8 Novel food packaging systems with antimicrobial agents from microbial source

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Chapter Contents

○ ABSTRACT

Antimicrobial packaging systems are used to control microbial growth in food products. Several antimicrobial packaging technologies have been developed employing different substances. This chapter focuses on those that use antimicrobial substances produced by microorganisms as active agents (bacteriocins, lipopeptides, and other metabolites). The most important antimicrobial compounds used in food packaging and the techniques employed to evaluate their antimicrobial action are described. Also, the forms in which the packaging materials contain and deliver these antimicrobial compounds, are presented. An extensive list of the scientific publications on the subject is detailed and their results are discussed. Current trends suggest a greater market emphasis on food quality requirements and safety features, associated with the addition of antimicrobial agents. This constitutes a challenge for the development and implementation of this type of packaging system, which requires the joint work of academia and industry.

○ KEYWORDS

Food packaging system, Controlled release, Delivery system, Food safety, Food quality, Antimicrobial action, Bacteriocins, Microbial metabolites, Spoilage microorganisms, Pathogens.

8.1 BACKGROUND

Food safety is a global priority since it constitutes a permanent and essential need for life. However, food microbial pathogens are still today one of the major causes of foodborne illnesses and food outbreaks remain among the main concerns related to public health (Nithya

and Halami, 2012; Achi and Halami, 2016; Jagus, Gerschenson and Ollé Resa, 2016; Quinto *et al.*, 2019). According to WHO, almost 600 million people in the world fall ill after eating contaminated food and 420,000 die every year (WHO, 2020). In addition, microbiological alterations significantly decrease the food shelf-life stability, causing considerable losses in the food industry (Quintavalla and Vicini, 2002; Jayant and Halami, 2020; Kourmentza *et al.*, 2021). To overcome this complex scenario, it is crucial to adopt measures that ensure food safety throughout the entire food chain, from raw material handling, processing, distribution, and storage, to end-products consumption (Said *et al.*, 2019). Despite several preservation methods have been developed to assure microbial food safety, globalization in food production, together with the growing consumer demand for minimally processed, more natural, highly nutritional fresh food products, demand major challenges to assure safe, health-promoting, and high quality food (Realini and Marcos, 2014; Jagus, Gerschenson and Ollé Resa, 2016).

Packaging is not only a way of protecting and preserving food during handling, transportation, and storage, but also has secondary functions such as sales promotion, customer service, and brand communication that have grown in importance over the years (Contreras *et al.*, 2021). In packaging development, it must be considered that the chosen design must satisfy the demands related to the product and its conservation, to marketing and sale, to consumer comfort and needs, and to environmental issues related to its disposal. In addition, the design and use of a suitable and convenient packaging system, that is, primary, secondary, and tertiary packages and accessories, is a critical issue in the food supply chain. The importance of packaging has increased notably in recent years, associated with the development of electronic commerce operations, which has been strongly boosted by the COVID-19 pandemic.

The European Organization for Packaging and the Environment stated in their New Circular Economy Action Plan that packaging is an integral and essential part of the product supply chain, from the production to the consumption stage. They consider that the packaging supply

chain plays a central role in contributing to a resource-efficient and circular economy by optimizing resource use, minimizing waste (food and packaging), and protecting products all along the value chains (EUROPEN, 2020).

Food products require adequate packaging and conservation conditions to move along this supply chain and therefore, the analysis of physical logistics flows and the role of packaging are essential and can influence the definition and design of manufacturing processes.

The characteristics of the packaging systems (e.g., shape, size, design, materials, introduction of active or intelligent components) must respond to the demands of the product (composition, shelf-life, and storage conditions) and its supply chain, to improve the performance of companies and minimize their costs. Environmental regulations and consumer demands for more environmentally friendly products are also factors that affect the formulation and design of food packaging systems. Some environmental aspects related to packaging systems should be considered for compliance with sustainability, environmental responsibility, and recycling regulations, such as the use of only the packaging strictly necessary to prevent waste, the minimization of mass and volume of packaging materials, the promotion of reuse (returnable packages) and recycling, and the evaluation of the final disposal (Cha and Chinnan, 2004).

Packaging systems generally consist of primary, secondary, and tertiary levels. The primary packaging provides the structure of the package, it is usually the smallest unit of use and distribution and is in direct contact with the product. The secondary package is related to the visual communication of the product and is used to group the primary packages. Finally, the tertiary package is used for storage and transport shipping. Therefore, packaging systems can fulfill varied general purposes such as physical protection of the content, hygiene and conservation, containment or agglomeration of small parts, information transmission, marketing, and security (traceability, real-time product tracking, etc.) (Robertson, 2013).

8.2 ACTIVE PACKAGING

Prof. Gordon L. Robertson defines active packaging as:

> Packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system (Robertson, 2013).

Active packaging is the packaging system that performs, in addition to the basic function of barrier to the external environment, some desired functions that are obtained through the incorporation of active components in the packaging or the use of functional polymers (Han, 2003; Ozdemir and Floros, 2004; Robertson, 2013; Realini and Marcos, 2014; Soltani Firouz, Mohi-Alden and Omid, 2021). These packagings interact with the food or the headspace between the package and the food to achieve goals such as increasing shelf-life, improving safety, preserving sensory properties, and maintaining the quality of the product. Active packaging is also used in the pharmaceutical industry and other consumer goods with similar objectives (Han, 2003; Silvestre, Duraccio and Cimmino, 2011).

The active packaging must fulfill the following conditions (Prasad and Kochhar, 2014):

a. The materials must be appropriate and effective for their intended use.

b. Good manufacturing practices should be used to manufacture active materials.

c. Active components and their released quantity to the food must be informed to the consumer. Its use must be permitted.

d. Mandatory “DO NOT EAT” labeling must be provided to enable consumers to distinguish non-edible parts when these may be perceived as edible. This information must be visible, legible, and indelible.

The active compounds commonly incorporated into an active packaging system are antimicrobials, preservatives, oxygen absorbers, water vapor absorbers, ethylene removers

(Brody *et al.*, 2008). According to the release mechanism of the active agent, active packaging can be classified into two main types, non-migratory active packaging, acting without intentional migration and active releasing packaging, allowing controlled migration of non-volatile agents or emission of volatile compounds in the atmosphere surrounding the food (Bastarrachea, Dhawan and Sablani, 2011; Contreras *et al.*, 2021).

Active packaging is a widely reviewed topic in the literature from different approaches (Rooney, 1995; Brody, 2004; Ozdemir and Floros, 2004; Hernandez-Izquierdo and Krochta, 2008; Kim *et al.*, 2008; Sztaki, 2010; Robertson, 2013; Prasad and Kochhar, 2014; Realini and Marcos, 2014; Bastarrachea *et al.*, 2015; Soltani Firouz, Mohi-Alden and Omid, 2021) and on which there is growing interest from the food industry as a strategy for food preservation.

