTRODUCTION

Surfactin is a cyclic lipopeptide generated from *Bacillus subtilis* secondary metabolites, which works as a biosurfactant to lower surface tension. The ability of surfactin synthesis is broadly dispersed among *B. subtilis* strains, as well as *B. licheniformis* and *B. amyloliquefaciens* strains [1]. Kluge *et al*. (1988) hypothesized a non-ribosomal mechanism catalyzed by multi-enzymatic thiotemplates comprising the surfactin synthetase in their study on surfactin biosynthesis [2]. Surfactin has anti-bacterial, anti-fungal, anti-viral, anti-cancer, anti-mycoplasma, anti-inflammatory, thrombolytic and hemolytic action, which is of particular importance given the interest in the creation of new peptide antibiotics [1, 3 - 9].

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Zhaoxin Lu & Yingjian Lu (Eds.)

Surfactin has a wide range of applications in petroleum recovery, biological pesticide production, cosmetics research and development, food processing, and pharmaceuticals, among others. According to the current notion of green sustainable development, chemically manufactured surfactants can be replaced by surfactin [10 - 12]. Surfactin has been examined extensively by researchers and experts since its discovery. To breed high-yielding strains and generate unique molecular structures of surfactin, researchers used physical, chemical, and genetic engineering strategies [13, 14]. Furthermore, improvement of the fermentation method for increasing surfactin yield has been extensively researched [15 - 21]. Surfactin synthesis, as well as its function and application, is increasingly concerned with rational genetic engineering of strains.

Surfactin transcriptional structural characterization and bacteriostatic mechanism are discussed in this review. In addition, the genetically modified method and novel molecular structure of surfactin are summarized briefly, as well as its function and application in agricultural production, livestock, aquaculture, and food field. This provides a better reference for further exploring and industrialization of surfactin.

STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES OF SURFACTIN

Surfactin, a 1036 Da amphipathic cyclic lipopeptide, is made up of a heptapeptide with the chiral sequence LLDLLDL connected with β-hydroxy fatty acid with a chain length of 12 to 16 carbon atoms to form a cyclic lactone ring structure. In addition, the carboxyl group of β-hydroxy fatty acid is linked to the N-terminus of the 7th amino acid of the peptide backbone by an amide bond, and a lactone bond connects the hydroxy group to the C-terminal carboxyl group of the heptapeptide, generating a closed ring structure [22 - 25]. L-Glu^{1}-L-Leu^{2}-D-Leu^{3}-L-Val^{4}-L-Asp^{5}-D-Leu^{6}-L-Leu^{7} is a typical chiral sequence of surfactin's heptapeptide-ring. The hydrophobic group of surfactin is attached to hydrophobic amino acid residues at positions 2, 3, 4, 6 and 7, as well as a length chain of β-hydroxy fatty acid. The heptapeptide's hydrophilic group is formed by the cyclic backbone and the amino acids at positions 1 and 5 (which introduce two negative charges to the molecule) [26, 27]. In addition, the amino acids at positions 2, 4, and 7 are highly variable, allowing them to undergo a variety of changes [28 - 31]. Natural surfactin is a combination of isoforms A, B, C and D obtained from the production strain with varied physiological properties [32]. Many surfactin variations and homologs with different fatty acid chain lengths, types, and positions of amino acids have been identified [30, 33 - 36].

Bonmatin *et al*. (1994) analyzed the three-dimensional configuration of surfactin using high-resolution two-dimensional nuclear magnetic resonance (^{1}H NMR) and molecular imaging techniques [37]. As a result, surfactin exists in the aqueous phase and at the water/air interface as a *β*-sheet structure with a characteristic horse-saddle conformation [37]. Residues 2 and 6 face each other on one side of the molecule, between the acidic Glu-1 and Asp-5 side chains, which define a minor polar domain [38]. On the opposite side, residue 4 faces the lipidic chain, which forms a large hydrophobic domain and incorporates the side-chains of residues 3 and 7 to a lesser extent, explaining its amphiphilic character and strong surfactant properties [39]. This conformation causes negatively charged amino acid residues on the ring to form potential divalent cation-binding holes. The fatty acid chain is fully extended on the other side of the ring, allowing it to participate in micelle production or penetrate the phospholipid bilayer [40]. Tsan *et al*. (2007) exploited NMR technology to characterize the structure of surfactin in polar and non-polar environments, obtaining a low-energy, stable 3D horse-saddle conformation, which differs from Bonmatin's [39]. In this conformation, the molecule residues 1 and 5 are located on the same side of the molecule, and the remainder of non-polar amino acids are on the other side except for the molecule residue 4 [39].

Surfactants with different chemical structures have different critical micelle concentration (CMC) values and self-aggregation forms, including micellar, hexagonal, cubic, and lamellar [31]. The self-aggregation structure of surfactants is also associated with intermolecular electrostatic interactions, ionic strength, polarity, and temperature of the solvent [41]. Surfactin is a powerful surfactant with a large molecular weight and complex conformation that can drop water surface tension from 72 $mN.m^{-1}$ to 27 $mN.m^{-1}$ at a concentration as low as 10 μM, far below the critical micelle concentration in water and about two orders of magnitude lower than most other detergents [11, 17]. Surfactin has the ability to form rod-like micelles with an aggregation number of ~170 [42]. Surfactin can also reduce the interfacial tension of water/dodecane from 52 mN m^{-1} to 2.45 mN m^{-1} at concentrations comparable to CMC [43].

ANTIBACTERIAL MECHANISM

Surfactin-membrane Interaction

Surfactin possesses considerable antibacterial effects on both gram-positive and gram-negative bacteria, and it is difficult to develop drug resistance due to its molecular structure. Surfactin's antibacterial mechanism is intimately linked to its physio-chemical properties, however, it has yet to be clarified. The antimicrobial peptide surfactin must first be attracted to the surfaces of the target bacterial cell

CHAPTER 2

Bacillomycin Production and Its Applications in Controlling Fungi and Mycotoxin in Agriculture and Food Systems

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Abstract: Fungi and mycotoxin contamination is one of the major concerns in agriculture as well as the food system, therefore, searching for environmentally friendly and efficient biogenic fungicides has become the path to ensure food safety. Bacillomycin is a new type of cyclic lipopeptide biogenic fungicide secreted by *Bacillus* sp. It not only has a strong antifungal function but also has the characteristics of green safety, high stability, and drug resistance. This chapter describes the structural types, biosynthesis and regulation, and culture optimization of bacillomycin in detail and introduces its applications in the protection of plant diseases and green preservation of fruits, vegetables, aquatic products, and cereal products.

Keywords: Anti-fungi, Agriculture and food system, Bacillomycin, Mycotoxins.

