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POLYTECHNIQUE MONTRÉAL

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Development of Antimicrobial Films for food Packaging Using melt Compounding

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Thèse présentée en vue de l'obtention du diplôme de *Philosophiae Doctor*

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Development of Antimicrobial Films for food Packaging Using melt Compounding

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DEDICATION

“To my cherished family”,

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RÉSUMÉ

De nos jours, la sécurité microbienne et la préservation de la qualité des aliments sont des préoccupations majeures. L'emballage antimicrobien est l'une de ces technologies prometteuses, qui réduit, inhibe ou retarde la croissance des micro-organismes pathogènes et d'altération et prolonge la durée de conservation des produits alimentaires. Divers composés antimicrobiens peuvent être directement ajoutés à la matrice polymère pour obtenir un emballage antimicrobien présentant l'activité biocide souhaitée ; toutefois, l'incorporation directe des agents antimicrobiens peut entraîner l'évaporation des agents antimicrobiens incorporés et leur migration vers les aliments.

Les nanoparticules d'oxyde de zinc (ZnONP), l'huile essentielle de limonène (LEO), et l'arginate d'éthyle lauroyle (LAE) qui ont été approuvés comme "généralement reconnu comme un matériau sûr" (GRAS) pour les applications alimentaires aux États-Unis par la FDA (Food and Drug Administration) sont considérés comme l'un des meilleurs candidats pour les systèmes d'emballage alimentaire actif. Dans cette thèse, la production de films antimicrobiens à base de polyéthylène (PE) traités avec trois classes différentes d'agents antimicrobiens susmentionnés a été étudiée. Le projet de recherche a été mené en trois phases.

Dans la première étape de ce travail, une étude détaillée de l'effet de l'épaisseur de la couche superficielle du Polyéthylène à basse densité linéaire (LLDPE) enrobée de ZnONPs produite par les techniques de coextrusion et d'enduction par immersion sur l'activité antimicrobienne a été réalisée. La performance antimicrobienne des films produits a été étudiée pour E. coli et S. aureus. Les films nanocomposites multicouches coextrudés LLDPE/ZnONPs ont montré des réductions de 0.5-1.5 log, tandis que la couche active sablée, a montré une réduction d'environ 4 log à la fois pour E. coli et S. aureus en raison d'un plus grand nombre de ZnONPs exposés. De plus, 2 nm de LLDPE ont été enduits de ZnONPs avec différentes concentrations en utilisant la méthode d'enduction par immersion, et le film résultant a montré des réductions de 3 log et 4 log pour E.coli et S. aureus respectivement. Ces résultats indiquent que les ZnONP non migrateurs qui ne sont pas à la surface du film ne sont pas disponibles pour fournir des ions zinc ainsi que des espèces réactives de l'oxygène (ROS) pour inhiber les bactéries. Ils confirment également l'importance du contact direct de l'agent actif avec les bactéries.

Dans la seconde phase de ce projet, les effets antimicrobiens synergiques de cinq supports minéraux différents (nanotubes d'halloysite (HNTs), kaolinite (Kao), nanoparticules de silice mésoporeuse (MSNPs), nanoparticules d'oxyde de zinc (ZnONPs) et tamis moléculaire de type 4A (Z4A)) chargés d'huile essentielle de limonène dans des films de polyéthylène à basse densité (LDPE) ont été étudiés. Tous les hybrides support-limonène ont été ajoutés dans des films LDPE par compoundage à l'état fondu et une caractérisation complète a été effectuée par l'évaluation des propriétés thermiques, mécaniques, optiques, de barrière et fonctionnelles. Les films obtenus présentent des propriétés antimicrobiennes exceptionnelles contre *E. coli*. L'analyse thermogravimétrique (TGA) a montré que 20-25% de la teneur initiale en limonène était conservée contre la dégradation thermique par le compoundage à l'état fondu. En ce qui concerne les propriétés mécaniques, les molécules de limonène libre agissent comme des plastifiants et, par conséquent, elles augmentent l'allongement et diminuent la résistance à la traction et le module élastique pour tous les films développés par rapport au LDPE pur. Les propriétés barrières à l'oxygène ont également diminué pour la même raison. En outre, les études de libération de limonène à court et à long terme ont indiqué que les nanotubes d'halloysite et les nanoparticules de silice mésoporeuse, en raison de leurs fortes interactions synergiques avec le limonène et de leur capacité à réduire le limonène libre dissous dans la matrice polymère, présentaient un profil de libération antimicrobienne soutenu dans des films de LDPE.