8.3 ANTIMICROBIAL PACKAGING SYSTEMS

One of the main causes of the decay in the quality and safety of food is the growth of pathogenic and spoilage microorganisms. Antimicrobial food packaging is a particular and innovative form of active packaging that reduces, inhibits, or stops the growth of food-borne microorganisms on food surfaces and conserves its quality. In this kind of packaging, the active agent that is deliberately incorporated into the packaging is a compound with antimicrobial activity released into the environment surrounding the packaged food to exert its antimicrobial action (Han, 2003; Malhotra, Keshwani and Kharkwal, 2015; Sofi *et al.*, 2018). Several substances with antimicrobial activity from different origins, such as essential vegetable oils, antibiotics, silver ions, inorganic gases, organic salts, enzymes, metabolites of bacterial origin (Appendini and Hotchkiss, 2002; Aider, 2010; Sung *et al.*, 2013), and viable cell of probiotic (Espitia *et al.*, 2016) have been used for this purpose. This chapter specifically addresses those compounds with antibacterial activity from microorganism sources.

The use of antimicrobial packaging has been driven by consumer interest in more natural and healthy foods with fewer preservatives (Appendini and Hotchkiss, 2002; Miranda *et al.*, 2016). In particular, the advantages of using antimicrobial packaging are:

* Prevent applying antimicrobials directly to food, reducing the total amount of antimicrobial in the product.

* Reduce the amounts of antimicrobials to be applied, as higher antimicrobial concentrations could be maintained on food surfaces compared to direct application to the food itself since it is applied only where it is required.

* Allow antimicrobials to slowly migrate from the packaging material to the food surface maintaining a high concentration of antimicrobials on the food surface, compensating for their consumption during storage.

Different strategies have been studied and implemented to control the amount of antimicrobial released from the container throughout the shelf-life of the product, reducing the amount of active agent required (Sung *et al.*, 2013; Chawla, Sivakumar and Kaur, 2021).

One of the main drawbacks of this technology is that the effectiveness of antimicrobial packaging can be seriously affected by several factors. In general, laboratory conditions cannot be reproduced on an industrial scale and the obtained results change significantly with scale change, (e.g., polymer-embedded antimicrobials). Additionally, research involving antimicrobials at levels well above what is sensory acceptable and feasible and above regulatory limits lacks applicability and is distracting. Besides, the main obstacle towards the commercialization and popularization of antimicrobial packaging use is the ability of the package to retain and release antimicrobials with reliable efficacy and compliance with regulatory constraints(López-Carballo *et al.*, 2012). Incorporating antimicrobials into packaging is an important opportunity that requires commitment and joint work from both academia and industry.

8.3.1 Design of antimicrobial packaging systems

An antimicrobial packaging can be formed as homogeneous or composite materials, micro or nanostructured, that consists of the combination of two or more phases separated by an interface. The active compounds can be integrated or form one of these phases (Azeredo, 2013). The release kinetics of the active compound is related to the interaction between the active agent and the matrix where it is incorporated as micro or nano structures (particles, capsules, droplets, or films), and with the material of the other phases (diffusivity, solubility, ionic charge, etc.).

Release kinetics of a compound depends on the packaging materials (polymer composition and glass transition temperature (Tg)), the storage conditions (time, temperature, and relative humidity), and composition and aggregation state of the food. Antimicrobial release from the polymer can be controlled by the Tg of the material, considering that the diffusivity of the antimicrobial compound in the polymeric material is generally greater in amorphous than in vitreous state (Robertson, 2013). This allows antimicrobial release to accelerate as the temperature approaches to favourable conditions for microbial growth.

The suitable antimicrobial packaging solution must be selected based on food product characteristics, the target spoilage microorganisms to be controlled, the specific requirements of the product conservation, and its conventional packaging systems. Once the microorganisms of interest for a particular food are identified, specific research must be carried out to obtain an antimicrobial packaging that is effective against them. Antimicrobial agents are selected based on food characteristics and a viable antimicrobial packaging solution is evaluated based on feasible modifications of the typical package of the food. Since, the antimicrobial compounds can migrate into the food, it is considered an indirect food additive. Therefore, it must be GRAS

or authorized material for contact with food. Also, it must be approved for its use in the estimated concentration that will be incorporated into the food by migration from the container (European Commission, 2004; Robertson, 2013). The choice of the antimicrobial compound is specific to the temperature the food will experience during its shelf-life, the microbiota of the product, regulations, and consumer sensory preferences.

As it was stated before, the design of an antimicrobial packaging system depends on the use for which it is intended. Han (2013) proposed the following factors to be considered in the development of antimicrobial packaging:

1) Chemical nature: chemical characteristics of the packaging material and the processes associated with its industrial production (e.g., extrusion, lamination, thermocompression, printing): considering their effect on residual antimicrobial activity of the active compounds after introducing and processing. Compatibility between the active substance and the packaging material (solubility, difference in polarity).

2) Interaction between antimicrobial substance and food: Characteristics of the food (composition, pH, water activity, aggregation state) can have a direct impact on the activity of the antimicrobial agent. The particular microbiota of each food must be considered when selecting and incorporating the antimicrobial agent (Quintavalla and Vicini, 2002).

3) Storage temperature: The temperature can affect the antimicrobial activity of the active compound and its solubility and diffusivity into the food. The temperature has also an important influence on the rate of microbial growth.

4) Functional and physical properties of packaging materials: the incorporation of active substances into the packaging material can modify its barrier and mechanical properties and its final performance.

Antimicrobial packaging is often combined with other active packaging technologies to achieve a synergistic effect (e.g., modified atmosphere, humidity controllers, gas absorbers) (Brody, 2004; McManamon *et al.*, 2019). Antimicrobial packaging is commonly used in processed meats and seafood, minimally processed fruits and vegetables, cheese, and baked goods. Antimicrobials are mainly incorporated into the material in direct contact with food and that is in turn inside containers that can be made of plastic, cardboard, glass, or multicomponent (Chawla, Sivakumar and Kaur, 2021).

Antimicrobial packaging systems can take the following forms (Appendini and Hotchkiss, 2002; Fernández *et al.*, 2008; Kim *et al.*, 2008; Robertson, 2013; Fang *et al.*, 2017; Sofi *et al.*, 2018):

1. Addition of sachets or pads containing volatile antimicrobial agents into packages. The sachets or pockets are enclosed into the inner part of the package and employ volatile substances that inhibit microbial growth when released into the food environment.

2. Incorporation of volatile and non-volatile antimicrobial compounds directly into polymers matrix. The active material is generally processed by extrusion, compression, or injection, which requires thermal and shearing forces, conditions that can affect the stability and activity of the antimicrobial agent.

3. Coating or adsorbing antimicrobials onto polymer surfaces. The polymeric material acts as a carrier of the antimicrobial additives, which are coated onto the film as a thin layer by soaking or spread. Volatile compounds are released into the package headspace through evaporation and non-volatile substances migrate into the food product through diffusion. Weaker unions such as hydrogen bonds, ionic or hydrophobic interactions, or the Van der Waals forces are responsible of the adsorption onto film surfaces.

4. Immobilization of antimicrobials to polymers by ionic or covalent linkages. This procedure reduces the amount of antimicrobial agent added to the polymer matrix, maintaining its

concentration and antimicrobial activity, and moderating migration into the food. The type of bonding controls the release of active agents from the matrix.