INTRODUCTION

Fungi have a crucial role to play in the decisions about food waste and food loss all over the world. Over 25% of fruits and vegetables are wasted mainly due to fungal contamination [1]. Cereals are also often contaminated with various microorganisms, especially fungi, while harvesting, transporting, storing, or distributing [2, 3]. Furthermore, certain fungi often produce toxic secondary products, mycotoxins, in the process of food contamination [4]. Mycotoxins can pose a serious threat to human health due to their carcinogenic, mutagenic, and genotoxic activities [5]. They can have deleterious effects even at low concentrations and have a wide range of toxicity [6]. Therefore, exploring more effective methods of fungal inhibition and mycotoxin reduction is a major concern

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Zhaoxin Lu & Yingjian Lu (Eds.)

for the agricultural and food industries. With the growing awareness of food safety, the commonly used chemical biocides are not meeting consumer concerns about food safety and quality due to possible toxicological risks and environmental contamination. At present, the microbicidal action of beneficial microorganisms and their active secondary metabolites is regarded as the most promising option in research and practice. Among them, nisin, a natural antimicrobial peptide produced by *Lactococcus lactis*, has been approved by the Food and Drug Administration as a GRAS-status food additive and is widely used in the storage and sterilization of food [7]. Therefore, it is necessary to develop effective biocides to prevent food spoilage caused by microorganisms and environmental pollution caused by chemical agents [8 - 10].

Bacillomycin, which belongs to the family of antimicrobial lipopeptides iturins, is mainly secreted by *Bacillus* spp. through the non-ribosomal peptide (NRPS) pathway [11]. Back in 1948, Landy *et al*. identified an antibiotic that showed spectral similarity in many respects to a concentrate of eumycin, but the limited available data on the chemical properties of eumycin and its specific action on *Corynebacterium diphtheriae* and acid-fast bacilli could distinguish it from this antibiotic [12]. They subsequently named this antibiotic substance as bacillomycin. Since then, the structure and properties of bacillomycin have been further investigated [11, 13 - 21]. Bacillomycin is characterized as an amphiphilic substance with hydrophilic amino acids and hydrophobic fatty acid chains, which can yield additional functions as an amphiphilic antibiotic, including antimicrobial, antiviral, anti-inflammatory, and anticancer activities [22, 23]. Therefore, it could be widely used in food storage, agricultural biological control, and medicine.

Bacillomycin has strong antifungal activity and also possesses low toxicity and environmental friendliness. It is considered as a potential natural bio-fungicide by inhibiting *Rhizopus stolonifer* of cherry tomatoes to prevent their spoilage [24]. In addition, it is also used for the control of fungi in crops and grains and the elimination of mycotoxins. Bacillomycin has been shown to inhibit contamination by *Fusarium graminearum*, *Aspergillus flavus*, and *Aspergillus ochraceus* in corn, wheat, oats, and rice, as well as to reduce the levels of mycotoxins such as deoxynivalenol, aflatoxin and ochratoxin [25 - 27].

In general, the inhibitory effect of bacillomycin is mainly to interact with the membrane to form a pore, which then destabilizes the microorganism internally, but of course, this is also related to the structure and type of bacillomyicn [27]. For example, C_{15}-bacillomycin D can effectively inhibit *Staphylococcus* species, including *Staphylococcus epidermidis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus*, while C_{14}-bacillomycin D has almost no effect on

Staphylococcus [28]. In addition, the low yield and the short validity of bacillomycin pose a challenge to its application in food preservation and agricultural control. Currently, there are various strategies to obtain higher yields through gene regulation, optimization of fermentation parameters, and addition of metal particles [29 - 32]. Sun *et al*. obtained a higher yield of bacillomycin D by knocking out the gene of *rapC*; however, due to the complexity of its regulatory mechanism, this approach is time-consuming [33]. The screening of fermentation substrates and optimization of fermentation parameters can be used to increase the yield of bacillomycin in a simple and efficient manner. Our previous study showed that the addition of inulin and L-Gln significantly increased the yield of bacillomycin D. Subsequently, the yield of bacillomycin D was up to 1.93 g/L using fed-batch fermentation [30].

Considering the unique properties of bacillomycin, this chapter systematically summarized the molecular structure and types, biosynthesis, production, and its applications in controlling fungi and mycotoxin in agricultural and food systems.

BACILLOMYCIN

Types and Molecular Structure of Bacillomycin

Bacillomycin is a class of lipopeptides of the Iturin family, first discovered and named by Landy *et al*. from *Bacillus subtilis* in 1948 [12]. Its variants mainly include bacillomycin D, bacillomycin F, bacillomycin L, bacillomycin Lc, bacillomycin Ls and bacillomycin Lb [16 - 19, 34 - 36] (Fig. **1**). Their molecules are composed of fatty acid of chain lengths from C_{14} to C_{17} and seven peptides acylated with β- amino acids [37]. Their differences lie in amino acid composition, amino acid position, and fatty acid chain length [13]. The precursor of the fatty acid chain can be normal, exclusive, or reversed, so each bacillomycin has its isomers. As previously mentioned for bacillomycin L, bacillomycin Lb, bacillomycin Lc, and bacillomycin Ls share the same peptide chain structure and differ only in the structure of the fatty acid chains. The slight difference may be related to the regulation and control pathways of fatty acid chain length [38].

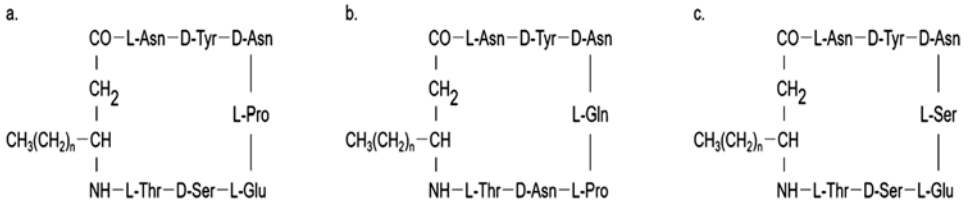

Fig. (1). Structure of bacillomycins.
Note: **a**) bacillomycin D.; **b**) bacillomycin F.; **c**) bacillomycin L.

CHAPTER 3

Fengycin Production and Its Applications in Plant Growth and Postharvest Quality

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Abstract: Fengycin is a cyclic lipopeptide produced mainly by the *Bacillu*s genus, which is structurally composed of a β-hydroxy fatty acid and 10 amino acids. The biosynthesis of fengycin is catalyzed by large non-ribosomal peptide synthetases. Fengycin is an amphiphilic molecule with strong surface activity and displays strong antimicrobial activity. In this chapter, the molecular structure and biological properties of fengycin, and the function and catalyzing mechanism of fengycin multienzyme were summarized. Multiple antimicrobial mechanisms of fengycin and the strategies for increasing the production of fengycin were introduced. Fengycin has the advantages of low toxicity, biodegradation and high stability. Its applications, including biological control of plant pathogens, bioremediation of a contaminated environment, postharvest disease control of fruit and vegetables, food processing and preservation, *etc.*, were reviewed finally.

