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Review

Efficacy of Antimicrobial Agents for Food Contact Applications: Biological Activity, Incorporation into Packaging, and Assessment Methods: A Review

AMIN MOUSAVI KHANEGHAH,[1]* SEYED MOHAMMAD BAGHER HASHEMI,[2] ISMAIL EŞ,[3] DANIELA FRACASSETTI,[4] AND SARA LIMBO[4]*

[1]*Department of Food Science, Faculty of Food Engineering, and* [3]*Department of Material and Bioprocess Engineering, Faculty of Chemical Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil (ORCID: http://orcid.org/0000-0001-5769-0004 [A.M.K.]);* [2]*Food Science and Technology Department, College of Agriculture, Fasa University, Fasa, Iran; and* [4]*Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan 20123, Italy (ORCID: http://orcid.org/0000-0002-4739-4751 [S.L.])*

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ABSTRACT

Interest in the utilization of antimicrobial active packaging for food products has increased in recent years. Antimicrobial active packaging involves the incorporation of antimicrobial compounds into packaging materials, with the aim of maintaining or extending food quality and shelf life. Plant extracts, essential oils, organic acids, bacteriocins, inorganic substances, enzymes, and proteins are used as antimicrobial agents in active packaging. Evaluation of the antimicrobial activity of packaging materials using different methods has become a critical issue for both food safety and the commercial utilization of such packaging technology. This article reviews the different types of antimicrobial agents used for active food packaging materials, the main incorporation techniques, and the assessment methods used to examine the antimicrobial activity of packaging materials, taking into account their safety as food contact materials.

Key words: Active packaging; Antimicrobial agents; Assessment of antimicrobial activity; Food contact materials

Active packaging (AP) offers a significant opportunity to extend the shelf life of many perishable products and to reduce food waste, in addition to providing food protection. The accepted primary characteristic of AP is that it aims "to retain and increase the shelf life of foodstuffs" *(9)* and improves "sensory or safety characteristics" *(71)*. This type of packaging solution is intentionally designed to include components that absorb agents from or release agents into the environment that surrounds the packaged food *(82)*. Oxygen scavenging, moisture adsorption, and control of ethanol, carbon dioxide, and ethylene production are just a few examples of AP functions *(10)*. Antimicrobial AP is a noteworthy approach to food safety and facilitates the control of specific alterations within packaged food *(25)*. The antimicrobial AP market size is estimated to grow from USD 7.28 billion in 2015 to USD 10 billion by 2021. Demand will primarily be driven by food safety and security concerns, together with the goal of reducing food loss and food waste along the supply chain. Food contamination can occur during different stages, such as harvesting, food processing, and distribution *(59)*. Packaging is the leading available tool to protect food products from external contaminants and prevents chemical, physical, and biological alterations (such as deterioration) during the preparation or storage of products. Conventional packaging materials have a passive function and are not able to control reactions within the packages in an active way. Advances in material science and engineering have resulted in the development of new materials, commonly known as antimicrobial AP, to help maintain quality and enhance food safety.

Synthetic antimicrobial agents that are used in AP applications include organic acids (i.e., citric acid, acetic acid, lactic acid, etc.) and their salts, sulfites, alcohols, and other organic preservatives that are often used in the formulation of foods. Plant extracts, such as olive leaf extract, essential oils (thyme, rosemary, sage, garlic), and bacteriocins, including nisin, sakacin, lysozyme, and pediocin, represent relatively new avenues for antimicrobial AP and have received substantial attention from the scientific community. Inorganic materials, such as copper, silver, zinc oxide, and titanium oxide, especially at nanodimensions, are also of great interest due to their extensive range of antimicrobial properties against a variety of bacteria *(27)*.

Previous reviews have discussed the application of antimicrobial agents in packaging materials, highlighting the effectiveness of the released substances to reduce food

* Authors for correspondence. Tel: +55(19) 3521-0091; Fax: +55(19) 3521-2153; E-mail: mousavi.amin@gmail.com (A.M.K.). Tel: +39 02 50316659; Fax: +39 02 50316670; E-mail: sara.limbo@unimi.it (S.L.).

spoilage and describing the incorporation of these techniques into packaging materials *(85)*. However, recent advances in the nanotechnology field, the development of biodegradable or biocompatible materials, and the discovery of stimuli-responsive materials have broadened the field of antimicrobial packaging.

In this review, the efficacy of antimicrobial agents for food contact applications will be discussed, to provide an overview of the primary substance categories and the mechanisms through which they prevent deterioration and inhibit spoilage and pathogenic bacteria. The advantages and limitations of traditional and more innovative techniques for the incorporation of antimicrobial agents into packaging materials will be presented, with a view toward identifying potential pitfalls to proactively consider during the design of an efficient packaging system. Finally, the efficacy, specifically in terms of legal requirements, and the assessment methods used to measure the inhibitory activity of antimicrobial agents are also examined to offer a more complete overview of the design process for modern AP.

ANTIMICROBIAL AGENTS FOR INCORPORATION INTO ACTIVE PACKAGING MATERIALS

Pathogenic and spoilage microorganisms may be controlled by the application of antimicrobial agents, which results in an extension of the lag phase or culminates in microbial inactivation. An antimicrobial compound is selected based on its activity against the target microorganism, its lethal concentrations and MICs, and its suitability for incorporation into the packaging material *(97)*.

Essential oils. Essential oils are secondary metabolites of aromatic plants. The aroma of an essential oil is composed of more than 70 different compounds; however, only a few of these compounds are bioactive agents, although they are present at high concentrations (20 to 70%). Essential oils are characterized by a chemical complexity that results in a variety of molecules with different volatility and odor profiles *(6, 37)*.

Essential oils are identified by two primary structures, commonly known as terpenoids and phenylpropanoids *(3)*. The former class is characterized by aromatic and aliphatic constituents, is derived from the isoprene units, and may contain oxygen (alcohols, ketones, aldehydes). Terpenoids are synthesized from a precursor, isopentenyl diphosphate, which is repeatedly added to the prenyl diphosphate precursor of various classes of terpenes. Then, the terpene skeleton is formed by a specific type of terpene synthetase, and the final functional properties of different terpenes are obtained through secondary enzymatic pathways *(6)*. Examples of terpenoids are carvacrol (commonly present in many plants), thymol (in origanum and thymus), eugenol (in clove oil), menthol (in peppermint oil), and citral (in lemon grass). Phenylpropanoids are synthesized from the amino acid phenylalanine, which is converted into cinnamic acid and subsequently reduced to an aldehyde (e.g., cinnamaldehyde). Compounds such as eugenol and safrole are produced from phenylpropenes that are derived from the further reduction of aldehydes *(23)*.

From a technical point of view, essential oils and plant extracts are commonly used in AP due to their ability to reduce or eliminate oxidative reactions and microbial spoilage (Table 1).

The large number of chemical compounds and the variety of molecules detected in essential oils and the multiple chemical-physical characteristics of essential oils are involved in their broad activity spectrum. Thus, the interactions among all components and, simultaneously, synergism with food ingredients, structure, and microbiological status also influence their activity and mechanisms of action *(36, 38, 39)*. The activity of essential oils against microorganisms has been extensively investigated *(32, 59, 86)*, and several mechanisms have been proposed for their antimicrobial activity, primarily based on the hydrophobic nature of these substances *(49)*. In the case of gram-positive bacteria, essential oils interact with the membrane and alter the permeability of cations, such as H^+ and K^+. Indeed, the higher acidity of some phenolic compounds allows for the establishment of ion exchange through the bacterial membrane.