5. Polymers that are inherently antimicrobial. This option requires direct contact between antimicrobial polymer material and the food product for effective inhibition. The solubility and diffusivity of the polymer in the food must be considered, which requires its approval as food additive.

For each application, the most suitable form must be evaluated.

8.3.2 Mechanism of antimicrobial action of the package

Films with antimicrobial activity used as a food packaging system can be classified into two groups: (I) films that allow the antimicrobial to migrate into food and (II) films that do not release antimicrobial substances and that inhibit microbial growth on the surface of food (Suppakul *et al.*, 2003; Contreras *et al.*, 2021). The antimicrobial substance must be in contact with the food surface and must be maintained above the minimal inhibitory concentration for the target microorganisms during the shelf-life of the product for effective biostatic or biocidal action.

Some factors can affect the performance of the antimicrobial packaging system such as the interaction between the antimicrobial substance and the packaging material or the effect of the film production process on the antimicrobial activity. In addition, the antimicrobial substance can interact with food components (protein, carbohydrate, lipids, etc.) causing the inactivation or elimination of the biocide activity. This was observed even when antimicrobial action was confirmed by in vitro analysis (Türe *et al.*, 2009). (Ahmed *et al.*, 2020)

The transference of the antimicrobial from the packaging material to a particular food and its mechanism of action must be studied in detail for each system to obtain a complete description of the phenomena involved in the inactivation of pathogens that can affect that food. Different parameters such as a) partition coefficient of the active agent in the different phases; b) diffusion phenomenon of the active substance through the packaging material; c) volatilization of the antimicrobial into the headspace (if the substance is volatile); d) solubility and diffusivity of the antimicrobial into the food, especially if it directly interacts with the food, must be considered in this study.

In the case of packaging materials formed by a film matrix and a coating or multilayer film that release the antimicrobial agent on the food and the headspace, the mass transport process involves equilibrium and kinetic phenomena. The equilibrium phenomena are related to the partition coefficient of the active compound between the film matrix and the coating, the phases of a multilayer film packaging, and the inner packaging layer or coating and the food. The partition coefficient is a quantitative parameter that represents the relative solubility of a given substance in a system composed of two phases that are immiscible with each other, at a specific temperature. Ideally, materials should be selected to reduce the loss of the antimicrobial agent by retention in the packaging material, to increase the concentration on the food surface (López-Carballo *et al.*, 2012). In addition, a the high solubility of the antimicrobial into the food matrix generates a rapid penetration into the food, producing a decrease of the active concentration on the food surface. On the contrary, a low solubility causes the accumulation of the antimicrobial on the food surface, improving protection and reducing the migration of the substance into the food matrix (Cutter, Willett and Siragusa, 2001).

On the other hand, the kinetic factors associated with the mass transference process involve an appropriate control of the antimicrobial compound diffusion from the packaging system to the

food surface. It can help to retard or inhibit the initial growth of the pathogens on the food surface, but can also create a prolonged release or a residual activity that remains during food storage and distribution (Han and Floros, 1997; Cutter, Willett and Siragusa, 2001; Nerin *et al.*, 2016).

The knowledge of the diffusion process can be used to determine the concentration of antimicrobial in the packaging system to guarantee the necessary amount to maintain the antimicrobial levels above the minimum inhibitory concentration (Contreras *et al.*, 2021). The composition of each phase and other chemical and physical factors affect the diffusion process. The hydrogen bonds, ionic bonds, ionic osmosis, hydrophobic interactions, electrostatic interactions are some of these factors that influence the antimicrobial diffusivity through the packaging systems and the food. The diffusion process also depends on some physical factors such as Tg, free volume, crystallinity, porosity, and the presence of micro or nanoparticles that provide a modification of the tortuosity in the diffusive pathway of the antimicrobial agent through the package. Moreover, due to the complicated composition of foods and the different environmental conditions to which they are exposed during the different stages of their storage (pressure, temperature, and time combinations), a critical study must be performed to evaluate the type and concentration of the appropriate antimicrobial substance for specific food (Chawla, Sivakumar and Kaur, 2021). The kinetic of the antimicrobial release can be decisive for the viability and application of the antimicrobial packaging system for a particular food. If the release of the antimicrobial substance is slower than the microbial growth rate, the active packaging system becomes useless. On the contrary, a fast release can exceed allowable levels of the antimicrobial in the food initially and shorten the period of the antimicrobial action of the container.

The migration of antimicrobial agents can be divided into two categories, controlled and uncontrolled release systems. Uncontrolled release packaging systems have widespread use in food applications. However, controlled release systems may be more relevant in these applications due to their ability to prevent sensory or toxicological problems or generate inefficiency of the system, caused by too high or too low concentration of the released substance. The main source of knowledge of controlled release technology comes from the pharmaceutical sector (Mastromatteo *et al.*, 2010). The release mechanics are based on different mechanisms such as diffusion, swelling, dissolution, or degradation of the substance through the matrix to the target point. Depending on whether the mechanism responds to Fick's laws of diffusion, the mechanisms are divided into Fickian and non-Fickian (Chawla, Sivakumar and Kaur, 2021).

Generally, the active agent release mechanism can be described through three phenomena (Langer and Peppas, 1983):

Reservoir system: The active agent is contained in reservoirs that work like a rate-controlling barrier of release. The barriers can present different morphologies like micro-porous, macro-porous, or non-porous (dense films). The release rate depends on several variables such as thickness, area, and permeability of the barrier. The rate-limiting step is the diffusion of the active substance through the polymeric barrier.

Swelling-induced release: These systems are characterized by the low diffusivity of the active substance in the polymeric matrix of the antimicrobial packaging. When the matrix is in contact with a compatible liquid medium, the film matrix swells due to the absorption of the fluid. The diffusion coefficient of the active agent in the swollen part of the matrix increases and then diffuses through the packaging system and can act on the food. In these cases, the antimicrobial

must have a high solubility in the fluid. Diffusion is possible due to the existence of a swollen zone of the polymer that allows the transfer of the antimicrobial. In swelling-controlled systems, the film must change from a glassy state to a rubbery state when it interacts with the food system. The release rate is determined by the glass-to-rubber transition process.

Degradation-induced release: Two different erosion mechanisms have been proposed: a) superficial or heterogeneous erosion and b) bulk or homogeneous erosion. The erosion is superficial when the degradation of the polymer is faster than the absorption of the solvent in the polymer mass. Degradation occurs mainly in the outer layers of the polymer, thus affecting only the surface and not the internal parts of the matrix (heterogeneous process). Instead, bulk erosion occurs when the absorption of the solvent (generally water) by the system is much faster than polymer degradation. In this case, the entire system is rapidly hydrated, and the polymer chains are cleaved. Thus, the erosion is not limited to polymer surfaces (homogeneous process). Generally, polymers that are built from highly reactive functional groups tend to degrade rapidly and to be surface eroded, whereas polymers containing less reactive functional groups tend to be bulk eroded (Langer and Peppas, 1983).