Keywords: Anticancer, Amphiphile, Biological control, Bioremediation, Food processing and preservation, Fengycin, Inhibition of filamentous fungi, Lipopeptide, Non-ribosomal biosynthesis, Postharvest disease control.

INTRODUCTION

Lipopeptides have been extensively reported from diverse microorganisms and predominantly from *Bacillus* species. Microbial lipopeptides have gained more attention owing to their attractive functional properties, efficient biological activities, biodegradability, and low toxicity when compared with other chemosynthetic surfactants. Fengycin belongs to one of the important members of the lipopeptide family, together with surfactin and iturin, which are the most recognized and extensively studied [1, 2]. Fengycin is a decapeptide linked with a β-hydroxyl fatty acid chain, and cyclization occurs between L-Tyr at position 3 and L-Ile atposition 10 [3, 4]. Fengycin was first discovered by Vanittanakom

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Zhaoxin Lu & Yingjian Lu (Eds.)

and Loefflerin from the strain *Bacillus subtilis* F-29-3. Fengycin A and B have been characterized in studies [5]. Fengycin is usually a mixture composed of a series of structurally close homologs, which differ in the length of the fatty acid chain. Fengycin consists of two main components differing by one amino acid exchange in position 6 of the peptide with D-Ala and D-Val for fengycin A and fengycin B, respectively [6].

Fengycin containing both hydrophobic and hydrophilic constituents makes the molecule amphipathic, thus presenting a strong surface activity and diverse functional properties [1]. Fengycin has shown antifungal, antibacterial, antivirus, antitumor and insecticidal activity [5, 7 - 10, 119]. In 1986, Vanittanakom and Loeffler primarily revealed the antagonistic action of fengycin against filamentous fungi such as *Pyricularia oryzae* (MIC 1.0 pg/ml), *Conidiobolus coronatu*s (MIC 3.16 pg/ml), *Fusarium* sp. (MIC 10 pg/ml), and *Rhizoctonia solani* (MIC 3.16 ug/ml). Natural antimicrobial agents are becoming an effective alternative to agricultural chemicals, but fengycin is still a hot area of concern. Fengycin isolated from *Bacillus amyloliquefaciens* strain ELI149 showed marked antifungal properties against several phytopathogens, including *Fusarium oxysporum*, *Fusarium avenaceum* and *Mucor* sp [11]. Although fengycins were well described as antifungal compounds against plant fungi, it has also been evidenced that fengycin has inhibitory activity against bacterial pathogens. A recent study in Nature by Piewngam *et al*. demonstrates that fengycin restricts human intestinal *Staphylococcus aureus* colonization by modulating quorum sensing to reduce the population of *Staphylococcus aureus* [7]. In addition, the antibacterial activity of fengycins produced by *Bacillus amyloliquefaciens* MEP218 was determined on a plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (Xav) causing the bacterial spot disease [12].

There are a few studies addressing the mechanisms of action of fengycin; it may destroy the cell membrane structure by interacting with the lipid bilayers and sterol molecules. Deleu *et al*. demonstrated that fengycin insertion into biological membranes caused cell disruption [13]. Wise *et al*. [14] highlighted that lower content in anionic phospholipids might increase fengycin insertion into the fungal cell membrane by reducing electrostatic repulsion with the negatively charged fengycin [14]. Other than that, it is well-known that fengycins have little or no effect on mammalian cells [15].

Like other lipopeptides, fengycins are biosynthesised by large, modular, multifunctional enzymes known as nonribosomal peptide synthetases (NRPS). The earlier studies characterized three fengycin synthetase genes, *fenC*, *fenE* and *fenB*, and determined the biological functions of the enzymes encoded by these genes [16, 17]. NRPS subunit, module, and domain exchanges or alternatives

theoretically can change the constituents of the core peptide themself and generate lipopeptides with structural diversity [18]. Researchers have reported the biosynthesis of novel lipopeptide products by the COM structural domain deletion and the thioesterase TE structural domain translocation [19, 20]. Additionally, molecular and metabolic regulation of synthetase and fermentation process optimization are important ways to promote the production of fengycin. Fructose was found to enhance fengycin production by raising the expression of the fengycin synthetase genes and regulatory genes [21]. By optimizing fermentation conditions and medium components, fengycin production increased from 24.70 to 130.10 mg/L [22].

Fengycins from various microbial sources have been studied for their unique properties and biological activities; their applications have been enhanced in different areas, including biological control of plant pathogens in agriculture, environmental protection and remediation of pollutants, food processing and preservation and medicinal fields [23 - 26].

SPECIES, STRUCTURE AND BIOLOGICAL PROPERTIES OF FENGYCIN

Nonribosomally synthesized lipopeptide substances are widely found in bacteria and fungi. In particular, *Bacillus subtilis* can produce a variety of lipopeptides during growth and metabolism [27]. Lipopeptides are amphiphilic surfactants with hydrophilic and hydrophobic properties that comprise both a fatty acid chain with a β-hydroxyl or β-amino groups and 7-10 amino acids to form a cyclic peptide. Lipopeptides generally have antimicrobial activity, and the formation of a series of homologs depends on the different lengths of fatty acids and amino acid compositions. Antimicrobial lipopeptides are synthesized by nonribosomal peptide synthetase (NRPS) and mainly include the three families surfactin, iturin and fengycin. In 1986, Vanittanakom from Germany and Nishikiori from Japan simultaneously discovered members of the third family of lipopeptides, the fengycin family, namely, fengycin produced by *Bacillus subtilis* and plipastatin produced by *Bacillus cereus* [5, 28]. The fengycin family exhibits superior antifungal activity and strong surface active properties; thus, it has promising applications in many fields, such as the food, agricultural, medicine and cosmetic industries.

TYPE AND STRUCTURE

Fengycin belongs to a class of small molecule secondary metabolites and cyclic decapeptides containing β-hydroxy fatty chains produced by *Bacillus spp*. Hydroxy fatty acid chains of different lengths are linked to N-terminal amino acid residues (Glu) by amide bonds. Generally, fengycin is a blending of two

CHAPTER 4

Brevibacillus sp. and Brevibacillin: Biosynthesis, Classification, Bioactivity, and Potential Applications

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Abstract: The bacterium *Brevibacillus laterosporus*, which forms spores, is found in various environments, including soil, water, plants, and food. Parasporal crystals of *B. laterosporus* are well known for their insecticidal properties against a wide range of invertebrate pests. In the chapter, the isolation and identification of various antimicrobials produced by *B. laterosporus*, such as lacterosporamine (C17H35N7O4), Basiliskamide A and Basiliskamide B, Tostadin, *Gramicidin* A-C, Gramididin S, *Tyrocidine* A-C, laterocidin, and Loloatin A-D, and the linear lipopeptides Bogorol A-E, Brevibacillin were reviewed. Furthermore, their antimicrobial mechanism, biosynthesis, and potential applications in food and agriculture were introduced.