La dernière phase a étudié les effets antibactériens et antiviraux de l'arginate d'éthyle lauroyle (LAE) sous sa forme commerciale LAM (10% LAE, 90% maltodextrine) incorporé dans des films LLDPE. La poudre de LAM a été ajoutée dans des films LLDPE en utilisant le mélange à l'état fondu à 6.8, 8.7, 14 et 16.5% (p/p). La stabilité thermique des films développés a diminué avec l'augmentation des concentrations de LAM. De plus, il a été montré que les films contenant 14 et 16.5% de LAM présentaient des propriétés antibactériennes intéressantes contre *E. coli*. Les résultats obtenus ont montré que les films susmentionnés ont perdu leur activité antimicrobienne après 10 jours d'immersion dans l'eau ; cependant, leur activité a été récupérée après 5 mois de stockage à température ambiante avec un taux de réduction de 2 log (UFC/mL) dû à la migration du LAE de la masse vers la surface du film. Le film contenant 14% (p/p) de LAM a montré une activité antivirale contre le *Coronavirus humain (HCoV-229E)* et des réductions de 0.7 et 1.18 log de la dose infectieuse de culture tissulaire 50 ($TCID_{50}$/mL) après 1 et 2 heures de contact, respectivement.

ABSTRACT

Nowadays ensuring microbial safety and extent in preserving the quality of food are major growing concerns. Antimicrobial packaging is one such hopeful technology, which reduces, inhibits, or retard the growth of microorganisms extends the shelf life in food products. A variety of antimicrobial compounds can be directly added into the films to achieve antimicrobial packaging with a desired biocidal activity; however, direct incorporating the antimicrobial agents may result in the evaporation of incorporated antimicrobial agents and migration toward the foods.

Zinc oxide nanoparticles (ZnONPs), Limonene essential oil (LEO), and ethyl lauroyl arginate (LAE) that were approved as "generally recognized as safe" (GRAS) for food applications within the United States by Food and Drug Administration (FDA) is considered as one of the best candidates for packaging systems with antimicrobial properties. In this dissertation, the production of polyethylene (PE)-based antimicrobial films treated with three different classes of aforementioned antimicrobial agents was studied. This dissertation was conducted in three stages.

In the first stage of this work, a detailed study on the effect of thickness of linear low-density-polyethylene (LLDPE) skin layer film embedded with ZnONPs produced by coextrusion and dip-coating techniques on antimicrobial activity was carried out. The antimicrobial performance of the produced films was studied for *S. aureus* and *E. coli*. The coextruded multilayer LLDPE/ZnONPs nanocomposite films showed 0.5-1.5 log reductions, while sandblasted the active layer, showed around 4 log reduction in both *S. aureus* and *E. coli* due to a higher number of exposed ZnONPs. In addition, 2 nm of LLDPE were coated on ZnONPs with different concentrations using the dip-coating method, and the resulted film showed a reduction of 4 and 3 log for *S. aureus* and *E. coli* respectively. These results indicate that non-migrating ZnONPs that are not at the film surface are not available to provide zinc ions as well as reactive oxygen species (ROS) to inhibit bacteria. It also confirms the importance of direct contact of the active agent with bacteria.

In the second stage of this project, the synergistic antimicrobial effects of five different mineral carriers (halloysite nanotubes (HNTs), kaolinite (Kao), mesoporous silica nanoparticles (MSNPs), zinc oxide nanoparticles (ZnONPs), and molecular sieve type 4A (Z4A)) loaded with limonene essential oil in low-density-polyethylene (LDPE) films were investigated. All carrier-limonene hybrids were added into LDPE films using melt compounding and thermal, mechanical, optical, barrier, and antibacterial properties were reported. The resulting films exhibit outstanding

antimicrobial properties against *E. coli*. Thermogravimetric analysis (TGA) showed 20-25% of the initial limonene content was retained against thermal degradation by melt compounding. Regarding mechanical properties, free limonene molecules had a plasticizers effect; consequently, soften the matrix, and improving the elongation property modulus for all developed films compared with neat LDPE. The oxygen barrier properties also decreased for the same reason. Furthermore, the short-term and long-term limonene release studies indicated that both halloysite nanotubes and mesoporous silica nanoparticles carriers due to their strong synergistic interactions with limonene and their capability to reduce free limonene dissolved in the polymer matrix exhibited sustained antimicrobial release profile from LDPE films.