Cox et al. *(20)* showed that the presence of lipoteichoic acids in the cell membrane of gram-positive bacteria might facilitate the penetration of the hydrophobic compounds in essential oils due to the lipophilic nature of acids. This penetration creates an imbalance within the microbial cell and results in coagulation of the cytoplasm, the denaturation of several enzymes and cellular proteins, and the loss of metabolites and ions, which can cause cell death.

Some essential oils are also able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP *(89)*. However, the presence of the extrinsic membrane proteins in gram-negative bacteria limits the diffusion rate of hydrophobic compounds through the lipopolysaccharide layer. Other mechanisms, such as the inhibition of protein synthesis, the coagulation of cell content, the inhibition of DNA and RNA synthesis, and the disruption of the proton motive force and electron flow, were also reported *(12, 81)*.

Thus, the effects of essential oils are attributable to complex and synergistic mechanisms, in which the hydrophobicity of an essential oil plays a significant role.

Scanning and transmission electron microscopy observations reveal cell ultrastructural alterations due to the exposure of microbial cells to essential oils. The plasma membrane, the cytoplasm, mitochondria, and the nucleus are the primary cellular ultrastructures that are sensitive to essential oils *(6)*. Bakkali et al. *(6)* hypothesized that essential oils are also effective in eukaryotic cells, in which they damage whole mitochondria and the mitochondrial DNA of mother cells, inducing respiratory alterations. Studies performed with pathogenic bacteria, such as *Escherichia coli, Bacillus cereus,* and *Staphylococcus aureus,* indicate a relationship between the activity of essential oils and bacterial shape, which explains the antibacterial role of certain essential oils. These studies showed that rod-shaped bacterial cells are more sensitive to essential oils than coccoid cells *(57, 58)*.

TABLE 1. *Antimicrobial activity of some essential oils and plant extracts added to different packaging materials*[a]

Plant antimicrobial agent	Target microorganism(s)	Packaging material	Reference
Oregano EO	*E. coli, Salmonella enterica, L. monocytogenes*	PP-EVOH	*64*
	S. aureus, E. coli	Plastic packaging	*8*
	Alternaria alternata	Paper	*77*
Linolool	*E. coli, S. aureus*	LDPE	*86*
Cinnamon EO	*A. alternata*	Packaging paper	*77*
Clove, oregano, and cinnamon EO	*E. coli, Y. enterocolitica, P. aeruginosa, L. monocytogenes, S. aureus, B. cereus, C. albicans, Debaryomyces hansenii, Zygosaccharomyces rouxii, Botrytis cinerae, A. flavus, Penicillium roqueforti, Pyrobaculum islandicum, Penicillium commune, Penicillium nalgiovense*	PP or PE-EVOH	*58*
Oregano and pimento EOs	*E. coli* O157: H7, *Pseudomonas* spp.		*70*
Thymol and carvacrol	*E. coli, S. aureus, L. innocua, Aspergillus niger, Saccharomyces cerevisiae*	LDPE	*80*
Carvacrol and linolool	*E. coli*	PA and LDPE	*75*
Thymol and carvacrol	*E. coli, L. innocua*	LDPE	*34*
Thymol, carvacrol, and cinnamaldehyde	*E. coli, Y. enterocolitica, P. aeruginosa, L. monocytogenes, S. aureus, B. cereus, Enterococcus faecalis, C. albicans, D. hansenii, Z. rouxii, B. cinerae, A. flavus, P. roqueforti, P. islandicum, P. commune, P. nalgiovense*	PP	*32*
Clove extract	*L. plantarum, Fusarium oxysporum*	LDPE	*40*
Grapefruit seed extract	Aerobic bacteria and yeasts	LDPE	*54*
Olive leaf extract	*S. aureus*	Methylcellulose	*4*

[a] EO, essential oil; PP-EVOH, polypropylene–ethylene vinyl alcohol; LDPE, low-density polyethylene; PP, polypropylene; PE-EVOH, polyethylene–ethylene vinyl alcohol; PA, polyamide.

Exposure to essential oils (both in solution and in the vapor phase) also influences the membrane roughness of gram-negative cells, as previously discussed *(2)*. After treatment with carvacrol, gram-negative cell membranes showed increased roughness compared with gram-positive cells. The authors suggested that carvacrol interfered with the outer membrane, resulting in the exposure of certain membrane components, such as proteins and lipids. In the same study, a cytoplasmic membrane of gram-positive bacteria was modified by carvacrol to increase fluidity; however, in this case, the roughness of the cell was not significantly altered.

Some components of essential oils appear to be more efficacious against bacteria; however, the inhibitory effects of the individual chemicals often differ. This behavior is due to the additive, antagonistic, and synergistic effects of the various components that constitute essential oils. Faleiro *(28)* explained that additive effects occur when the combined effect is equal to the sum of the individual effects. However, antagonistic effects are evident when the activity of the components in combination is reduced compared with their individual effects. Synergism is noted when the activity of the combined substances is greater than the sum of the individual activities. Various techniques have been proposed to explore such relationships, including the fractional inhibitory concentration index, which is calculated as the ratio between the MICs obtained for the compounds that are components of a mixture and the MICs for pure compounds. In a study by Lopez et al. *(58),* cinnamaldehyde-fortified cinnamon essential oil and thymol-fortified thyme essential oil were evaluated against different microorganisms *(57)*. *Aspergillus flavus* was inhibited by the synergistic effect of cinnamaldehyde and cinnamon essential oil, whereas *Listeria monocytogenes* and *Candida albicans* experienced an additive effect of the same substances. The antagonistic effect of these two substances on the growth of *Salmonella* Choleraesuis was evident. The combination of cinnamaldehyde-fortified cinnamon essential oil and thymol-fortified thyme essential oil showed a synergistic effect on *L. monocytogenes, Salmonella* Choleraesuis, and *A. flavus* and an additive effect on *C. albicans*.

Carvacrol is one of the primary components that exerts antimicrobial activity, both due to its high abundance in certain oils and its high specific activity *(95)*. The activity of carvacrol is related to its ability to interact with the bacterial membrane; the presence of a hydroxyl group and the delocalized electrons of the benzene ring play significant roles in this mechanism *(95)*.

Ultee et al. *(94)* demonstrated that carvacrol and its precursor cymene, which lacks the hydroxyl group, have different membrane affinities. In particular, carvacrol has a preference for liposomal membranes, causing more expansion, whereas cymene does not influence the pH gradient or the ATP pool. Owing to the presence of a hydroxyl group located at the meta position, thymol did not affect the growth of *B. cereus* compared with the effects of carvacrol. The growth of *B. cereus* in the presence of menthol, which lacks a delocalized electron system, was 10 times greater than the growth in the presence of carvacrol.

The effectiveness of carvacrol may be attributable to its chemical structure; Veldhuizen et al. *(95)* determined that compounds such as 3-isopropylphenol, *o*-cresol, and *p*-cymene were the primary substances involved. The removal of ring substituents from carvacrol reduced its antimicrobial

activity, which shows the critical role of these components in its biological function; this is likely due to a decrease in the amphipathic features of these molecules, which may affect the initial interactions of these compounds with the bacterial membrane.