Keywords: Antimicrobial peptide, Application, Biosynthesis, *Brevibacillus*.

ANTIMICROBIAL PEPTIDES AND MECHANISM

Linear Peptide

Tostadin

B. laterosporus XDH was isolated from the soil of Taishan mountain (Taian, China), which produced an antimicrobial peptide containing nine amino acids, whose sequence is Ser-Leu-Tyr-Lys-Leu-Thr-Cys-Lys-Phe. Among these, Lys4 and Phe9 are D-type of amino acids. Furthermore, it had antimicrobial activity against *E. coli* and *S. aureus*, the minimum inhibitory concentration (MIC) of which is 16 μg/mL and 32 μg/ mL, respectively. The minimum bactericidal concentration (MBC) of tostadin is two times its MIC. Then, the terminus of tostadin is modified by acetyl and amide groups, showing higher antimicrobial

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Zhaoxin Lu & Yingjian Lu (Eds.)

activity than the wild type. The modified and wild tostadin is stable at 100 °C for 20 minutes, with more than 95% activity [1].

Gramicidin

Gramicidins are the most famous antimicrobial peptides produced by *B. laterosporus*, which show activity against gram-positive bacteria (Fig. **1**) [2]. Then the crystal structure of *Gramicidin* A. and B was identified in 1953 and 1965 [3, 4].

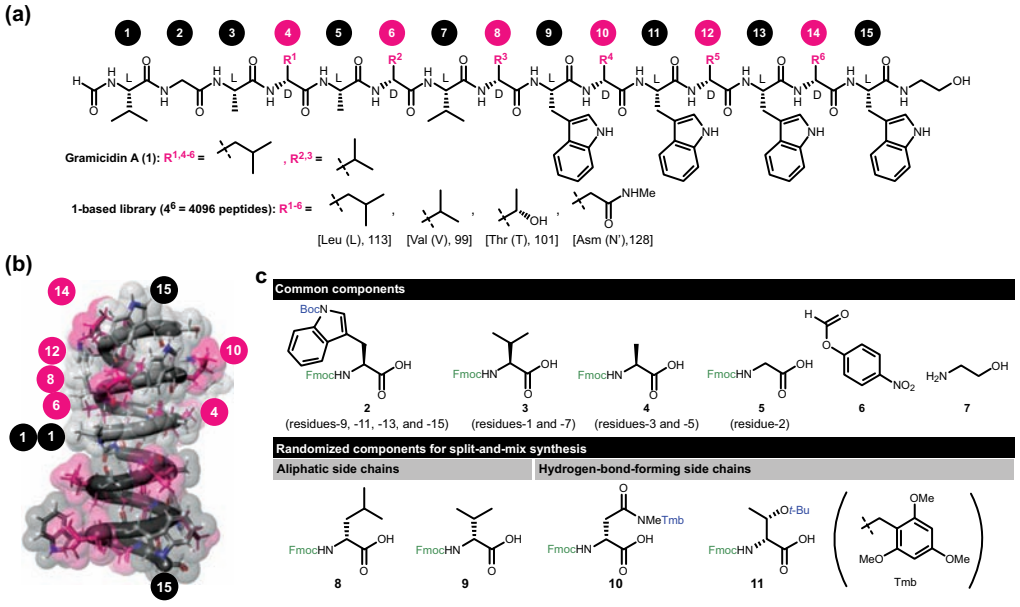

Fig. (1). Chemical structure of *Gramicidin* A. [5].

Gramicidin is a linear peptide of 16 amino acids whose amino terminus is modified to CHO, and the carboxy terminus of glycine is modified to -NHCH$_2$CH$_2$OH, which improves the tolerance to proteases. The amino acids at positions 4, 6, 10, 12, and 14 are D-type amino acids, whose sequences are shown in Table **1** below:

Table 1. *Gramicidin* classification and sequence.

Gramicidin	Sequence
Gramicidin A.	For-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-Gly-ol
Gramicidin B.	For-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-Val-Trp-D-Leu-Phe-D-Leu-Trp-D-Leu-Trp-Gly-ol
Gramicidin C.	For-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-Val-Trp-D-Leu-Tyr-D-Leu-Trp-D-Leu-Trp-Gly-ol

Further, *Gramicidin* A. is a lipophilic left-handed helix (β-helix) that all hydrophobic side chains display on the outside of the helix, which binds to the hydrophobic tail of phospholipids in the cell membrane through hydrophobic interactions on each leaflet of the lipid bilayer, and two helices dimerize to form a complete transmembrane channel. Alternating C=O groups achieve hydrogen-bonding dimerization through head-to-head and tail-to-tail linkages. The amino-terminal formyl group has head-to-head linkage properties without loss of structural continuity [6]. Unlike in lipid bilayers, in organic solvents, two *Gramicidin* A. molecules do not form a head-to-head connection but form an antiparallel double helix.

Beaven *et al*. analyzed the effect of lipids on the lifetime of channels formed by *Gramicidin* A. using lipids of different chain lengths to mimic cell membranes (Fig. **2**) [8]. In this experiment, 3 sets of combinations with the same average chain length but different lipid compositions were set up: A mixed dioleoyl-cholesterol and dioleoyl-cholesterol in an equimolar ratio (dC 18:1 + dC 22: 1:1), B an equimolar mixture of dipalmitoleoyl-cholesterol and diacetyl-cholesterol (dC 16:1 + dC 24:1) and control C eicosanoyl-PC (pure dC 20:1). The molecular dynamics simulations and a simple lipid compression model determined the distribution of lipids around *Gramicidin* A. channels. The results showed that the channel lifetime of control group C was 65 ± 10 ms, but the channel lifetime of combination A was 95 ± 10 ms. The lifetime of the B combination channel was 195 ± 20 ms. It indicates that the shorter the lipid carbon chain in the cell membrane or the simulated membrane, the longer the channel's lifetime formed by *Gramicidin* A.

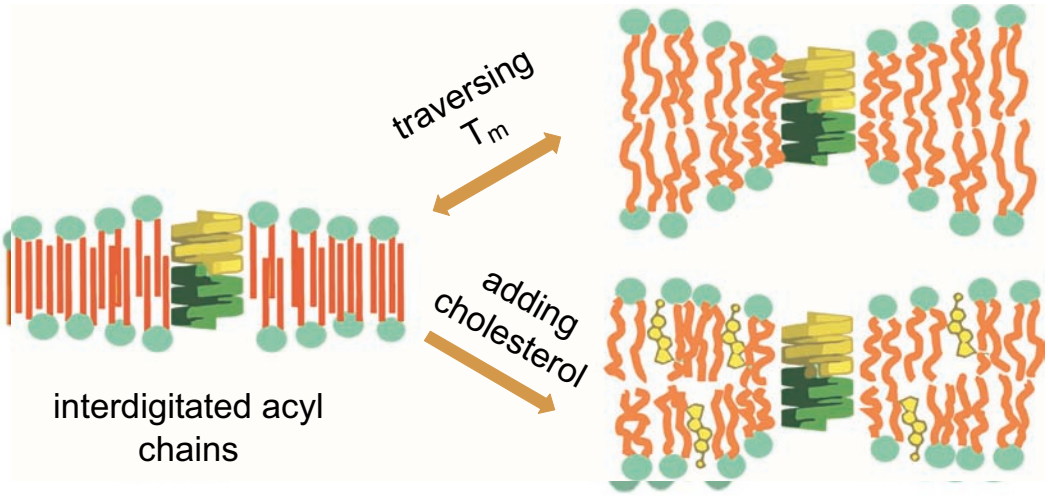

Fig. (2). Schematic diagram of the helical structure of *Gramicidin* A. in the cell membrane [7].