The last phase investigated the antibacterial and antiviral effects of ethyl lauroyl arginate (LAE) in its commercial form as LAM (10% LAE, 90% maltodextrin) incorporated into LLDPE films. LAM powder was added into LLDPE films using melt compounding at 6.8, 8.7, 14, and 16.5% (w/w) which cause decreasing of the thermal stability. Moreover, it has been shown that the films containing 14 and 16.5% LAM showed interesting antibacterial properties against *E. coli*. Immersion the aforementioned samples in water for 10 days caused decreasing antimicrobial properties; however, their activity was recovered after 5 months of storage at room condition with a reduction rate of 2 log (CFU/mL) due to LAE migration from bulk to the film surface. The film containing 14% (w/w) LAM has proven to exert antiviral activity against *Human Coronavirus (HCoV-229E)* and reductions of 0.7 and 1.18 log tissue culture infectious dose 50 ($TCID_{50}$/mL) were shown after 1 and 2 hour contact time, respectively.

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
AMPs	Antimicrobial proteins peptides
ATCC	American type culture collection
CDC	Centers for disease control and prevention
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
CNTs	Carbon nanotubes
CS	Chitosan
DTAB	Dodecyl trimethylammonium bromide
EFSA	European food safety agency
EOs	Essential oils
EPA	Environmental Protection Agency
EVOH	Ethylene vinyl alcohol
FCV	Feline calicivirus
FDA	Food and drug administration
FTIR	Fourier transform infrared spectroscopy
GOs	Graphene oxides
GRAS	Generally recognized as safe
HDPE	High-density polyethylene
HNTs	Halloysite nanotubes
Kao	Kaolinite
LAE	Ethyl lauroyl arginate
LAM	LAE in combination with Maltodextrin
LB	Luria bertani

LbL	Layer by layer
LDPE	Low-density-polyethylene
LEO	Limonene essential oil
LLDPE	Linear low-density-polyethylene
MIC	Minimum inhibitory concentration
MMT	Modified montmorillonite
MNV	Murine norovirus
MOFs	Metal–organic frameworks
MSNs	Mesoporous silica nanoparticles
NPs	Nanoparticles
OML	Overall migration limit
OTR	Oxygen transmission rate
PBS	Phosphate-buffered saline
PE	Polyethylene
PEG	Poly(ethylene glycol)
PET	Polyethylene terephthalate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVA	Polyvinyl alcohol
QACs	Quaternary ammonium compounds
RH	Relative humidity

ROS	Reactive oxygen species
SARS	Severe acute respiratory syndrome
SEM	Scanning electron microscope
SML	Specific migration limit
$TCID_{50}$	Tissue culture infectious dose 50
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
Z4A	Molecular sieve type 4A
ZnONPs	Zinc oxide nanoparticles

CHAPTER 1 INTRODUCTION

The microbial contamination resulting in the growth of microorganisms in/on foods is a constant concern all over the world, leading many countries to find ways to increase the shelf life. Despite all preventive measurements, the increased number of outbreaks of foodborne diseases still remains a global public health threat. For example, the United States Centers for Disease Control and Prevention (CDC) reported that foodborne diseases account for about 48 million illnesses, leading to over 130,000 deaths and hospitalizations in the USA alone [1, 2].

The Canadian government has also estimated that over 10% of the people has been affected result in 105 death every year [3]. Among the different types of foodborne pathogens in Canada, *E. coli*, *Salmonella*, *Listeria monocytogenes*, and *Norovirus* are responsible for about 77% of total food-related deaths due to known causes [3].

On the other hand, almost yearly thirty percent of foods wasted by microbial spoilage through the supply chain [4]. Therefore, food producer are pushing to develop new technologies to control foodborne microorganisms over time in order to improve food shelf life and freshness [5].