8.4 ANTIMICROBIAL SUBSTANCES FROM MICROBIAL SOURCES

Natural bioactive compounds are produced by living organisms either as primary metabolites, with an essential function in cell life processes (growth, development, and reproduction), or as secondary metabolites, which are not directly involved in primary metabolic processes but usually have other important function, as defense compounds, signaling molecules, among other (Ali, Siddiqui and Khan, 2018). Some of these naturally occurring metabolites can inhibit microbial growth, being considered natural antimicrobials. They have been recovered from different sources including plants (fruits, vegetables, seeds, herb, and spices), animals (eggs,

milk, and tissues), and microorganisms (bacteria, fungi, and viruses) (Lucera *et al.*, 2012; Pisoschi *et al.*, 2018; Quinto *et al.*, 2019).

In general, the term "preservatives" refers to food-grade compounds that inhibit or prevent microbial detrimental growth in food products (Jayant and Halami, 2020). Particularly, the use of natural preservatives for the removal of undesirable microorganisms in food with the aim of reducing its level of processing, improving its safety, and extending its useful life, is known as "biopreservation" (Gyawali and Ibrahim, 2014; Pisoschi *et al.*, 2018; Quinto *et al.*, 2019). In turn, about antimicrobial compounds of microbial origin, Montville & Chikindas (2007) define "biopreservation" as the use of microorganisms (including bacteriophages), their metabolic products, or both to preserve foods that are not generally considered fermented (Montville and Chikindas, 2007).

Hence, antimicrobials of natural origin emerge like a new perspective for food preservation, since they can exert specific inhibition against foodborne pathogens, not only ensuring food safety and quality but also extending product shelf-life and reinforcing consumers' confidence. These natural compounds also arise like harmless substitutes to detrimental chemical synthetic preservatives, which in some cases can be carcinogenic and mutagenic, among other negative effects (Achi and Halami, 2016; Pisoschi *et al.*, 2018). Furthermore, natural antimicrobials strengthen as a viable option to microbial resistance caused by antibiotics misuse, that led to the development of multidrug-resistant microorganisms, including foodborne pathogens which become resistant to commonly used antibiotics and to conventional food processing and preservation techniques (Gyawali and Ibrahim, 2014; Pisoschi *et al.*, 2018; Quinto *et al.*, 2019).

The mode of action observed for natural antimicrobials include cell lysis, cell membrane rupture, interference of nucleic acids mechanisms, the decay of the proton motive force, and

depletion of adenosine triphosphate (ATP) (Pisoschi *et al.*, 2018; Quinto *et al.*, 2019). When selecting natural antimicrobials as food biopreservatives, their inhibitory spectrum should be considered, since it is desirable, they exert a specific antagonistic effect against foodborne pathogens, with minimal consequences on desirable or beneficial food microorganisms. Additionally, nowadays there is growing concern about bioactive compounds not to influence negatively the consumer's own microbiome (Pisoschi *et al.*, 2018).

The complexity of food matrices can affect or interfere with natural antimicrobial compounds´ activity (Quinto *et al.*, 2019). Therefore, biopreservation techniques could render more effective when a mixture of two or more natural antimicrobials is applied in combination with other food preservation techniques, achieving a synergistic effect. “Hurdle technology” has gained attention in the food industry since final costs production get diminished and minimal impact on the nutritive value and sensory properties of foods is accomplished, together with enhanced food safety (Deegan *et al.*, 2006).

Although the natural origin of biopreservatives positions them as generally recognized as safe (GRAS), their use in several countries remains unregulated and their incorporation as an ingredient in food will require, in many cases, legal approval (Lucera *et al.*, 2012; El-Saber Batiha *et al.*, 2021). Hence, proper safety regulations should be established and uniformed worldwide to benefit natural preservatives application with a growing and expanded market in the food industry (Pisoschi *et al.*, 2018).

8.4.1 Classification, sources, and inhibitory spectrum

Most commonly antimicrobials synthesized by different microorganisms are described below, with special attention to those compounds that have been immobilized in the polymeric matrix and/or applied as food bioactive packaging or coatings.

Bacteriocins

Bacteriocins comprise a heterogeneous group of small ribosomal synthesized proteinaceous molecules excreted by a wide variety of Gram-positive or Gram-negative bacteria that inhibit or stop the growth of other bacteria at precise concentrations (Chikindas *et al.*, 2018). These antimicrobial peptides have a bacteriostatic or bactericidal effect mainly against bacteria closely related to the producing strain or more rarely against other groups of bacteria (Cotter, Ross and Hill, 2013; Soltani *et al.*, 2021).

Bacteriocins are mostly synthesized as non-biologically active precursor peptides that undergo significant posttranslational modifications before cleavage of the leader region and extracellular release (Soltani *et al.*, 2021). These modifications also differentiate their antimicrobial spectrum of activity (Jayant and Halami, 2020). Besides, bacteriocin-producing cells are immune to themselves, since they also set up the production of an “immune protein” (de Freire Bastos, Varella Coelho and da Silva Santos, 2015).

Most bacteriocins act by forming selective pores or channels in target microorganism membrane cells, resulting in increased permeability and consequently leakage of low molecular mass intracellular components and ions with the disruption of the proton motive force and depletion of intracellular ATP, and eventual death (Pisoschi *et al.*, 2018; Kumariya *et al.*, 2019).

Several bacteriocins have been described and shown to be effective against many foodborne pathogenic bacteria. However, bacteriocins synthesized by lactic acid bacteria (LAB) find particular interest as natural and safe biopreservatives in the food industry, due to the GRAS status of the producing strains (Balciunas *et al.*, 2013). Bacteriocin information has been assembled in different databases, like Bactibase, Bagel, or LABiocin, which are available online and facilitate comparison between different bacteriocins (peptide sequence, inhibitory spectrum, physicochemical properties, etc.) (Said *et al.*, 2019).

Among the most relevant bacteriocins produced by LAB in food preservation, the following can be mentioned:

Nisin: Nisin is synthesized by certain *Lactococcus lactis* subsp. lactis strains. This positively charged heat-stable peptide belongs to Class I bacteriocins and has 34 amino acids with a 3.5 kDa molecular mass. Nisin has antimicrobial activity against a broad spectrum of Gram-positive bacteria, including lactic acid bacteria, pathogens such as *Listeria*, *Staphylococcus*, and *Mycobacterium*, and spore-forming bacteria, such as *Bacillus* and *Clostridium*. However, it is less effective against Gram-negative bacteria, yeasts, and molds (Jagus, Gerschenson and Ollé Resa, 2016; Pisoschi *et al.*, 2018; El-Saber Batiha *et al.*, 2021). Its antimicrobial action is related to the adsorption of the negatively charged phospholipids on the cell membrane and subsequent rupture of the membrane by the formation of pores. Thus, only Gram-positive bacteria are affected by nisin. However, Gram-negative bacteria can be inhibited by nisin when they are affected by some processing technologies like heating, freezing, or chelating agents that may cause the permeabilization of their outer membrane (Campos *et al.*, 2016).

Nisin A was the first isolated form of this antimicrobial peptide, while other natural variants have been described differing by up to 10 amino acids from the nisin A sequence (nisin Z, Q,

F synthesized by *L. lactis*; nisin U1, H, P, produced by *Streptococcus* strains, and nisin O detected in *Blautia obeum*) (Jayant and Halami, 2020). Currently, nisin represents the only bacteriocin approved as a food additive in more than 50 countries (Delves-Broughton and Weber, 2011).