When bacteria reach a specific cell density (i.e., a quorum concentration), they coordinate both bacterium-bacterium interactions and associations with higher organisms. This kind of communication is known as quorum sensing (QS). Through QS activity, virulence factor expression, bioluminescence, sporulation, motility, swarming, biofilm formation, and mating are controlled and regulated. The expression of QS genes results in the production of chemical signaling molecules known as autoinducers or bacterial pheromones *(48)*. In particular, gram-negative bacteria use acylated homoserine lactone (AHL), whereas gram-positive bacteria use modified oligopeptides. These molecules are produced as the bacterial population grows until a threshold concentration, which is perceived by the bacteria, is reached, resulting in the activation or repression of specific genes. Various pathogenic bacteria, such as *Pseudomonas aeruginosa, Vibrio* ssp., and *Yersinia enterocolitica,* use QS to regulate their virulence and pathogenicity *(48)*. Therefore, the inhibition of QS is a new challenge, and identification of various natural compounds that offer anti-QS properties will be of interest. Bacterial QS may be inhibited through different mechanisms, including (i) the inhibition of AHL synthesis, (ii) the inhibition of AHL transport and/or secretion, (iii) the sequestration of AHLs, (iv) antagonistic action, and (v) the inhibition of targets downstream of AHL receptor binding. Few studies have examined the role of essential oils in the inhibition of QS; however, the results of preliminary studies are encouraging. Different essential oils from ornamental plants, such as rose, geranium, lavender, rosemary, and clove, have been shown to be effective against biofilms formed by *Salmonella, Listeria, Pseudomonas, Staphylococcus,* and *Lactobacillus* spp., whereas orange and juniper essential oils appear to have no anti-QS properties *(28)*. The most well-studied compounds found in essential oils are cinnamaldehyde and its derivatives, which affect a wide range of QS-regulated activities, such as biofilm formation in *P. aeruginosa* and the regulation of certain signaling compounds (AI-2–mediated QS) in another *Vibrio* spp. In a recent study by Khan et al. *(48),* 21 essential oils were tested for their anti-QS activity using strains of *Chromobacterium violaceum* (CV12472 and CVO26 strains) to evaluate the inhibition of QS-controlled violacein production. The study found that cinnamon, lavender, and peppermint inhibited the production of violacein in CV12472, whereas this inhibition was reduced when using the CV026 strain. The application of clove oil, at the highest concentration, resulted in both antibacterial and anti-QS activity. Thus, to assess the mechanism underlying the anti-QS activity of clove oil, the effects of sub-MICs of clove oil on swarming motility were investigated in *P. aeruginosa* (PAO1 strain). A concentration-dependent reduction in swarming motility was observed. Undoubtedly, additional studies are necessary to further understand this critical mechanism and to further investigate the effects of multitarget actions of various ingredients in essential oils on the bacterial QS system.

Bacteriocins. Bacteriocins are compounds produced by, among other bacteria, lactic acid bacteria that primarily exhibit inhibitory activity against gram-positive microorganisms. The use of bacteriocins in antimicrobial packaging has attracted considerable attention due to their natural origins, their activity against gram-positive bacteria, and their resistance to elevated temperatures and acidic environments *(85)*.

Nisin is active against an extensive range of gram-positive bacteria *(46),* and its inhibitory activity is attributable to multiple actions. Interference in cell wall biosynthesis is achieved via binding to the precursors of peptidoglycans and lipid II. Then, nisin forms pores in the cell membrane, which leads to the release of essential ions and, ultimately, cell death *(47)*.

Chen and Hoover, in 2003 *(14),* proposed that the effectiveness of nisin is greater against bacilli and clostridial spores than against their vegetative cells; inhibition involves a sporostatic mechanism that requires the continuous presence of nisin to inhibit the outgrowth of the spores, due to the binding of nisin to the sulfhydryl groups of protein residues. Heat has a positive effect on nisin activity against spores; following thermal treatment, in a low-acid medium, the antimicrobial activity of nisin is enhanced. Nisin is commonly used in food manufacturing as a natural preservative and shelf life extender, but the possibility of immobilizing it offers a real opportunity for bioactive food packaging applications *(13)*.

Sakacin A is a bacteriocin produced by *Lactobacillus sakei* DSMZ 6333 that inhibits several lactic acid bacteria, as well as *L. monocytogenes*. Interest in sakacin A activity against *L. monocytogenes* in the field of AP has been thoroughly described *(90–92)*. Indeed, the risks associated with this pathogenic microorganism and strict safety concerns with ready-to-eat foods have supported research into the antimicrobial properties of sakacin A.

Sakacin A consists of 30 to 60 amino acids (<10 kDa), is heat stable, and as with all bacteriocins belonging to subclass IIa, does not undergo posttranslational modifications. It is characterized by an amphiphilic or hydrophobic C-terminal domain and a highly conserved hydrophilic N-terminal domain that binds to target cells through electrostatic interactions *(76)*. This binding also facilitates anchoring of the C-terminal domain to the hydrophobic core of the target cell membrane, leading to membrane leakage. Sakacin A contains cysteine, allowing it to create disulfide bridges that enhance its bacteriocin activity. The number of disulfide bridges formed is directly related to the antimicrobial activity of this type of peptide. In a study by Fimland et al. *(30),* the introduction of a second disulfide bridge within the C-terminal domain of sakacin P (a 43-amino-acid, class IIa bacteriocin produced by several strains of *L. sakei*) enhanced the activity of recombinant sakacin P (a bacteriocin containing one disulfide bridge) by 10- to 20-fold.

Generally, bacteriocins are susceptible to interactions with food macromolecules and proteolytic degradation. Thus, in AP systems where the release of such bioactive compounds is desired, the medium plays an important role; however, few studies have addressed this issue. Partitioning of bacteriocins between polar and nonpolar food components and their interactions with fats and proteins may cause significant reductions in the bioactive compounds in foods. Indeed, bacteriocins contain a high concentration of hydrophobic amino acids and are positively charged. Therefore, their interactions with charged food components are expected. In a study performed by Aasen et al. *(1)*, certain aspects of the interactions of sakacin P and nisin with food constituents were thoroughly explored. In a system where fat, water, and bacteriocins are well mixed (such as in homogenized whole milk or forcemeat), the bioactivity of sakacin P is less efficient. It appears that bacteriocins adhere to the fat-water interface and that sakacin P activity decreases when it is bound to lipids. When the fat-water phases are permitted to separate, this activity is entirely or partially restored. This behavior suggests that bacteriocins are more efficient when applied to the food surface than in bulk, which indicates great potential for use in AP release systems. The effectiveness of bacteriocin also decreases during food storage due to the proteolytic activity of food proteases *(1)*. Therefore, if enzymatic activity is partially or completely inactivated by thermal processes or other technological treatments, the rate of bacteriocin efficacy is reduced. Thus, in investigations of AP applications, thermally processed foods are considered better targets than nonprocessed foods, and the concentration of proteases released in fresh foods may be greater than that in processed foods.

pH modifications also contribute to the amendment of bacteriocin activity. In meat, a low pH breaks ionic bonds by neutralizing the negative charges of muscle proteins and preventing the binding of positively charged bacteriocins, thus increasing their activity. A study by Aasen et al. *(1)* showed that nisin adsorption is more affected by pH reduction than sakacin P adsorption, possibly due to stronger hydrophobic interactions in sakacin P than in nisin. However, bacteriocins that are largely affected by ionic bonds are more susceptible to pH changes. Therefore, for AP applications, it is critical to verify the activity of a bacteriocin in medium.

Organic acids and their salts. Some organic acids, such as propionic acid, benzoic acids, sorbic acid, lactic acid, and acetic acid, and their salts have strong antimicrobial activity and can be utilized as antimicrobial agents in packaging materials *(26)*. These substances are considered to be generally recognized as safe (GRAS) additives and are active against yeasts, molds, and many bacteria; thus, their use is widely accepted in many food formulations. Salts of lactic acid, such as sodium lactate and potassium lactate, exert greater inhibitory effects against gram-positive bacteria than against gram-negative bacteria and offer antifungal activity against certain *Aspergillus* species.