One of the promising approaches that emerged as a viable technology is adding the antimicrobial additive to the package while melt compounding is the most ordinarily used technique [6]. However, this technic has some drawbacks like degradation of the active additive during the process causing poor antimicrobial performance [7]. In addition, it may affect the mechanical, thermal properties of food packaging structure [8]. Thus, analyzing all properties to reduce these undesired changes in the active film is necessary.

Three types of additives were considered in this study. Antimicrobial performance, availability, and nontoxicity were the main reason to consider zinc oxide nanoparticles (ZnONPs), limonene essential oil (LEO), and ethyl lauroyl arginate (LAE).

In the first stage of this study, the antibacterial performances of an linear low-density-polyethylene (LLDPE)-based film containing ZnONPs generated by coextrusion and dip-coating processes were investigated.

To date, there was a great deal of interest in embedded ZnONPs in film [9, 10]. However, there is no published information investigating the effect of antimicrobial properties and ZnONPs positions from the surface of the film. Therefore, the global research hypothesis of this part is that the

antimicrobial performance of ZnONPs on the surface is higher than particles embedded inside and cover by the polymer matrix.

In second stage, the possible synergistic effects of several mineral carriers with volatile limonene in melt compounded low-density polyethylene (LDPE) films were reported. The release behavior of limonene from LDPE films in presence of carriers is subjected to consideration. The high volatile nature of limonene is the main challenge for its successful incorporation into a polymer matrix via melt blending [11]. Thus, in order to develop a sustainable antimicrobial packaging system, a synergistic combination with other carriers is required to retain the maximum amount of limonene in the matrix during processing and storage.

In the third part comprises the antibacterial and antiviral effects of ethyl lauroyl arginate (LAE) in its commercial form as LAM (10% LAE, 90% maltodextrin) incorporated into LLDPE films. The ability of LAE migration to the film surface is also subjected to assessment. LAE as a cationic surfactant exhibited remarkable antimicrobial properties against an extensive range of microorganisms [12]. The compatibility of LAE with polymer matrix will control the amount and rate of LAE released from the film surface. Several studies reported the advantage of the adding of LAE in polymer matrices in the solution state [13, 14]. However, using LAE in combination with a carrier incorporated in polymer matrices by melt compounding is lacking in the literature.

The following chapters make up this dissertation:

- Chapter 1: Introduction
- Chapter 2: Literature review
- Chapter 3: Objectives and ordering of the articles
- Chapter 4 to 6: Reports of three articles
- Chapter 7: General discussion
- Chapter 8: Conclusion and suggestion

CHAPTER 2 LITERATURE REVIEW

2.1 Antimicrobial Substances

Many synthetic and natural antimicrobial agents have been reported to be used in food packaging. The safety and nontoxicity of the final structure containing antimicrobial additive is the main point to consider. Therefore, using synthetic or natural GRAS material is the best choice [15].

Antimicrobial substances because of their diverse physiologies have a specific action against microorganisms. However, the exact mechanism(s) of action is often not defined. For example, some antimicrobial additives prevent metabolic pathways, and some target to destroy the cell membrane of microorganisms. In addition, the final performance of antimicrobial film will be affected by the type of the target microorganism. Some characteristics of microorganisms such as gram-negative and gram-positive of the cell wall, acid/osmosis resistance, mesophilic, thermophilic, and psychotropic can be very helpful for the choice of an antimicrobial agent [16].

The use of an antimicrobial additive is controlled by factors such as its thermal stability during extrusion, the compatibility, solubility of additive in the polymer, release kinetics, and its broad-spectrum of inhibition to the range of microorganisms [17-19].

The antimicrobial substances used to activate packaging materials can be categorized into natural and synthetic substances as shown in Figure 2-1 [20]. Nature is a wealthy resource of antimicrobial substances to prevent a wide spectrum of microorganisms. While being more preferred is healthier than synthetic substances. Natural antimicrobial substances are further divided into three categories: antimicrobial compounds from microorganisms (e.g., nisin, pediocin, reuterin, plantaricin, helveticin, and fungicides), animals (e.g., chitosan and lysozyme, lactoperoxidase), and plants (e.g., plant extracts and essential oils (carvacrol, oregano, thymol, Eugenol, limonene, etc.) [21, 22].