Pediocin: Pediocins are synthesized by *Pediococcus* strains (*P. acidilactici* and *P. pentosaceus*). They are thermostable small peptides and maintain their functionality over a broad pH range. Pediocins primarily inhibit the growth of Gram-positive pathogens, such as *L. monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Clostridium perfringens* (Pisoschi *et al.*, 2018; Villalobos-Delgado *et al.*, 2019; El-Saber Batiha *et al.*, 2021). Pediocin PA-1/AcH is commercialized as ALTATM2341 and MicroGARDTM, a fermented lactic acid bacteria dried powder with GRAS character, although not approved as a food additive (Balciunas *et al.*, 2013).

Reuterin: Reuterin (β-hydroxypropionaldehyde), is produced by *Lactobacillus reuteri*. It is water-soluble, thermoresistant, active over a broad pH range, and resistant to proteolytic and lipolytic enzymes (Gyawali and Ibrahim, 2014; Pisoschi *et al.*, 2018; Villalobos-Delgado *et al.*, 2019). Reuterin modifies thiol groups in protein and small molecules, inducing oxidative stress in cells by eventually causing cell death (Gómez-Torres *et al.*, 2016). This bacteriocin presents a broad spectrum of antimicrobial activity towards several pathogenic and spoilage microorganisms, including Gram-positive and Gram-negative bacteria, yeasts, and molds (Montiel *et al.*, 2016). Inhibitory activity has been reported against *L. monocytogenes*, *Campylobacter jejuni*, *S. aureus*, *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, and *Yersinia enterocolitica* (Pisoschi *et al.*, 2018; El-Saber Batiha *et al.*, 2021).

Natamycin

This antimicrobial, also known as pimaricin, is a macrolide antifungal polyene with a molecular weight of 665.7 Da, produced by fermentation of *Streptomyces* species (*S. natalensis*, *S. chmanovgensis*, and *S. Gilvosporeus*) (Hammond and Lambert, 1978; Duchateau & van Scheppingen, 2018). Natamycin acts by binding irreversibly to ergosterol, thus disrupting specifically the fungal cell membrane leading to cytoplasm efflux (Carocho, Morales and Ferreira, 2015). Therefore, it is active against almost all foodborne yeasts and molds, but not against bacteria, viruses, or protozoa (Jagus, Gerschenson and Ollé Resa, 2016). It has approval and is used as a food additive in over 40 countries, mainly in dairy-based food products, especially in hard cheese and salami-type sausages for preventive control of fungi spoilage (Carocho, Morales and Ferreira, 2015; Pisoschi *et al.*, 2018).

Bacteriocins and lipopeptides from Bacillus

Bacillus genus is known to produce a large scale of bioactive molecules including enzymes, antibiotics, insecticides, and antimicrobial substances, like lipopeptides and bacteriocins (Stein, 2005; Abriouel *et al.*, 2011; Meena and Kanwar, 2015; Achi and Halami, 2016). Despite its technological potential, the genus *Bacillus* has not been widely used in the food industry since not all species are recognized as GRAS. However, some representatives, such as *B. subtilis* and *B. licheniformis* are found safe for use in the food and agricultural industry (Abriouel *et al.*, 2011; Nithya, Murthy and Halami, 2013; Kourmentza *et al.*, 2021). Moreover, antimicrobials from *Bacillus* are gaining significance and becoming increasingly important due to their broader spectrum of inhibition (in comparison with nisin or other LAB bacteriocins), with activity against Gram-positive and Gram-negative bacteria, yeast, and fungi; also, they have better heat and pH stability (Stein, 2005; Abriouel *et al.*, 2011; Achi and Halami, 2016).

Based on the biosynthetic pathway, *Bacillus* antimicrobials can be classified into two categories. The first group includes ribosomally synthesized peptides, among which are bacteriocins such as subtilin, ericin S, and subtilisin (Stein, 2005; Abriouel *et al.*, 2011; Achi and Halami, 2016; Jayant and Halami, 2020). While the second group comprises non ribosomally synthesized peptides, such as lipopeptides, constituted by amphipathic cyclic structures of hydrophilic peptide sequences, of usually 7 to 10 amino acids long, with a hydrophobic C13–C18 fatty acid chain (Achi and Halami, 2016; Villalobos-Delgado *et al.*, 2019). The better-characterized lipopeptides are surfactin, iturin, and fengycin (Meena and Kanwar, 2015; Kourmentza *et al.*, 2021). The use of these cyclic ring-structure lipopeptides can reduce protease sensitivity affecting antimicrobial peptides and advantages their future application in the food additives market (Meena and Kanwar, 2015).

ε-Polylysine

This antimicrobial is a cationic poly-amino acid formed by 25–30 lysine residues produced by different species belonging to *Streptomyces*, *Kitasatospora* genus, among others (Shukla *et al.*, 2012; Zhang *et al.*, 2019). It has FDA approval as a natural antimicrobial food additive according to GRN Nº 336 as an antimicrobial agent in a wide variety of food categories at levels of up to 0.025%$_{w/w}$ since 2010 and with GRN Nº 135 for use as an antimicrobial agent in cooked rice and sushi rice at levels from 5 to 50 ppm since 2003 and has been mainly used in Japan, Korea, and USA (Chheda and Vernekar, 2015). ε-Polylysine is common in food applications like boiled rice, cooked vegetables, soups, noodles, and sliced fish (sushi).

The e-polylysine has a strong inhibitory activity against a wide range of Gram-positive and Gram-negative bacteria, including *Bacillus coagulans*, *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium, yeasts, and molds, such as *Aspergillus niger*, *Trichophyton*

mentagrophytes, *Candida* spp., and *Phaggia rhodozyma*, and against bacteriophages (Lopez-Pena and McClements, 2015). The high cationic charge density of this polymer allows its absorption onto anionic microbial cell surfaces due to electrostatic interactions, damaging the outer membrane of bacteria, leading to the disruption of the cytoplasm, cellular content efflux, and eventually cell death. Nevertheless its good water solubility, these highly charged molecules can also interact with various components of complex food matrices, such as binding with acidic polysaccharides, hydrochlorides, phosphates, copper ions, or others, reducing their antibacterial activity and restricting their range of application in the food industry (Lopez-Pena and McClements, 2015; Villalobos-Delgado *et al.*, 2019). On the contrary, its activity is not affected by pH and is stable when heated (120°C for 20min). Therefore, it can be sterilized along with the raw material. ε-Polylysine antibacterial activity can be enhanced with hydrochloric acid, citric acid, malic acid, glycine, and higher fatty glycerides.

8.4.2 Techniques for measuring antimicrobial packaging action

Different methods have been used to assess the antimicrobial efficiency of active films. Generally, this antimicrobial activity is determined in culture media and/or in food models to establish the effect of the active film against foodborne pathogens and spoilage microorganisms. Even though each author applies different procedures or analytical conditions according to their own antimicrobial-packaging-target microorganism-food system, and there are no standards established to test the antimicrobial performance in vitro or in food systems (Abdollahzadeh, Nematollahi and Hosseini, 2021; Moradi *et al.*, 2021). The most used techniques and some analytical variables are presented below.