CHAPTER 5

LAB Bacteriocin-Based Strategies for Food Preservation

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Abstract: Bacteriocins are ribosomally-synthesized peptides or proteins with broad or narrow-spectrum antimicrobial activity. Bacteriocins produced by lactic acid bacteria (LAB) are considered natural preservatives with safe and green properties, and their use in food preservation meets consumer demand. In this paper, the classification of LAB bacteriocins and their antimicrobial mechanisms are described in detail. Its application in the preservation of food products such as meat, dairy, seafood, fruits and vegetables is reviewed. The application of bacteriocins in hurdle technology is also presented, including their combination with other antimicrobial agents such as essential oils, bacteriophages, lysozymes, chemical antimicrobial agents, as well as thermal and non-thermal processing technology. To sum up, this review will provide insights for researchers working with lactobacillus bacteriocins as well as for industry personnel looking for new methods of natural and safe food preservation.

Keywords: Bacteriocin, Lactic acid bacteria (LAB), Preservation.

INTRODUCTION

With rapid globalization and industrialization, the worldwide demand for processed food items is estimated to be around 7 trillion dollars [1]. Maintaining food safety and quality from storage to delivery to the end user is a difficult task. Furthermore, the rising demand for delectable and well-preserved food items devoid of chemicals has enticed the exploration of inventive methods to safeguard food, guaranteeing its safety, excellence, freshness, and sensory attributes. Among the nature preservative compounds, bacteriocins have caught great attention due to their remarkable ability to effectively prevent food spoilage and the growth of harmful bacteria.

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Zhaoxin Lu & Yingjian Lu (Eds.)

Bacteriocins are ribosomally-synthesized peptides or proteins that exhibit antagonistic effects on groups of bacteria that are either closely related or unrelated to them [2]. With broad-spectrum or narrow-spectrum antimicrobial activity, bacteriocins can be diverse, varying in size, structure, and specificity. LAB-derived bacteriocins have gained considerable attention as natural food preservatives owing to their GRAS certification from the U.S. Food and Drug Administration. Since bacteriocins are thermally stable and have a wide pH tolerance, they are able to withstand heat treatment during food storage, as well as changes in acidity and alkalinity. The proteolytic activity of bacteriocins allows for their inactivation by digestive proteases, with minimal impact on the gut microbiota.

In preservation, bacteriocins are used in two main ways: either the bacteriocin-producing strains are inoculated into foods as initial cultures or protective cultures, or purified or semi-purified bacteriocins are added as an additive ingredient to foods. Despite the fact that only nisin and pediocin produced by *Lactococcus lactis* have been given the go-ahead as food additives, there is still considerable interest in investigating the examination and separation of LAB strains that produce bacteriocin, apart from the characterization and utilization of bacteriocins in food products.

LAB BACTERIOCIN

Bacteriocin Produced by LAB

LAB is a heterogeneous assemblage of gram-positive, non-spore-forming microorganisms, coccobacilli, or rods that can ferment carbohydrates and whose main fermentation product is lactic acid, including *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, *Sporoloctobacillus*, *Enterococcus*, *Lactococcus*, *etc* [3]. LABs are widely used for food fermentation, and their colonization in the intestines after ingestion can regulate intestinal flora balance as probiotics [4]. They can also be used to prevent food corruption as they synthesize lactic acid, hydrogen peroxide, and bacteriocins, which efficiently impede the proliferation of detrimental microorganisms.

Bacteriocin of LAB is a protein or polypeptide that has antibacterial properties and is produced through the metabolic process of ribosome synthesis. It is characterized by its high efficiency, non-toxicity, thermal resistance, absence of residue, and no drug resistance. It can inhibit a variety of bacteria, fungi, and even viruses and can be very useful in preserving food [5]. It is expected to be an alternative to chemical preservatives and has always been a focus of scientific attention. Although many bacteriocins have been isolated and purified, and their properties have been studied, only Nisin A and Pediocin have been approved by

the Food and Drug Administration (FDA) and are suitable for preserving heat-sensitive foods during cold-chain transport [4].

Classification

Bacteriocins have been categorized into various groups according to their characteristics, including molecular weight, chemical structure, thermal stability, enzyme stability, mode of action, antibacterial activity, and the presence of post-translational modified amino acid residues. Klaenhammer proposed the first classification system for LAB bacteriocins and it has been reclassified and modified by many researchers, resulting in no consensus [2, 6, 7]. The following classification is revised by Heng *et al*., who revised the classification scheme proposed by Klaenhammer (Fig. **1**).

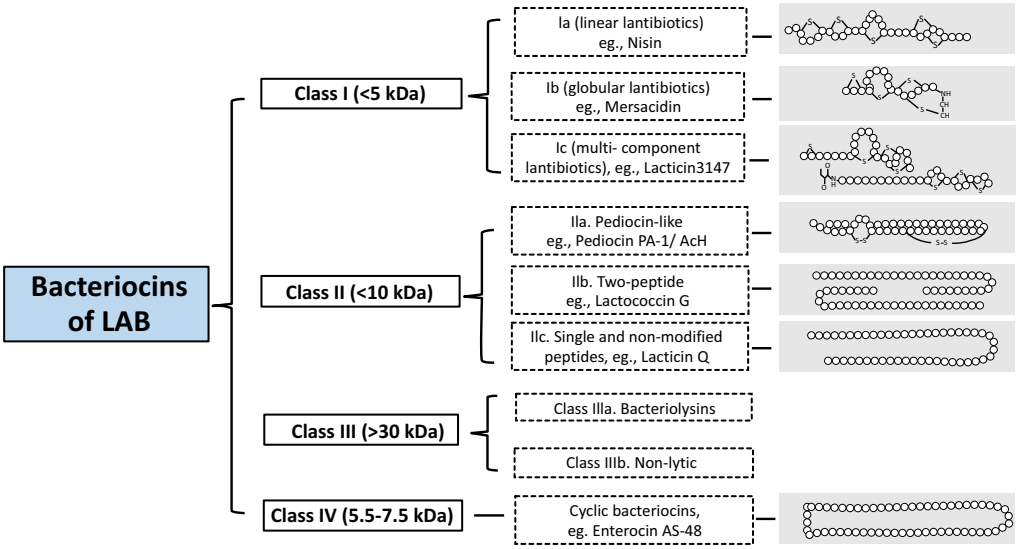

Fig. (1). Classification scheme of bacteriocins proposed by Heng et al. and structure of representative bacteriocins from each class [22].