The concentration of organic compounds must be taken into consideration because some organic acids, such as lactic acid, citric acid, and acetic acid, reduce the pH of foods. The ability of these acids to lower the pH of the medium depends on the number of dissociable functional groups and their acid constants, *K*. Antimicrobial activity is facilitated not only by the ability to reduce the pH but also by molecule-specific mechanisms. Some weak organic acids, such as sorbic acid, tend to remain in their undissociated form even under moderately acidic conditions. In this form, these organic acids are uncharged, and their lipophilicity facilitates passage through the lipid membrane of microorganisms. In the cytoplasm, they induce metabolic disorders, contributing to cellular death. Indeed, in the neutral pH of the cytoplasmic environment, organic acids are dissociated into charged protons and anions that remain in intracellular compartments without having the opportunity to pass through the membrane. The increase in the H^+ concentration disturbs cellular homeostasis by exhausting the bacteria, which continually expel H^+ ions from the cell. In fact, the acidic pH inside the cell causes damage to enzymatic activities, proteins, and DNA structure, thereby damaging the extracellular membrane.

In some cases, the use of a mixture of organic acids at different concentrations is more efficient against microbial growth than a single organic acid. Hence, their use is limited to certain foods, and they are most suitable for low-acid foods, such as cold-pack cheese and bologna *(26)*. In other cases, organic acids are incorporated into biopolymers, such as chitosan, to control their release rate.

Enzymes and proteins. For in-package applications, an enzyme may be added directly to a food or may be impregnated into the packaging material. Lysozyme is an enzyme that lyses sensitive bacteria through the breakdown of the peptidoglycan polymers of the bacterial cell wall. This enzyme has been shown to exert antimicrobial activities against bacteria, fungi, protozoans, and viruses; thus, it is widely used as a food preservative. Gram-positive bacteria are susceptible to lysozyme because the target cell-wall component (peptidoglycan) is freely accessible to the enzyme, whereas the lipopolysaccharide layer of the outer membrane of gram-negative bacteria offers a formidable barrier against lysozyme attack.

Mecitoğlu et al. *(63)* reported that moderately purified lysozyme from hen egg albumen impregnated into zein films had antimicrobial activity against various bacteria, including *Lactobacillus plantarum* and *Bacillus subtilis*. Furthermore, films fabricated using a combination of lysozyme and EDTA exerted an antimicrobial effect against *E. coli*. Conte et al. *(19)* prepared and tested cross-linked films using five different ratios between the lysozyme concentration and the bonding agent concentration, and all were efficient at inhibiting *Micrococcus lysodeikticus* growth. Moreover, the antimicrobial efficiency of the introduced film is enhanced by increasing the quantity of the surface-bound enzyme. Lysozyme combined with nisin was relatively ineffective against *Pseudomonadaceae* in pork meat *(65)*. Lactoferrin is another protein molecule that has attracted attention in food

packaging applications. Lactoferrin is an 80-kDa whey glycoprotein that binds two ferric ions for each protein molecule. It binds ions reversibly and, therefore, exists as a mixture of iron-free (apo-lactoferrin) molecules and molecules that contain either one or two iron atoms (holo-lactoferrin) *(7)*. The antimicrobial activity of lactoferrin is not unique and is associated both with iron deprivation, which inhibits bacterial growth, and large cationic patches display on the lactoferrin surface that interact with a specific component of the lipopolysaccharides in gram-negative bacteria. In this latter mechanism, the permeability of the outer membrane is strongly compromised. Mascheroni et al. *(61)* developed a paper material that contained lysozyme and lactoferrin to enhance antibacterial efficiency against gram-negative bacteria. Lysozyme was more efficient when lactoferrin and lysozyme were impregnated into paper that contained carboxymethylcellulose; in 2016, Rollini et al. *(78),* developed and tested a lysozyme-lactoferrin–coated PET film. In this application, the synergism between lysozyme and lactoferrin was evidenced by the reduction of H_2S-producing bacteria in fresh salmon fillets.

Inorganic nanoparticles or microparticles. Inorganic nanoparticles have widely been applied as antimicrobial agents due to their high thermal stability, longevity, and a broad spectrum of antifungal and antibacterial activities. By combining nanoencapsulation and biopolymer immobilization, it became possible to fabricate antimicrobial biodegradable films *(43)*. The inorganic nanoparticles that are most widely incorporated into packaging systems include silver, titanium dioxide (TiO_2), zinc oxide (ZnO), copper and gold nanoparticles, nanoclay, and carbon nanotubes. Some of these nanosubstances present significant antimicrobial properties. Generally speaking, it is essential to evaluate the features of nanocomposites for use in packaging because they may preserve food and increase shelf life, in addition to decreasing the necessity for plastic use *(62)*.

Silver nanoparticles have low toxicity in eukaryotic cells *(67)*. In recent years, many studies have examined the antimicrobial activity and other functional properties of silver nanoparticles. In a recent study, the applied silver nanoparticles embedded into an edible coating for chicken sausages were able to inhibit lactic acid bacteria for 30 days, increasing the shelf life of the sausages *(60)*. Moreover, the synergistic antimicrobial activity of silver nanoparticles with other substances used in films, such as chitosan and titanium oxide, against both gram-positive and gram-negative bacteria has previously been demonstrated *(51, 56)*. The antibacterial activity of metal oxide forms of nanoparticles, such as titanium dioxide (TiO_2) and zinc oxide (ZnO), was also investigated *(22)*. TiO_2 has photocatalytic activity, which leads to multifunctional properties, such as self-cleaning, as well as antibacterial and UV protection properties *(34)*. Threepopnatkul et al. *(87)* reported that thin blended films composed of polyethylene terephthalate and polybutylene succinate combined with TiO_2 exhibited greater antibacterial properties against *S. aureus* and *E. coli* bacteria than those combined with ZnO.

Because zinc oxide is listed as GRAS, its potential use in food packaging is intriguing. A prepared AP made of calcium alginate loaded with ZnO nanoparticles was utilized against *S. aureus* and *Salmonella* Typhimurium *(27)*. ZnO nanoparticles are more efficient against *S. aureus* and other gram-positive bacteria than other metal oxide nanoparticles *(5)*.

Although many studies related to inorganic nanoparticles have been performed, microcomposites can also be used for AP. The principal difference between nanoparticles and microcomposites is the ion release rate. Usually, the release rate from a nanocomposite is rapid, and the maximum release rate is reached during the first day of packaging. This rate is considerably lower for microcomposites, and this interesting property allows for a more controlled release. However, studies have revealed that nanocomposites show greater antibacterial activity against *S. aureus* and *P. aeruginosa (72)*. It can be concluded that the concentrations of inorganic compounds incorporated into packaging material are a significant parameter in the design of an AP agent. For example, Ag-zeolite is an antimicrobial agent that inhibits a broad range of metabolic enzymes in several microorganisms. Ag-zeolite has been examined in a laminated form, with a thin layer that varies from 3 to 6 μm and is primarily incorporated into plastics *(96)*. Moreover, the addition of Ag-zeolites does not alter the critical properties of packaging materials (such as thermal degradation and transparency) *(11)*.

Other chemicals. In foods, the presence of transition metals may result in the breakdown of specific chemical compounds into simpler compounds via degradative reactions. This situation might favor the growth of certain microorganisms that use the resulting simple compounds as substrate sources. To prevent such metal-promoted reactions, which might produce compounds that are easily utilized by foodborne pathogens, chelating agents can be used *(31)*. Synthetic materials, such as EDTA, have significant potential to inhibit iron-promoted oxidation and prevent microbial growth indirectly; they can be incorporated into the material by covalent immobilization. For this purpose, nonmigratory AP agents are of great interest in the food packaging field because they prevent active substances from migrating from packaging materials into food products; moreover, they are not classified as a direct food additive, and their further application in the control of microbial contamination has been approved by regulatory bodies *(88)*.