In vitro assays in culture media

***Agar-based methods**: Disc diffusion* and *well diffusion* are among the most common methods, in which the antimicrobial activity is assessed against sensitive or target cells grown on an agar layer (Moradi *et al.*, 2021). These are low-cost, simple, and flexible methods, but they only detect the presence or absence of antimicrobial activity, hence they are considered qualitative procedures (Abdollahzadeh, Nematollahi and Hosseini, 2021). Briefly, in the *disc diffusion* technique, a sterile plate with a solidified general non-selective agar is inoculated with the target microorganisms, and a piece of sterile disk or square-shaped film, aseptically cut, is placed on the agar. The plates are incubated and the development of zones of inhibition on the lawn surrounding film discs is observed (Abdollahzadeh, Nematollahi and Hosseini, 2021; Moradi *et al.*, 2021).

The *well diffusion* technique is preferably used when assessing the antimicrobial activity of antimicrobial polymer casting solutions. The target microorganisms are inoculated on the surface of a sterile agar plate and 5-8 mm diameter wells are cut on the agar with a sterile punch. The antimicrobial studied diffuses through the agar; so, after incubation, inhibition zones are measured around the wells (Balouiri, Sadiki and Ibnsouda, 2016; Abdollahzadeh, Nematollahi and Hosseini, 2021).

Also, the *disk volatilization method* can be used when volatile antimicrobials are incorporated in the active films, the *agar spot diffusion* technique is appropriate for high viscosity antimicrobial solutions, while the *parallel streak method* can be used when handling leaching antimicrobials (Moradi *et al.*, 2021).

The choice of the procedure depends on the antimicrobials and packaging matrix under study. Several parameters should be considered, such as quantity or size of antimicrobial material, incubation time/temperature, target microorganism, culture medium, and inoculum

concentration (Dafale *et al.*, 2016). Regarding the target microorganisms, different culture agar media have been used (TS, nutrient agar, BHI, or LB), but MH (for bacteria) and YGC (for fungi) agar are preferred for antimicrobial assays (Moradi *et al.*, 2021). The agar plate is generally seeded with an inoculum of ca. 6 log CFU mL^{-1}, however, doses between 5-8 log CFU mL^{-1} have been used (Abdollahzadeh, Nematollahi and Hosseini, 2021).

Biopolymeric antimicrobial matrices are aseptically cut generally into 6 to 16 mm diameter discs or 1 cm x 1 cm squares and sterilized prior to being placed on the agar surface. According to the antimicrobials and polymer characteristics, the active packaging can be sterilized by autoclaving (121 °C for 15 min), exposing both sides of the films under UV light (254 nm for 2–10 min) or immersing in ethanol (15 min), in the case of synthetic polymers. Casting solutions also can be sterilized by filtration (0.45 mm sterile syringe filter) (Abdollahzadeh, Nematollahi and Hosseini, 2021; Moradi *et al.*, 2021). After incubation, the inhibition zones surrounding the films loaded on the agar surface are observed and measured in millimeters (mm). Some authors subtract the whole inhibition diameter from the diameter of the film or well, while others report the whole inhibition zone diameter (Moradi *et al.*, 2021). Also, the Antimicrobial index calculated as Z – F / F, where Z and F are inhibition zone areas surrounding and under the film discs, respectively, can be determined (Ahmad *et al.*, 2012). The antimicrobial effect of the active films can be classified according to the size of the inhibition diameter, but there is no consensus in this classification and the results will vary according to each experimental condition. For example, the response to edible coatings con be classified as: not sensitive (-) for diameters < 8 mm; sensitive (+) for diameters 9–14 mm; very sensitive (++) for diameters 15–19 mm; and extremely sensitive (+++) for diameters > 20 mm (Ponce *et al.*, 2003).

***Viable cell count method**:* This method allows the study of the antimicrobial release profile as well as the effectiveness of the inhibitory activity in direct contact with target cells in aqueous media. Again, there are several factors to consider (bacterial/fungal type and inoculum, type of culture medium, incubation time, film characteristics, among others), and although this is an in vitro assay, experimental conditions should be settled as similar as possible to mimic the food matrix in which the active film could find application.

In general, a volume of broth medium (ca. 1-50 mL) and appropriate pieces of antimicrobial film cut aseptically (for example, 1×1 cm) are inoculated with ca. 6 log UFC mL^{-1} of target microorganism and incubated at the optimum temperatures and time for the microbial growth (up to 48 h for bacteria and yeast and 4-7 days for mold). Incubation under agitation is recommended, and samples are taken out at different time intervals to prepare serial dilutions and determine cell viability by agar plate counting (Moradi *et al.*, 2021).

This is considered a semi-quantitative method when it is conducted in a broth culture medium, but more accuracy can be obtained when a neutral solution (e.g., saline solution, peptone water, or PBS buffer) in the absence of a nutrient source is used. Also, the dimension of the antimicrobial film disposed for the assay, as well as the ratio between the antimicrobial film surface and the culture media volume are important parameters. The assay can be aseptically conducted using tubes, Erlenmeyer flasks, or well plates. The more common ratio used is 1x1 cm films per 1-10 mL of broth, but another film/culture media ratio was analyzed; even, the desired amount of active film is sometimes weighted (Moradi *et al.*, 2021). The inclusion of microbial suspension controls, alone and in contact with films without antimicrobial addition, is mandatory. In some cases, free antimicrobial solutions are also included as controls to compare the effect of free against immobilized-released antimicrobial inhibition capacity. This assay renders log CFU mL^{-1} values, and growth/survival curves can be plotted to analyze

results. Also, viable cell reductions or antimicrobial activity can be determined as follows (Moradi *et al.*, 2021):

- Cell viability reduction (%) = $[(V_0 - V_t) / V_0] \times 100$, where V_0 and V_t are average viable CFU mL^{-1} at t_0 (initial time) and t (time in which the % is calculated), respectively.
- Antimicrobial activity (%) = $[(V_c - V_t) / V_c] \times 100$, where V_c and V_t are average viable CFU mL^{-1} in the inoculum control sample and treatment at the time t, respectively.

Optical density-based methods: In the spectrophotometric method, the growth of the target bacteria in contact with the active film in broth culture media is monitored by optical density measurements (OD; 600 nm). Even this methodology is considered faster and less expensive than viable cell count, it presents some disadvantages. Both dead and live bacteria are detected by spectrophotometric determinations, and certain colored antimicrobials or polymer film matrices can interfere with the measurements (Abdollahzadeh, Nematollahi and Hosseini, 2021).

Food models

Although in vitro tests of the inhibitory effect of bioactive coatings are a necessary first stage of the study, it is desirable to test the application of the coating or packaging directly on the food to analyze all the factors that complex food matrices can introduce on the antimicrobial activity of the metabolite under study.