With the increasing demand for food production and demand for packaging, natural antimicrobial agents would not be enough; therefore, inorganic or organic antimicrobial materials have been more considered. Synthetic antimicrobials are widely considered for food packages due to their availability, cost-effectiveness, thermal stability, and physical properties [23].

The synthetic antimicrobial substances used to activate packaging materials can be included in the groups of metals (e.g., silver, gold, zinc, and copper), metal oxide nanoparticles (like TiO_2, CuO,

and ZnO, etc.) [24], metal-based complexes (e.g., silver zeolites, zinc zeolites, copper zeolites, metal-organic frameworks) [25], organic acids (such as acetic, benzoic, sorbic, lactic, malic, citric, etc.), and cationic surfactants (e.g., lauric arginate or LAE, etc.) [26].

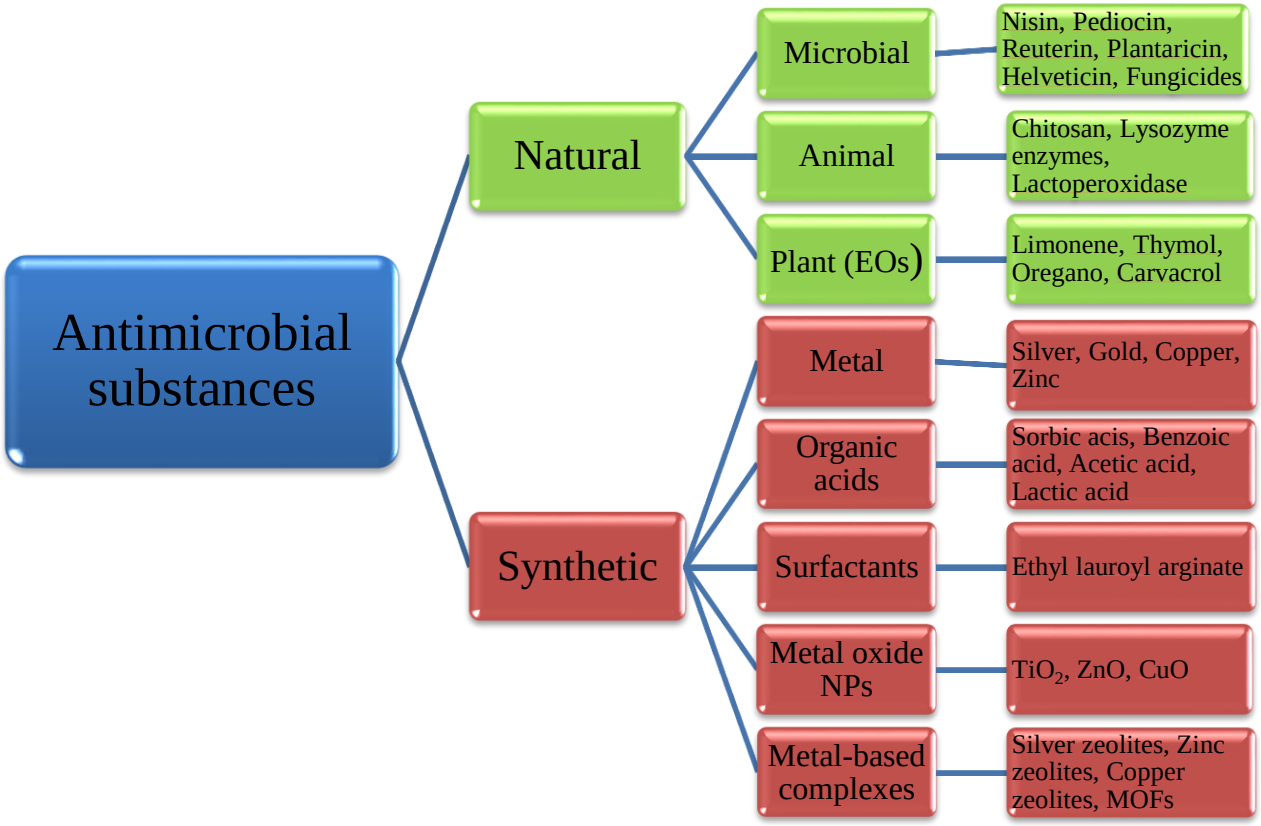

Figure 2-1 Classification of antimicrobial substances regarding the origination [20].