Class I

Class I bacteriocins, which have been modified to be smaller molecules (< 5 kDa), consist of 19 to 50 amino acid residues. They are called lantibiotics because they have unusual post-translational modified residues, including thioether amino acids lanthionine and methyllanthionine [8]. Modification of these amino acids leads to the formation of many thioether rings in the peptide, which make lantibiotics structure-stable and heat-stable [9]. Lantibiotics can be further classified into three subclasses according to the ring rigid structure formed by these special amino acids.

CHAPTER 6

Application of ε-poly-L-lysine in Improving Food Quality and Safety

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Abstract: Each year, economic losses in the food industry due to spoilage of grain, aquatic products and fruit are huge. People now express more concern about food safety and nutrition, therefore, the need for green preservatives is also growing. Epsilon-poly-L-lysine (ε-PL), a cationic polyamino acid with 25–35 L-lysine residues, possesses broad-spectrum antimicrobial activity, biodegradable properties, resistance to high temperature, and non-toxicity and can dissolve in water. So, it has been extensively applied in the field of preservatives for foodstuffs, agriculture and biomedicine. Thus, the chapter mainly focuses on the recent research on microbial synthesis, production enhancement, and antimicrobial mechanism, as well as improving food safety, its utilization in food packaging materials and agriculture of ε-PL .

Keywords: Agriculture, Antimicrobial activity, Antimicrobial mechanism, Food preservative, Food packaging materials, Food safety, ε-poly-L-lysine, Microbial synthesis, Natural amino-acid homopolymers, Production enhancement.

INTRODUCTION

Biopolymers, as the preeminent molecular entities in living matter, play a pivotal role in maintaining the structural and functional integrity of biological systems. Microorganisms, characterized by their remarkable adaptability and metabolic versatility, exhibit a notable capacity for the synthesis of a diverse array of biopolymers. Among these, microorganisms demonstrate proficiency in producing various types, such as polynucleotides, polyesters, and polyketides. However, homopolymers, denoted by the recurrence of a singular amino acid type, are not as ubiquitously encountered. Nature has yielded two documented occurrences of

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Zhaoxin Lu & Yingjian Lu (Eds.)

amino-acid homopolymers, namely, γ-poly-glutamic and ε-poly-L-lysine (ε-PL) [1]. ε-PL is a cationic polyamino acid containing approximately 30 L-lysine residues bound by complex linkages between the α-carboxyl and ε-amino groups. (Fig. **1**) [2]. The homopolymer ε-PL is distinguished by its inherent properties, encompassing biodegradability, water solubility, thermostability, and non-toxicity [3, 4]. Besides, ε-PL shows antiphage and anti-obesity activity [5]. It can selectively remove endotoxin and be an excellent carrier for drug or gene delivery, which may have potential use in the pharmaceutical and biomaterial industries.

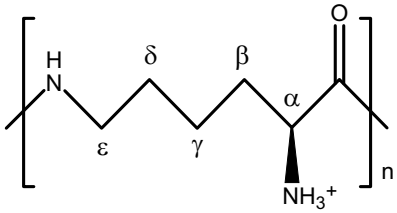

Fig. (1). Chemical structure of ε-PL biosynthesized in microorganisms.

Each year, economic losses in the food industry due to spoilage of grain, aquatic products and fruit are huge. Meanwhile, the widespread practice of using synthetic ingredients to prolong the shelf-life of food has created a host of health risks and hidden problems. All of these go against the great demands of safe and healthy food. Thus safe and effective food additives without side effect need to be explored and fill the gap in the traditional antiseptic preservation technology. ε-PL , a natural preservative, is safer compared with traditional chemical preservatives and thus can meet well with the growing need of green food additives. This chapter is primarily dedicated to exploring the potential of ε-PL in the enhancement of food security and safety. This exploration extends to its diverse uses in agriculture and the formulation of food packaging materials.

MICROBIAL PRODUCTION OF E-PL

In bacterial organisms, the synthesis of lysine takes place *via* the diaminopimelate pathway (DAP) (Fig. **2**). Within this metabolic pathway, diaminopimelate (DAP) is synthesized through the amalgamation of aspartate (Asp), derived from oxaloacetate (OXA). It actively engages in the tricarboxylic acid cycle, fulfilling its role as a vital component, while concurrently, the ammonium ion plays a pivotal role as a nitrogen source. The first sorts of enzymes in the cascade, namely aspartokinase (Ask) and aspartate semialdehyde dehydrogenase (Asd), assume a pivotal role in orchestrating the production of the ultimate amino acids [6].

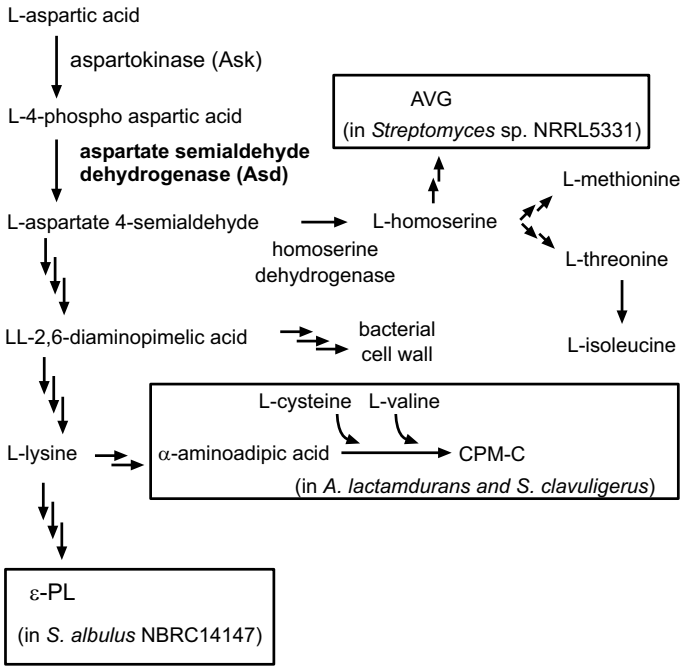

Fig. (2). The biosynthetic pathway starting from L-aspartic acid, known as the aspartate pathway. ε-PL : ε-poly-L-lysine; AVG: aminoethoxyvinylglycine; CPM-C: Cephamycin C [6].