When designing an active food packaging, in advance it will be oriented to be applied on a specific food product or category and for the inhibition of certain pathogen or spoilage microorganism. Generally, the antimicrobial film will be applied or introduced to the selected food in several ways, including spraying, immersion, panning, and brushing of the coating solution. The antimicrobial effect can be monitored by artificially contaminating the food

systems with pathogens or spoilage microorganisms, applied individually or in pools of strains. Usually, microbial suspensions can be added to food by mixing, dipping, or surface inoculation (Hu and Gurtler, 2017). Although a microbial inoculum of 5-7 log CFU g^{-1} is generally used in this type of assay, to work with a more realistic contamination grade occurring in food, an inoculum of ca. 2 and 3 log CFU g^{-1} is recommended (National Advisory Committee on Microbiological Criteria for Foods, 2010). The incubation conditions and the duration of the experiment will be determined by the active antimicrobial matrix and the food characteristic and shelf-life. The procedure basically consists of sampling a portion of the contaminated coated food in time intervals and preparing a homogenate, serially diluting, and determining the survival of each microorganism by plate coating in an adequate agar medium. A reduction of at least 2 log in the viability of the target microorganism is desired and indicative of potential practical significance for the system under study (National Advisory Committee on Microbiological Criteria for Foods, 2010). Controls of food systems without antimicrobial or non-active coatings should be included. Asepsis and biosafety procedures may be maintained in all stages of the experiment (Moradi *et al.*, 2021). Some examples of the application of antimicrobial packaging, including antimicrobials of microbial origin as active components, in food models are listed in Table 1.

8.5 APPLICATIONS

In the scientific literature, there are numerous studies of the performance of antimicrobial substances of microbial origin in materials or containers intended for food packaging, mainly based on the use of authorized and commercially available compounds, such as nisin and natamycin (Table 1).

Nisin is a bacteriocin commonly used in food packaging because of its GRAS status. Nisin is frequently used within polymers, however, its antimicrobial effect can be reduced due to the processing temperatures of polymer manufacturing. The decreased mechanical resistance (tensile strength) of polymers containing nisin could be compensated by using stronger outer packaging layers.

Nisin is more effective in processed foods with a low charge of proteolytic enzymes, such as dairy products and processed vegetables. This antimicrobial is a cationic and hydrophobic bacteriocin that is most stable in high-acid foods. Other preservative hurdles such as heat treatment, low water activity, modified atmosphere, refrigeration, low pH, and the presence of other natural or chemical preservatives such as lysozyme, chitosan, and acids, can enhance antimicrobial activity (Delves-Broughton and Weber, 2011).

The use of nisin-activated plastic packaging combined with chill temperatures proved to be effective in enhancing the microbiological quality of beef cuts by reducing the spoilage populations but without affecting the species diversity (Ercolini *et al.*, 2010). Similarly, it was observed that the application of nisin through plastic interleavers combined with 400 MPa high hydrostatic pressure treatment on cooked ham stored at 6ºC, appears as an effective combination of hurdles to obtain value-added ready-to-eat products with safe long-term storage (Jofré, Garriga and Aymerich, 2007; Jofré, Aymerich and Garriga, 2008). On the contrary, Marcos *et al.*, (2013) found that when *L. monocytogenes* was inoculated on the surface of sliced fermented sausages without added sodium salt, antimicrobial packaging (PVOH films containing nisin) induced a pronounced reduction in pathogen counts during refrigerated storage. However, the high hydrostatic pressure treatment did not have an antimicrobial effect against *L. monocytogenes* by itself, nor did it improve the performance of the antimicrobial

packaging, under the conditions studied. The authors attributed this to the protective effect exerted by the low water activity of the product and its lactate content (Marcos *et al.*, 2013).

Nisin has also shown to be effective in overcoming the problems associated with *L. monocytogenes* contamination after processing vacuum-packaged cold-smoked salmon and reducing initial spoilage microbiota counts (aerobes, anaerobes, and lactic acid bacteria) of samples that were vacuum packed with nisin-coated LDPE plastic films and stored at 4°C (Neetoo *et al.*, 2008; Ye, Neetoo and Chen, 2008). On the other hand, the incorporation of nisin and oyster lysozyme into edible calcium alginate films was able to retain the effectiveness of the antimicrobial agents in the smoked salmon during 35 days of storage, making it more effective than when they have applied alone on the samples (Datta *et al.*, 2008) .

Pediocin is a bacteriocin particularly effective against *Listeria monocytogenes* and which has stability across wide pH and temperature ranges. Applying combined bacteriocins onto plastic food-packaging films was an effective way to inhibit *L. monocytogenes* in fresh and processed meat and poultry. Nisin and pediocin were successfully applied to cellulose casings without diffusing out during frankfurter processing. The bags coated with pediocin completely inhibited the growth of inoculated *L. monocytogenes* through 12 weeks of storage at 4ºC (Ming *et al.*, 1997). The antimicrobial efficacy of cellulose-based films containing pediocin (25 and 50%) against *Listeria innocua* and *Salmonella sp.* in sliced ham was tested by means of an experiment that consisted of overlapping the slices of contaminated ham with the antimicrobial film. The 50% pediocin-film showed a reduction of 2 log cycles in the growth of *L. innocua* and 0.5 log in the growth of *Salmonella sp.* in relation to the control treatment after 15 days of storage at 12ºC in vacuum package (Santiago-Silva *et al.*, 2009). In that regard, a U.S. patent 5,573,797 assigned to Viskase Corp. describes a packaging film formed by a polymeric film or a regenerated cellulosic film, containing a heat-resistant *Pediococcus*-derived bacteriocin in a

synergistic combination with a chelating agent to inhibit or kill *Listeria monocytogenes* on contact with food (Darrel, 1996).

Natamycin is widely used in the food industry. It has reduced diffusivity from traditional packaging into food. Thus, its use is limited to structures with minimal barrier properties like biopolymers. In this sense, Fajardo *et al*. (2010) evaluated the effect of the application of chitosan coating containing 0.50 mg mL^{-1} of natamycin on the physicochemical and microbial properties of semi-hard cheese. They found a decrease in molds/yeasts of 1.1 log (CFU g^{-1}) compared to control after 27 days of storage and an increase in O_2 and CO_2 permeabilities (Fajardo *et al.,* 2010). Furthermore, Egyptian Romy cheese wrapping with cellulose sheets fortified with Natamycin-loaded alginate nanoparticles was evaluated as a way of controlling the growth of toxigenic *Aspergillus flavus* and subsequent aflatoxin production. The antimicrobial sheets were sufficient to complete the elimination of 5 log CFU g^{-1} *A. flavus* initially inoculated and the reduction of aflatoxin production by 79%, without affecting the original flavor, color, and overall appearance of traditional Romy cheese (Fayed, Elsayed and Ali, 2021).

Bacteriocins produced by *Lactobacillus curvatus* were incorporated in plastic packaging for the preservation of different meat products (pork steak, ground beef, frankfurters, wieners, and smoked salmon) to control *Listeria monocytogenes* contamination. The results showed that the films activated by soaking, spraying, or coating with these bacteriocins were effective in inhibiting the growth of the pathogen on the food surface (Mauriello *et al.*, 2004; Ercolini *et al.*, 2006; Ghalfi *et al.*, 2006; Blanco Massani *et al.*, 2014).