In this context, chelating agents have been covalently bound to the packaging material. The potential use of iron-chelating AP modified with hydroxamate to retain its iron-chelating capacity was previously demonstrated *(68)*. In 2014, Roman et al. *(79)* developed a chelating film through the polymerization of acrylic acid on polypropylene (PP). In this study, it was possible to engineer the chelating activity of PP by altering the graft conditions and manipulating the thickness of the film.

ANTIMICROBIAL ACTIVE PACKAGING: TECHNOLOGICAL AND SAFETY REQUIREMENTS

The construction of an antimicrobial packaging system is based on the nature and the chemical-physical properties of both the active substance and the food, which determines the mode of interaction with the food microbiota. Commonly, the transfer of antimicrobial agents from packaging into food is classified, based on migration mechanisms, as either nonvolatile migration or volatile migration. However, a third class of antimicrobial agents contains substances that show antimicrobial activity without true migration into foods. These substances are not mobile and exert antimicrobial activity only on the surface of the food, primarily providing sufficient energy to form ions and radicals that are capable of controlling microbial growth *(42)*.

Nonvolatile migration involves the diffusion of molecules incorporated into the packaging thickness, immobilized onto the surface, or coated onto the food contact layer. This migration requires direct contact between the packaging material and the food, and the transfer is primarily based on Fick's diffusion law. The difference in the antimicrobial concentration between the packaging and the food represents the driving force of diffusion. The velocity of mass transport is subject to the diffusion coefficient, D, which represents a kinetic dimension of migration and is dependent on layer temperature. However, the quantity of antimicrobial substance that migrates into the food is also dependent on the partition coefficient between packaging and food, $K_{p/f}$ (p, plastic; f, food), and on the solubility of the substance itself. In fact, this coefficient varies from $K_{p/f} = 1$, which indicates good solubility, to $K_{p/f} = 1{,}000$, which represents poor solubility. In the case of antimicrobial release and packaging design, it would be useful to estimate both D and $K_{p/f}$. Factors such as the identity of the substance (initial concentration, molecular weight, chemical structure, polarity, solubility in the media, etc.), the identity of the packaging material and its chemical structure, the nature of the food in contact with the AP (water, acid, alcoholic, fatty), and the storage conditions of the material and the packed food, specifically time and temperature, influence both D and $K_{p/f}$. Han and Floros *(33)* studied the diffusivity of potassium sorbate through different plastic films (low- and high-density polyethylene [LDPE and HDPE], polypropylene [PP], polyethylene terephthalate [PET]) with thicknesses that ranged from 12 to 20 μm. The experiments were performed at different temperatures and revealed that LDPE had the fastest diffusion and the highest D among the tested films *(33)*. This result indicates that LDPE may be a good material for use as a reservoir system for potassium sorbate, whereas HDPE, PP, and PET can be used to modulate diffusion, reducing the rate of migration.

Volatile-migration mode involves the release of volatile molecules, characterized by high vapor tension, that tend to be easily released into foods. Essential oils are typical antimicrobial agents that use this diffusional mechanism. Factors such as temperature and interactions between the volatile agent and the packaging material influence the rate of release. The mass transferred to the headspace produces a concentration in the headspace, reaching the food surface and fostering adsorption into foods. In this situation, an equilibrium between the concentrations in the packaging (C_p), in the headspace (C_h), and in the food (C_f) should be reached to guarantee that the concentration is maintained above the MIC for a specific target microorganism. The advantage of this kind of mechanism is its ability to reduce microbial growth on the surface of porous and irregularly shaped foods, such as ground beef, shredded cheese, and others *(25)*. The partition coefficient is also of great importance for volatile antimicrobial agents. In this case, $K_{p,f}$ and also the partition between the headspace and the packaging matrix ($K_{h,p}$) help us understand the mechanism of action of these substances against spoilage microorganisms. Indeed, as previously discussed, antimicrobial efficiency via headspace is obtained from the combined effects of direct vapor absorption on microorganisms and indirect effects through a medium that absorbs the vapor, i.e., the food *(33)*.

Kurek et al. *(52)* reported the results of a study that examined the antimicrobial efficacy of carvacrol vapor related to the mass partition coefficient following its incorporation in a bio-based polymer (chitosan). In this study, five formulations of chitosan films were used as media to incorporate carvacrol, a volatile agent. The addition of plasticizers (such as glycerol and lecithin) influenced carvacrol retention. Those samples that showed the highest carvacrol retention in the film had the lowest $K_{h,p}$ values. In particular, $K_{h,p}$ was inversely proportional to the retention capacity and, thus, directly affected the carvacrol vapor phase concentration. The presence of plasticizers, such as arabic gum, glycerol, and lecithin, altered the overall thermodynamic behavior, and the carvacrol concentration above the food product was better maintained. Under these conditions, dry films composed of chitosan-arabic gum-glycerol and carvacrol had the lowest carvacrol concentrations (0.09%, w/w) and the highest $K_{h,p}$ (1.01×10^{-4}). The efficacy of this packaging solution was tested in vitro, revealing its ability to inhibit the growth of recently inoculated gram-positive *B. subtilis* and *Listeria innocua* and the gram-negative bacteria *E. coli* and *Salmonella* Enteritidis. However, the film with the lowest $K_{h,p}$ (6.81×10^{-8}) was inefficient against all tested microorganisms. Thus, during the lag phase, the carvacrol exploited its activity by inhibiting cell growth. This inhibition appears to be attributable to a synergistic effect between direct vapor absorption on microorganisms and an indirect effect through the medium that absorbed the vapor *(44)*. The partitioning of the antimicrobial between the headspace and the food matrix explains the activity during the exponential phase of bacterial growth. Indeed, to be active, an antimicrobial agent must damage bacterial cells before they begin to divide. The time delay for an active substance to reach cells must be shorter than the cellular generation time *(52)*, which requires good repartitioning and solubility in the food. For volatile antimicrobial agents, special attention must be paid to the nature of the food. In liquid and homogenous foods, the diffusion and repartitioning of antimicrobial substances is facilitated by thermal effects that maintain a constant concentration gradient at the interphase. Where some

dispersed phases are present (such as in emulsions), the fatty and aqueous phases define the partitioning of the absorbed antimicrobial. In this type of food, migration of the antimicrobial in the fatty phase is not of practical importance because microbial growth occurs in the aqueous phase. Therefore, the partition coefficient between the fatty and aqueous phases ($K_{f,w}$) also plays an important role, and those antimicrobial agents that display the lowest partition coefficients between the two phases are more effective.

The rate of diffusion into food should be modulated, taking into account the real shelf life of a food. In fact, if an antimicrobial agent is highly soluble in food, the diffusion will be unconstrained and, thus, ineffective in terms of protection during shelf life. If release from the package and migration into food are well-regulated and the constant flux of release is combined with a slow diffusion rate into the food, the probability of maintaining an antimicrobial surface concentration above the MIC increases, yielding a well-designed AP.