Research findings have elucidated that metal ions, specifically manganese, cobalt, and iron, function as regulatory entities in modulating the expression of genes integral to ε-PL synthesis. Specifically, the ferric uptake regulator protein (Fur) has been postulated to either stimulate the expression of genes associated with facilitating ε-PL synthesis or suppress the expression of genes that hinder the process of ε-PL synthesis [7]. Furthermore, studies have shown that ferrous ions stimulate proteinase activities and promote amino assimilatory enzyme activity, facilitating the conversion of ammonium ions to amino acids. Consequently, the promotion of L-lysine production results in an accelerated synthesis rate of ε-PL [8].

At an equilibrium of approx. 9.0, ε-PL is positively charged at pH<9.0 and can be electrostatically repelled by the agar medium or basic dyes such as methylene blue to form visual transparent zones around the strain [9]. Meanwhile, the color reaction between ε-PL and Dragendorff can also be used to detect ε-PL producing strains [10]. The concentration of ε-PL can be easily and rapidly quantified using the spectrophotometric method. In this approach, ε-PL is treated with an excess of the anionic dye methyl orange, and the unbound dye is then determined through spectrophotometry [11]. The development of a more responsive alternative was

CHAPTER 7

Bacteriophage Control of Foodborne Pathogens in Food Production

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Abstract: The application of bacteriophages (phages) that target and kill bacteria to safeguard foods and food production facilities has attracted attention over the last decade. As phages are often already present in foods and food production settings, their specificity and antimicrobial activity against foodborne bacterial pathogens can be harnessed to affect biocontrol/bio-sanitization with minimal risk to the product or the consumer. Efficacy studies on foodborne bacterial pathogens have established the utility of the approach, and these, coupled with the inherent safety of phages, have led to regulatory approvals and the marketing of phage products for food safety. Here, we review the supporting research that demonstrates the effects of phage on foods and food contact surfaces with specific reference to the challenges of controlling bacteria that can resist conventional cleaning processes either due to adaption and/or refuge in microbial biofilms.

Keywords: Bacteriophage, Bio-sanitation, Biofilms, Biocontrol, *Campylobacter*, Enterovirulent *E. coli*, Food surfaces, Food production, Food safety, Food processing, Foodborne pathogens, *Listeria*, Phage biocontrol, Ready-to-eat foods, *Salmonella*.

INTRODUCTION

Microorganisms can adversely affect food production with respect to food safety, food preservation and storage. Food manufacturers have the duty and responsibility to eliminate or minimize the microorganisms that affect human health from their products to ensure that the products they deliver are microbiologically safe. Biological controls have been developed to complement physical processes that remove pathogens and spoilage agents from raw and processed foods. A variety of microorganisms can survive harsh conditions either

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Zhaoxin Lu & Yingjian Lu (Eds.)

as short-term persister cells or within biofilms that form protected microenvironments.

Persister cells were described first by Joseph Bigger in his study analyzing the action of penicillin [1]. After antibiotic treatment, the author observed that a small proportion of *Staphylococcus* spp. cells were not only penicillin-resistant but, more importantly, dormant. Moyed and Bertrand continued this work investigating the persister genes for *Escherichia coli* cells post ampicillin treatment and described the *hipA* gene – a gene that plays a significant role in affecting the frequency of generating persister cells [2]. Although persister cells are frequently associated with multi-drug resistance, their formation may be triggered not only by antibiotics but also by other factors. Of specific relevance are the environmental stresses imposed in food production that provoke the formation of persister morphologies of pathogenic bacteria.

Although Antoine Van Leeuwenhoek first described the observation of biofilms on his own teeth in the 17th century, the properties of biofilms were not investigated in depth until recently with the availability of advanced scanning electron microscopy and molecular genetic approaches. Biofilms are now characterized as microbial ecosystems formed by single or multiple species of bacteria or fungi, which are embedded in an Extracellular Polymeric Substances (EPS) matrix composed mainly of polysaccharides, proteins, and extracellular DNAs [3]. The EPS generally contributes 50% to 90% of the organic carbon of biofilms and is suggested as the main matrix materials. The EPS provides structural protection for the embedded cells that supports their persistence against unfavorable pH, temperature, osmosis shock, antibiotics and host immune cells. Although the precise structure of the biofilm and its physiology is highly dependent on the resident microbes and the environment in which they are exposed, the formation of biofilm generally follows four distinct steps regardless of the founding bacterial species [4]. This includes the initial attachment to the target surface, reversible binding to the surface, which subsequently switches to irreversible binding, development of the microcolonies and maturation of the biofilm architecture. Preformed biofilm can disperse under unfavorable conditions when the biofilm matrix compounds are reduced and/or when the matrix structures are cleaved enzymatically.

Pathogen-Associated Biofilms and Food Safety

Compared to planktonic bacterial populations growing in nutritionally rich environments, cells lodged in biofilms are generally less active due to a combination of nutrient limitation and environmental stresses, but nevertheless, they can act as a reservoir for the spread of viable organisms. Bacteria within

biofilms are frequently physiologically distinct from planktonic bacteria that have formed much of the basis of experimental investigation. However, human consumption of pathogenic bacteria within biofilms can cause infection. In fact, estimates of infection sources suggest most human infections are associated with biofilms [5 - 7]. The observation that biofilms are more difficult to eradicate than free bacterial cells *via* host defensive mechanisms and antimicrobials is probably the reason why biofilms are frequently implicated in infection. Studies indicate that biofilm associated bacteria exhibit 10 to 1,000 times higher antibiotic resistance when compared with planktonic cells [8, 9]. This is not surprising as the physical barrier formed by biofilms can effectively reduce the performance of antibiotics by lowering the effective drug concentration below the lethal threshold and promoting the selection of antibiotic-resistant subpopulations. The close proximity of bacteria within biofilms can foster horizontal gene transfer of mobile genetic elements that confer resistance determinants. Notably, the high densities of bacteria enable the widespread conjugal transfer of DNA [10].

Despite the rapid development of modern technologies, food industries are under continuous challenge from the threat of bacterial contamination. Industrial food-producing lines are commonly considered a suitable environment for biofilm formation. Biofilms can form from founding bacteria, not necessarily pathogens in themselves, adhering to solid structures, or the whole biofilm matrix sloughed from elsewhere can adhere to hard surfaces of industrial infrastructures, for example, stainless steel, wood, glass, polypropylene, polyethylene, and rubber. They can also attach to the biotic surfaces of a wide range of food products. Within a food production plant, the sources of contamination commonly include contaminated raw materials, water, processing surfaces, handling personnel and animal carcasses. Detailed information on the content and dispersion of bacterial biofilms are not readily available and are frequently not given sufficient consideration in the setting of critical control points in HACCP (Hazard Analysis and Critical Control Points) systems. The disinfection protocols employed may not routinely reach inaccessible areas of the plant to eliminate biofilms or the bacteria therein, such that they remain a source of contamination at multiple stages in food processing and delivery. Food industries across different categories can be affected by biofilms, for example, meat, seafood, fruit and vegetable processors. The presence of biofilms in industrial settings also represents a structural cause for concern as they can block filters, reduce thermal transfer, and damage equipment by bio-corrosion. However, critically, biofilms harbor pathogenic bacteria that pose significant risks to public health worldwide and the incumbent economic losses. Common bacterial foodborne pathogens include *Salmonella,* enteropathogenic *E. coli*, *Campylobacter* and *Listeria monocytogenes.* These bacteria can form or be resident within mixed-species biofilms that are frequently associated with antimicrobial resistance. Efforts are