Enterocins synthesized by *Enterococcus faecium* and *Enterococcus avium* have also been used to control *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Bacillus*

cereus in cheese and ham using activated plastic and edible film packaging. The results showed an effective control and delay of the growth of pathogens using enterocin concentrations ranged between 250 AU cm^{-2} and 2000 AU cm^{-2} of the film (Jofré, Garriga and Aymerich, 2007; Marcos *et al.*, 2007; Ibarguren *et al.*, 2015; Guitián *et al.*, 2019).

Another approach to food protection involves incorporating live bacteriocin-producing bacteria into the package. In a study conducted with smoked salmon, films with LAB strains or a combination of both strains and nisin had a bacteriostatic effect on *L. monocytogenes* during 28-day refrigerated storage. (Concha-Meyer *et al.*, 2011).

The overall antimicrobial packaging market size is estimated to grow from USD 9.57 billion in 2018 to USD 17.55 billion in 2026, according to the Fortune Business Insights report on this topic (Fortune Busines Insights, 2021). This is driven by the growth of the packaging market, globally. Antimicrobial packaging is widely used in the food industry because of the rising consumer demand for products that are perishable, preservative-free, and minimally processed, and due to the need to increase the shelf-life of the products. The main companies in the market are BASF SE (Germany), The Dow Chemical Company (U.S.), Mondi Plc (South Africa), PolyOne Corporation (U.S.), Biocote Limited (U.K.), Dunmore Corporation (U.S.), Linpac Senior Holdings (U.K.), Microban International (U.S.), Oplon Pure Sciences Ltd. (Israel), and Takex Labo Co. Ltd.

Despite the numerous publications on antimicrobial packaging based on antimicrobials of microbial sources, the commercial applications of this type of active packaging systems are limited. Among them, Standa Laboratories offers antifungal coating for the food industry (cheese and sausage rinds) through the Sanico® brand. The product is based on natamycin as active compound (Standa, 2021). Also, JANSSEN PMP (https://www.janssenpmp.com), a

division of Janssen Pharmaceutica NV, has announced the signing of a collaboration agreement with Lipofabrik SAS (http://www.lipofabrik.com), a French company specialized in the production, purification, formulation, and commercialization of lipopeptides. Janssen PMP will develop products containing Mycosubtilin, a lipopeptide from Lipofabrik SAS, to preserve the quality of fresh produce in the global market.

8.6 REGULATIONS

In developing a new antimicrobial packaging system, regulatory compliance is almost as important as its effectiveness. The United States (USA) and the European Union (EU) have established strict regulations related to active food packaging. Active packaging is considered active material in the UE and is subjected to regulations EC 1935/2004 (European Commission, 2004) and EC 450/2009 (European Commission, 2009). In addition, the active substances used in the active packaging materials must be identified and the information on their permitted uses must be presented together with the data related to the maximum amount of substance released from the packaging material (European Commission, 2009). In the USA, the FDA must approve the food packaging system prior to marketing when it is intended to be used in contact with food. This normative is also valid when the active substance is considered a food additive. Title 21 of the Code of Federal Regulations (21 CFR) Part 172 and 173, includes approved food additives that are derived from microorganisms (FDA, no date b).

Nisin and natamycin are among the few antimicrobials of microbial origin accepted for use in food contact applications in the USA and EU. Nisin is considered by the U.S. Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) since 2000 according to GRAS Notice GRN Nº 65 (FDA, 2000) for intended use on casings for frankfurters and on

cooked meat and poultry products as an antimicrobial agent. FDA also authorizes the use of nisin preparations as antimicrobial in cheese up to a concentration of 250 ppm (FDA, 2021). According to European Union food safety regulation authorities (European Commission), nisin is accepted as a food preservative (E234), and it is recognized as a safe additive in food.

Natamycin has GRAS status from the FDA, with GRN Nº 578 for use in ready-to-drink tea beverages, fruit-flavored energy drinks, sport, and isotonic drinks, and fruit-flavored beverages at levels not to exceed 5 ppm; with GRN Nº 517 for use as an inhibitor against yeast and mold growth in yogurt at levels not to exceed 5 ppm in the finished product; and authorizes the use of natamycin on cheese as antimycotic in a maximum concentration of 20 ppm in the finished product (FDA, 2021). Natamycin is also recognized as a natural preservative by the European Union (EEC N° 235).

Regarding antimicrobial content, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) allow an acceptable daily intake (ADI) for nisin of 2 mg/kg bw and for natamycin of 0.3 mg/kg bw (FAO/WHO, no date).

For materials in contact with food, a special control of the content of the substance that can migrate into the food is required. In addition, an assessment of the potential hazards of ingesting substances that migrate into food should be carried out to ensure consumer safety and protection. In the case of other antimicrobial agents, trials regarding safety and allergy concerns must be applied to assess the safety, delivery mode, and dosage of the antimicrobial agents in contact with food or as food additives. Toxicity is even more important in the case of edible coatings because they should be considered part of the food and not just material in contact with food (Böhme *et al.*, 2016)

Furthermore, active food packaging systems must also comply with environmental legislation related to its disposal. This legislation is related to recycling and reuse and is linked to the

composition of materials. In the USA, the packaging waste directive must be complied with, and in the EU the packaging waste directives of the European Commission.

8.7 CONCLUDING REMARKS AND FUTURE TRENDS

In recent years, innovative and disruptive packaging technologies have been implemented that play an important role in food preservation strategies. Antimicrobial packaging systems based on antimicrobial substances of microbial origin have the advantage of their natural origin and highly specific action against other microorganisms, contributing to the development of the concept of hurdle technologies in food preservation. Advances in these antimicrobial packaging are strongly related to biopolymers and biodegradable polymers, along with a more sustainable policy in food packaging.

There are good perspectives for the improvement of the retention or controlled release of the antimicrobial agent in food packaging when used directly in contact with the product or as part of multilayers. Innovative solutions emerged to functionalize polymers, using for example nanostructured materials such as nanocomposites, nanolaminates, or electrospun fibers that can support the active agent. However, all these technologies must be studied in detail for each application to evaluate their scalability at an industrial level. Furthermore, additional research should be addressed to evaluate the performance of antimicrobial packaging systems in combination with other stress factors, such as high pressure, light pulses, electric fields, among others, in the context of the hurdle technology concept.

A wide variety of studies of possible applications of antimicrobial packaging systems based on substances of microbial origin can be found in the literature. However, the commercial implementation requires exhaustive further assessment. On the one hand, huge and expensive

studies are required to demonstrate the safety and compliance with regulatory requirements by the antimicrobial compound. On the other hand, the development of simple, robust, and inexpensive manufacturing processes with a reliable efficacy of the packaging to retain and release the antimicrobial is necessary.

Due to the complexity of the food-antimicrobial packaging system and the variables that can influence its adequate performance, collaborative work between researchers and industry is necessary to achieve useful products and develop cost-effective production methods for them. A multidisciplinary team, including experts in microbiology, food science, sensory evaluation, packaging design and manufacturing, and materials science, is needed for successful implementation.

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TABLE 1. Applications on foods of antimicrobials food packaging with antimicrobial agents of microbial sources