The technology used to incorporate and/or immobilize the active substance plays a pivotal role in the efficacy and effectiveness of an antimicrobial packaging (Fig. 1). The active substance must be incorporated into a packaging material such as synthetic polymers (i.e., LDPE, PP, etc.), bio-based materials (cellulose, alginate, polylactic acid), and paper or paperboard. Thus, a relationship between the material and the antimicrobial agent should be facilitated to create the appropriate compatibility to guarantee the maintenance of packaging functionality throughout the storage time for the material and the shelf life of the food. The incorporation of antimicrobial agents into synthetic polymers and certain biopolymers primarily occurs through thermomechanical processes, such as extrusion, which create flexible active films, or through the dissolution of the polymer and the antimicrobial substance into an appropriate solvent, followed by the evaporation of the solvent (casting procedure). Thermally stable antimicrobials are incorporated through the hot melting process; polymers, such as LDPE, and copolymers, such as ethylene vinyl alcohol (EVOH), have been used as packaging materials for the incorporation of antimicrobial functions. The incorporation of antimicrobial agents into plastics by melting, compounding, and processing is accomplished by directly adding the active substance into the melted polymer; organic acids and their salts and some essential oils have been incorporated using this approach. The high volatility of essential oils leads to partial exhaustion of antimicrobial activity during extrusion, which reduces the efficiency and effectiveness of these substances. Different studies have discussed the loss of antimicrobial activity from various substances following extrusion. In 2008, Suppakul et al. *(86)* prepared LDPE-blown films containing constituents of basil essential oil. Losses in agent concentrations due to volatilization during extrusion resulted in the partial exhaustion of antimicrobial activity in tests with cheese samples.

Casting technology, via solvent evaporation, is a widely applied technique used for more thermal-sensitive molecules. EVOH copolymers, produced via the casting technique, have been studied for use as films to incorporate antimicrobial agents *(64)*. EVOH was dissolved in a 50% (v/v) 1-propanol–water mixture at 50°C. Oregano essential oil was added to the film-forming solution at a concentration of 5% (w/w), with respect to the dry polymer weight. The results showed that release of the active agents depended on their affinity for the food simulants used and the ability of the simulant to swell the polymer *(64)*. In particular, the presence of strong binding forces between water and EVOH when exposed to foods with high water activity confer antimicrobial properties upon the film. Similarly, oregano essential oil and green tea extract were impregnated with EVOH copolymers; these mixtures resulted in the inhibition of microbial growth in vapor phases and liquid media, revealing the potential of their application as antimicrobial packaging films.

Some interesting results have been obtained from the extrusion of volatile antimicrobial agents with LDPE, as discussed by Solano and de Rojas Gante *(83)*. These authors incorporated known concentrations of oregano essential oil (*Origanum vulgare*) into LDPE via extrusion and observed that a low concentration of essential oil (1%, w/w) was solubilized and migrated to the amorphous region of the polymeric structure due to the nature of the oil. However, the addition of a higher concentration of essential oil (4%, w/w) saturated the amorphous region and interfered with the polymer–polymer interactions, resulting in increased oxygen permeability of the LDPE films.

In addition to hot melting procedures, coating technologies have emerged as useful and alternative incorporation systems for thermal-sensitive antimicrobial agents. Coating is defined as the process of applying at least one layer of fluid onto the surface of a material (paper, plastic, metal) in the form of a film, a sheet, or shaped structures *(29)*. Usually, coatings with a thickness of 1 μm have the ability to maintain the technological functionalities of the substrate (gas permeability, transparency, etc.), presenting a good substrate in which an antimicrobial substance can be dissolved or incorporated. These coatings are often bio-based polymers (alginate, pectin, starch), and the pH or water content triggers the release mechanism during contact with food.

The effectiveness of chitosan-coated plastic films incorporating antimicrobial agents to inhibit *L. monocytogenes* on cold-smoked salmon was evaluated by Ye et al. *(98)*. Chitosan-coated films containing 500 IU/cm^2 nisin and other antimicrobial agents were the most effective treatments against *L. monocytogenes* at an ambient temperature and showed long-term antilisterial efficacy during refrigerated storage of vacuum-packaged cold smoked salmon *(98)*.

A new mild technology for incorporating antimicrobial agents is layer-by-layer assembly. This technique consists of the fabrication of multicomponent films on solid supports, via controlled adsorption from solutions or dispersions, and is considered a potential means for implementing new and versatile surface applications, including antimicrobial packaging *(55)*. This approach has been very poorly studied and applied in the field of food packaging but has high potential. In a study by Dubas et al. *(24),* antimicrobial silver nanoparticles were immobilized on nylon and silk fibers by following the layer-by-layer assembly method. The

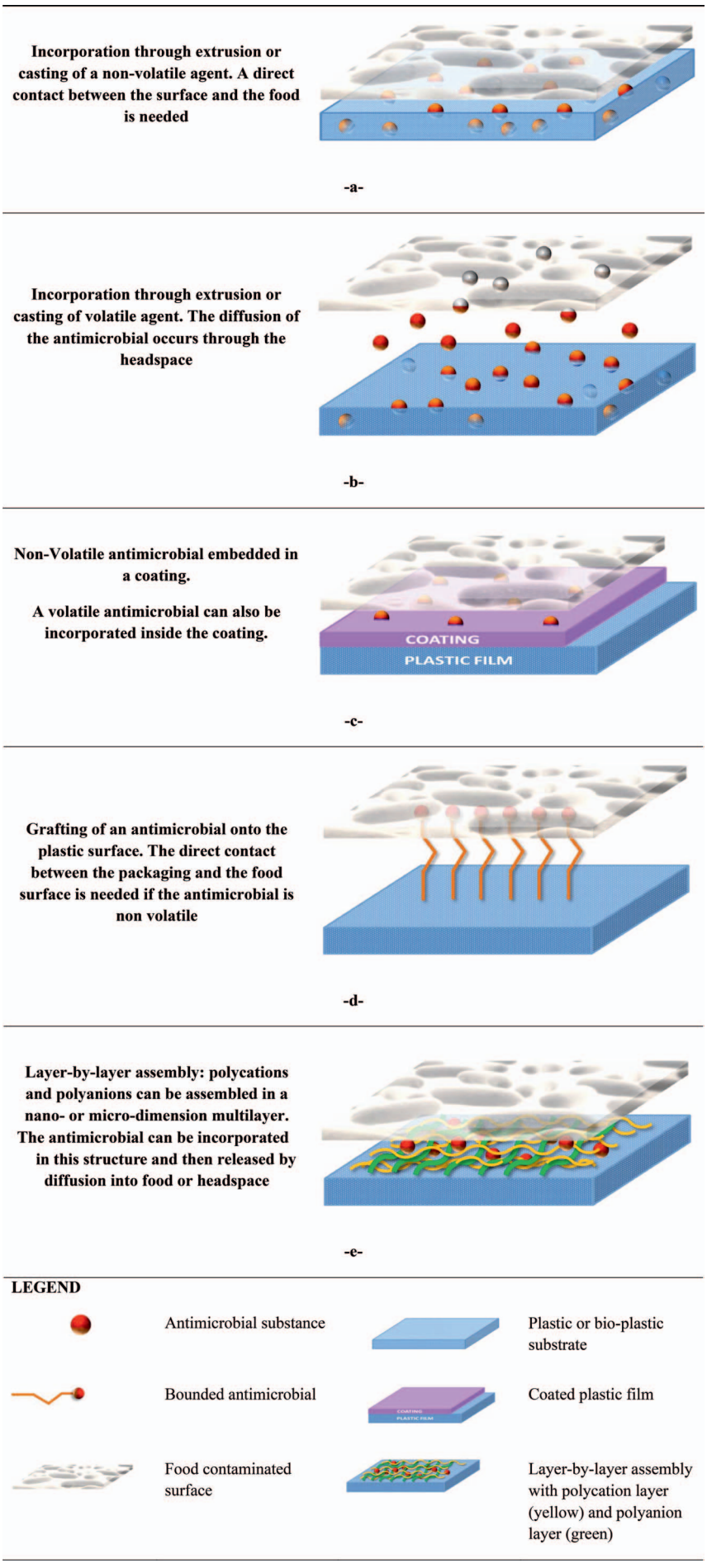

FIGURE 1. *Methods of antimicrobial incorporation into packaging materials.*

deposition of 20 layers onto the fibers resulted in an 80% reduction in pathogenic bacteria (*S. aureus*) for silk fibers and a 50% reduction for nylon fibers. Podsiadlo et al. *(73)* also demonstrated antimicrobial coating using a layer-by-layer assembly of silver nanoparticles. The authors prepared a nanostructured, hybrid, multifunctional composite that contained clay layers implanted with starch-stabilized silver nanoparticles. The tailor-made structure facilitated the release of silver equal to 0.5 to 3.0 μg L^{-1} over 1 month and inhibited *E. coli* growth within 18 h of contact. The possibility of modulating and creating a tunable reservoir of antimicrobial agents in a thin-layer structure, without negative modifications to the substrate, represents a substantial opportunity in the field of AP, and further study is required.