CHAPTER 8

Plant-Based Antimicrobials-Innovative Natural Food Preservatives

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Abstract: Plant-based antimicrobials have been intensively studied in response to consumers' need to reduce the use of synthetic chemical antimicrobials, as well as the global antibiotic resistance crisis. Bioactive compounds extracted from plants exert potential antimicrobial activities. In this chapter, recent research on their antimicrobial activities against foodborne pathogens in planktonic or biofilm state, antimicrobial mechanisms, their applications and limitations in food were reviewed. Additionally, the delivery methods for plant-based antimicrobials, including multi-hurdle, nanoemulsions, and edible coating/film technologies, were summarized. Lastly, the future research needs on plant-based antimicrobials were discussed.

Keywords: Antimicrobial, Food preservation, Food safety, Plant-based.

INTRODUCTION

Chemical antimicrobials have been used in the food industry for decades as preservatives (*e.g.*, sulfites, sodium nitrite) or disinfectants (*e.g.*, sodium hypochlorite). They have been proven effective against a broad range of spoilage microorganisms and foodborne pathogens in foods and/or on food contact surfaces [1].

However, negative concerns about these chemicals have been raised regarding their potential adverse impact on humans, animals, and the environment. For example, sulfites are considered anti-nutrients because they have been associated with the degradation of thiamine or vitamin B1 in food [1]. Epidemiological studies have revealed some evidence to link dietary nitrite with gastric cancers and the combination of nitrite plus nitrate from processed meat with colorectal

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Zhaoxin Lu & Yingjian Lu (Eds.)

cancers [2]. Antimicrobials and their metabolites, such as triclosan and triclocarban accumulating in wastewater, were proven to have significant impacts on aquatic ecosystems and may alter the function of soils [3]. With increasingly negative concerns, it is natural for regulators to question and reexamine the usage of chemical antimicrobials in the food industry. Regulation (EU) No 528/2012 of the European Parliament recognized that biocidal products such as disinfectants can pose risks due to their intrinsic properties and associated use patterns [4]. Conversely, alternatives to chemical antimicrobials, such as plant-based essential oils, have been gaining support from regulatory agencies [5, 6].

Besides the safety concerns of chemical antimicrobials, the global antibiotic resistance crisis has also drawn great attention. Both misuse and overuse of antibiotics have led to the development of antimicrobial resistance (AMR) in microorganisms, which has become a global problem and a growing threat [7]. Resistance of the microorganisms towards traditional antibiotics makes it more difficult to treat certain diseases, which in turn leads to higher medical costs, prolonged hospital stays and increased mortality [8]. It is estimated by the World Health Organization (WHO) that by 2050, drug-resistant diseases will cause 10 million deaths each year [8]. One of the strategies to tackle the AMR issue is to combine or replace traditional antibiotics with natural antimicrobials to reduce the risk of resistance development.

Over the years, health- and environmental-conscious consumers developed an increasing demand for clean-label food products. Even though it is a consumer term, clean label has been broadly accepted by the food industry, academics, and even regulatory agencies. It seeks out foods with easy-to-recognize ingredients and no artificial ingredients or synthetic chemicals, which has become associated with the trust toward food manufacturers [9]. One of the important movements is to replace synthetic chemical preservatives with natural antimicrobials. These new natural antimicrobials are expected to not only mitigate foodborne pathogen risks in processed foods but also to extend the shelf-life of raw foods (*e.g.*, fresh produce) or minimally processed foods, which is another trend started by health-conscious consumers. Under the pressure of consumers wanting clean label, raw foods, and reducing food waste by extending the shelf life of highly perishable foods, the search for natural antimicrobials has been the subject of intensive research during the last decade [10].

The source of the natural antimicrobials can be categorized into three categories: plant (*e.g.*, plant-based essential oils), microorganisms (*e.g.*, bacteriocin and bacteriophage) and animal origin (*e.g.*, lactoferrin). Plant-based natural antimicrobials have gained tremendous attention among researchers because they are also reported to have proven health benefits, such as high antioxidant activities

and their ability to reduce cardiovascular diseases and cancer [11]. This chapter focuses on plant-based natural antimicrobials.

CRUDE PLANT EXTRACT

The debate on whether to study crude plant extracts or single compounds existed decades ago when we started to scientifically explore the bioactivity of plants. Some researchers prefer to study pure compounds isolated from these plants because it gives a quantitative correlation between the dose of the compounds and the bioactivities, including antimicrobial capabilities. Researchers with a holistic perspective, however, argue that humans consuming edible or medical plants by consuming their crude extracts and crude plant extracts often have greater activity than isolated constituents at an equivalent dose. To this day, interestingly, the debate remains. However, regardless of which perspective one chooses to approach to explore this topic, screening the crude extracts from a variety of plants for their antimicrobial activities is always a logical first step.

Antimicrobial Property of Crude Plant Extract

Thanks to their ubiquitousness and a long history of being used by humans, plants have been studied by researchers around the world. To name a few, Cock and van Vuuren investigated 66 extracts from 29 native South African plants with a history of medicinal usage and reported antimicrobial activities from all extracts [12]. Bampali and colleagues evaluated three herbal tea samples from different areas of Lesvos Island in Greece and shared positive results of their antimicrobial effect [13]. Naaz and colleagues extracted essential oil from 12 medicinal plants and spices from Fiji and revealed high to moderate antimicrobial activities against *Escherichia coli* O157:H7 [14]. Driven by the passion to share their findings on functional native plants with the world, this type of screening research is expected to continue in the future.

Instead of seeking plants unique to specific areas, some researchers focused their attentions on plants widely consumed by humans across different regions-such as fruits, vegetables, herbs, and spices. Examples include mango extract against *E. coli* and *Salmonella* Typhimunium [15]; sweet potatoes against *Staphylococcus aureus* [16]; berries, pomegranates, and grapes against *S. aureus*, *Bacillus subtilis*, *Enterococcus faecium*, *P. aeruginosa*, and *E. coli* [17]. Sage, mint, thyme, rosemary, lemon balm, oregano were popular plants to study as well [16, 18, 19]. Crude plant extract contains a mixture of phytochemicals, which may act synergistically. However, mixtures are more likely to contain toxic constituents, and they must be thoroughly investigated and standardized before being approved for large-scale usage [20].

SUBJECT INDEX

A

B

C

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W

Zhaoxin Lu

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Yingjian Lu

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