Another technique used in the field of antimicrobial materials is immobilization, which refers to the covalent immobilization of the active molecule to a specific surface. Antimicrobial enzymes (e.g., glucose oxidase, lysozyme) and enzymes suitable for "in-package" processing and production of functional foods (e.g., naringinase, invertase, lactase, cholesterol reductase) can be used with this type of technology. Covalent immobilization provides increased enzyme stability over wider temperature and pH ranges and increased storage stability, and it enables the repeated use of immobilized enzymes *(35)*. Hanušová et al. *(35)* prepared polymer films with immobilized glucose oxidase and lysozyme and evaluated their enzyme activity under various conditions (pH, temperature). These enzymes were immobilized in polyamide and ionomer films because these materials have free functional groups in their structures that are necessary for binding enzymes following surface activation. The covalent bonds between enzymes and polymers were formed by a specific reaction (the Ugi four-component reaction). The in vitro antimicrobial activity of the immobilized enzymes on agar media was verified against *E. coli* CNCTC 6859, *Pseudomonas fluorescens* CNCTC 5793, *Lactobacillus helveticus* CH-1, *Listeria ivanovii* CCM 5884, and *L. innocua* CCM 4030. Conte et al. *(19)* presented a new technique for the immobilization of lysozyme onto the surface of polyvinyl alcohol films. The enzyme was sprayed, along with a suitable bonding agent, onto the surface of the cross-linked polymeric matrix, creating an active packaging to inhibit the growth of certain food spoilage microorganisms.

Antimicrobial agents that contain reactive functional groups, such as hydroxyl, carboxyl, or amino groups, can be covalently linked to a wide variety of polymerizable derivatives, which allows the production of polymerizable monomer-bearing groups with antimicrobial activity. This approach is widely utilized for the fabrication of biocide materials used in the biomedical and pharmaceutical fields. In such cases, the antimicrobial agent is characterized by chemical and thermal resistance to support a specific polymerization reaction. Most synthesized drug monomers and their polymers are acrylic types of pharmaceutically active compounds. Kenawy et al. *(45)* performed a literature survey for these types of substances, highlighting the advantages that this technique offers for the preparation of different hydrophilic or hydrophobic functionalities in polymer drug conjugates. The substances used as monomers in the polymerization process are not always safe for food contact applications. However, as a concept, antimicrobial polymers might be particularly useful for self-sterilizing packaging, reducing the need for peroxide treatment in aseptic packaging.

The safety issues associated with antimicrobial AP must be discussed. In the European Union (EU), the Framework Regulation (EC) 1935/2004 on Food Contact Materials (FCM) establishes that active materials and articles, as with all other FCM, shall be "manufactured in compliance with good manufacturing practice so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: (a) endanger human health; or (b) bring about an unacceptable change in the composition of the food; or (c) bring about a deterioration in the organoleptic characteristics thereof" *(16)*.

AP is defined as a material or article that "deliberately incorporates components that would release or absorb substances into or from the packaged food or the environment surrounding the food," as expressed in Regulation (EC) 450/2009 *(16)*. In terms of safety concerns, the term "active" applies to only an individual substance or a combination of individual substances that provide the active function, including the products of an in situ reaction of these substances. Therefore, attention will be focused on the substance or combination of substances that perform a specific function for the food and not on the material and/or the article in which these substances are included or contained. The material into which the active substance is added or incorporated is considered a "passive" part of the active solution. Special considerations must be given for antimicrobial substances; this category is covered under a different legal framework. The EU Guidance to the Commission Regulation (EC) 450/2009 *(16)* specifies certain subcategories of antimicrobial agents and their legislative references (Table 2).

In particular, only a preservative that is intentionally incorporated into a food contact material and is intended to be released into food is considered an active substance, falling under the specific regulation for active and intelligent materials (Reg. EU 450/2009) *(16)*. Because the released substance (which has a technological effect on foods during or after its release) will be eaten by the consumer, it falls under existing food laws governing toxicity and restrictions of use. However, the stability of these substances under AP manufacturing and processing conditions must be verified by the manufacturer, and a dossier must be submitted for safety evaluation by the European Food Safety Agency under Reg. EU 450/2009 *(16)* if it is demonstrated that chemical reactions, or the degradation or decomposition of the substances, can occur.

In the United States, materials that exert antimicrobial effects on food through migration must be processed through the U.S. Food and Drug Administration (FDA) food additive petition process. FDA approval requires a demonstration that the active compounds are not significantly degraded or converted to potentially toxic products. Antimicrobial agents intended to have an effect on a food

TABLE 2. *Classification of antimicrobial substances based on the legislative field of application under EU frameworks*

Subcategory	Function	Site of action	Release into food	Legislative reference(s)
Surface antimicrobial agents	Maintaining the surface of the food contact material free from microbial contamination	Surface of the FCM[a]	No	*18*
Process antimicrobial agents	Maintaining the components (ingredients, preparations, etc.) free from microbial contamination during production, storage, or handling of FCM	In the components used for the manufacture of FCM	No The substance should not be present in the final FCM	*17*
Preservatives	Prolonging the shelf life of foods from spoilage microorganisms Protecting the safety of foods from pathogenic microorganisms	On or into the food (surface, bulk)	Yes	*15, 16*

[a] FCM, food contact materials.

contact surface (e.g., maintaining the food free of microorganisms) are regulated by the U.S. Environmental Protection Agency. However, the FDA maintains jurisdiction when no lasting effect is intended.

Complications in the approval pathways in both Europe and the United States limit the application of these solutions in the field of food packaging, creating a technological and economic barrier to the circulation of antimicrobial solutions for food contact. This situation also creates a gap between the results of efficacy tests performed in the lab and those obtained from effectiveness tests that replicate the usual circumstances for food storage and distribution, better matching expectations with reality.

METHODS TO MEASURE THE ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL PACKAGING MATERIALS

The preservation of foods has been important for humanity since the beginning of civilization. People have developed many methods to protect their food against microbial degradation. Moreover, the use of new materials with specific antimicrobial properties, together with preservation techniques, provides additional long-term protection by preventing postharvest contamination without interfering with food quality *(21)*.

The evaluation of antimicrobial materials using different analyses has been a critical issue in food safety. Notably, during the development of antimicrobial AP, adequate methods, either in vitro or in situ, to quantify the antimicrobial effects of antimicrobial packaging materials are a prerequisite. Conclusions regarding the antimicrobial efficiency of a particular material vary depending on several factors. For example, the volatility of a material is an important parameter because nonvolatile material directly contacts the surface of foods. In this case, surface characteristics and diffusion kinetics become dominant factors for activity analysis. Another important property of AP is the controlled release of a material to achieve a critical inhibitory concentration against microorganisms. The number of layers used in packaging film is another factor that affects the evaluation method because the sensitivity of the technique must be improved for adequate detection. Thus, different assays have been applied to determine the viability and effectiveness of these methods against microbial degradation.

Direct contact assays: agar diffusion assays. After the discovery of agar as a solidifying agent, culturing of microorganisms on solid media increased dramatically, and consequently, several microbiological analyses became feasible *(84)*. The agar diffusion assay, also known as the agar plate test, is one of the easiest and most rapid analytical methods for measuring antimicrobial activity. In this assay, the antimicrobial film is positioned on a solid agar medium inoculated with the target microorganism. After incubation, a zone will appear surrounding the antimicrobial agent or film (halo or inhibition zone), which indicates diffusion of the antimicrobial compound through the agar. However, it is crucial to use an appropriate control group with packaging materials that have no antimicrobial activity *(84)*. The effectiveness of the agar diffusion assay depends on several factors that directly affect the size of the inhibition zone *(41)*. Because this assay is widely used for toxicity studies, the toxicity of the tested substance influences the inhibition zone, as does the resistance of bacteria to the tested substance. In addition to toxicity, the diffusion of an element on agar also influences the inhibition zone. Therefore, the concentration of agar included in the medium should confer adequate solidity to permit diffusion. Because the hydrophilicity or hydrophobicity, size, and release rate of a substance are related to diffusibility, these parameters must be taken into consideration during this test.

Each bacterium has a specific growth rate on a culture medium; consequently, growth rates on solid agar may differ in velocity. However, some bacteria are considered fastidious; therefore, their growth rate is slow. Due to their requirement for highly specific growth conditions, they cannot be tested by using this assay. Certain antimicrobial packaging materials might be difficult to test due to their highly specific physicochemical properties, such as molecular weight. Antimicrobial materials with high molecular weights diffuse slowly in a medium that contains agar. Therefore, they are not suitable for this assay.

A clear zone surrounding the examined film indicates antimicrobial diffusion and the subsequent inhibition of a target microorganism. Certain control experiments must be performed to control for oxygen restriction. This method can be used quantitatively because the diameter of the clear zone around the film can be measured.

Dual culture assay. Another relevant assay used to screen antimicrobial agents and materials is the dual culture assay. In this assay, two microorganisms, either bacteria or yeasts, are cocultured on agar in the presence of a combinatorial chemical library that contains specific chemicals. When both microorganisms are exposed to compounds from this library, it is determined whether a compound inhibits bacterial growth (an antibacterial agent) or yeast growth (an antifungal agent). In cases in which a compound inhibits both microorganisms, the compound is generally considered a potential toxin *(69)*.

The dual culture assay is often used to evaluate fungal antagonism and is highly dependent on the slow growth of fungi. This situation is a common limitation of the assay because it requires a long culture time to detect an antagonistic relationship between organisms. The dual culture assay can be used as a standard test that allows for the selection of a biocontrol agent. Use of this test on packaging materials makes it possible to analyze the cumulative influence of all mechanisms occurring for biocontrol, such as the presence of diffusible or volatile antibiotics and lytic enzyme production.

Broth dilution assay. In this assay, an antimicrobial solution is prepared by using alcohol, and small-volume aliquots are mixed into a culture medium that contains an inoculum solution with a specific microbial density (CFU). At this point, it is essential that the alcohol used for dilution not show antimicrobial activity. Because certain alcohols, such as ethanol, do exhibit antibacterial properties, control groups that use ethanol should be prepared to observe its antibacterial effects. Following the incubation period, microorganismal growth is measured by determining the optical density. Then, aliquots are diluted and cultured in petri dishes to assess the CFU. Optical densities below a specific value (generally 0.02) show no microorganismal growth. It is always essential to prepare inocula with different densities for proper calibration of the assay *(8)*.

Indirect contact assays: vapor phase assay. Evaluation of antimicrobial activity in the vapor phase is often difficult. Many assays have been performed; however, not all of them can be modified for the quick screening of multiple antimicrobial agents *(66)*. In this technique, a specific solidified medium is prepared in a plastic petri dish and inoculated with a bacterial suspension that contains a specific concentration of the target microorganism. Dilutions are performed by using different solvents, such as ethyl acetate *(8)* or ethyl ether *(58)*. Then, each aliquot of a dilution is added to sterile, blank filter disks and placed on the medium-free cover of each petri dish. Afterward, the petri dishes are sealed using sterile adhesive tape. Following an incubation period in an atmosphere with a high probability of microorganism contamination, the MIC that results in inhibition is measured. To ensure the viability of this assay, it is crucial to incubate the dishes for weeks under continuous temperature conditions, without eliminating the antimicrobial atmosphere, to verify whether the protective effects of antimicrobial agents are temporary or prolonged *(58)*. Therefore, the assay is highly time dependent and requires continuous-control equipment that maintains atmospheric conditions during analysis.

Disc volatilization. One of the most promising assays for antimicrobial activity screening appears to be the disc volatilization assay. In this method, the medium is dispensed into a petri dish and its cover; after solidification of the medium, the desired microorganisms at specific CFUs are inoculated on the medium. The medium in the cover of the petri dish serves as a seal and prevents the adsorption of the antimicrobial agent onto the plastic material of the petri dish *(53)*. Afterward, different concentrations of antimicrobial agent are prepared and added to sterile, blank paper discs, which are placed on the medium in the cover of each petri dish. The discs are left to dry and then incubated. Then, the MICs are determined. The MIC is defined as the lowest concentration of an antimicrobial agent that results in visible inhibition zones *(58, 66)*.

Although the disc volatilization assay is a valuable tool for the simple screening of antimicrobial activity, it requires a relatively high level of material consumption and labor because it is usually necessary to test each concentration of an agent on a separate petri dish for complete analysis. Therefore, further improvements to the assay are crucial to reduce the labor cost and the use of samples. To eliminate this disadvantage, a modified disc volatilization method was performed using a four-section petri dish (90 mm in diameter) and a large filter paper disc (85 mm in diameter). At the end of incubation, the MICs were recorded. The results showed that the use of a four-section petri dish reduced labor and material requirements severalfold *(66)*.

In another comparison study, the antimicrobial activity of an essential oil was tested against 14 microbes by using the agar diffusion (as a direct contact assay) and disc volatilization assays (as a vapor phase test). Antimicrobial activity was observed to be considerably higher in the disc volatilization assay than in the agar diffusion assay *(93)*. This result clearly showed that the disk volatilization assay is more efficient and precise than the agar diffusion assay. However, the number of antimicrobial agents that can be tested in this assay is limited, and the precision may vary depending on the material.

Time-kill assay. Kill time is a significant factor in determination of the effectiveness of an antimicrobial agent for AP. Kill time is described as the minimum time required for the inhibition of microbial growth. In this technique, the initial inoculum is prepared in fresh broth that contains a known concentration of the test microorganism. The antimicrobial agent of interest is added at different concentrations for each microorganism, and a negative

control is also prepared (growth medium without either the antimicrobial agent or the microorganism). Afterward, aliquots are removed at predetermined time points after inoculation, further diluted, and plated on agar plates to enumerate viable microorganisms. At this point, antimicrobial activity is controlled by streaking the transferred aliquot over the agar plate, and growth inhibition is observed at the site of the first streak. In this assay, antimicrobial activity is defined as the absolute decrease in total CFU from the initial inoculum *(50, 74)*. Because it requires the subculture of media at specific times, this assay is time-consuming and laborious.

CONCLUSIONS

Food products are prone to spoilage due to microbial proliferation that makes them unfit for consumption. Antimicrobial packaging technologies present an opportunity for both scientists and manufacturers to establish methods that decrease the hazards and improve the safety and quality of food products by reduction or inhibition of microbial growth. The use of plant extracts, essential oils, organic acids, bacteriocins, inorganic substances, and enzymes in packaging materials appears to be a promising avenue for food preservation. Additionally, measurement of the antimicrobial activity of packaging materials will successfully offer solutions to current producer and consumer problems.

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