As some synthetic antimicrobials like organic acids can be naturally present in some foods, then the classification of the antimicrobial substance based on natural and synthetic compounds cannot be established [27]; and those can be available commercially [28]. In addition, although metal oxide particles or metal particles naturally would be available, their corresponding nanoparticles (NPs) are synthesized through physical and chemical processes. Thus, they are considered synthetic antimicrobial substances.

Antimicrobial substances can be also classified into organic and inorganic regarding their chemical structure. Most organic antimicrobial agents are extracted from synthetic materials or natural resources, while inorganic antimicrobial additives are obtained from metal-based elements [29].

The organic antimicrobial substances are generally less stable than inorganic substances under conditions of high temperatures and/or high pressures that presents a great obstacle in the product formulation [30, 31]. For providing a continuous antimicrobial effect and preventing the growth of microorganisms, the antimicrobial concentration of a packaging system should be always above minimum inhibitory concentration (MIC) during periods of extended exposure [32].

2.1.1 Zinc oxide nanoparticles

Zinc oxide, as an inorganic and non-toxic antibacterial agent [33-38] is used widely in different applications like in the cosmetics, pharmaceutical, dentistry, food, and glass industries [39]. Figure 2-2 shows the various shapes of ZnONPs obtained from the emulsion method with a surface area of 20 m^2/g and a size of 164-2670 nm [40].

ZnO as a multifunctional material is considered GRAS by the FDA [41]. In addition, food industries use ZnONPs in various types of nutritional products to provide essential dietary zinc for humans and animals growth [42, 43].

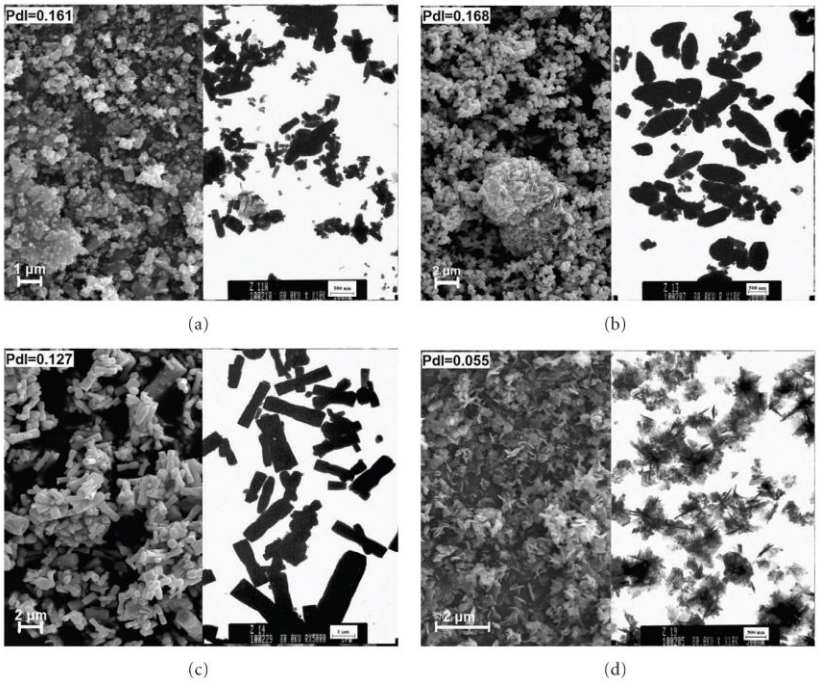

Figure 2-2 Different ZnONPs structures: (a) solids; (b) ellipsoids; (c) rods; and (d) flakes [40].

Below mechanisms are mentioned in the literature as methods for the exertion of antimicrobial properties: (i) the creation of reactive oxygen species (ROS), resulting in membrane damage or death [44]; (ii) dissolution of ZnONPs into Zn ions and interferes with protein metabolisms of the cells [45]; (iii) and destructing bacterial cell membrane through electrostatic forces due to direct interaction between ZnONPs and cell membrane [46]. The mechanism of ZnONPs antibacterial activity is demonstrated in Figure 2-3.

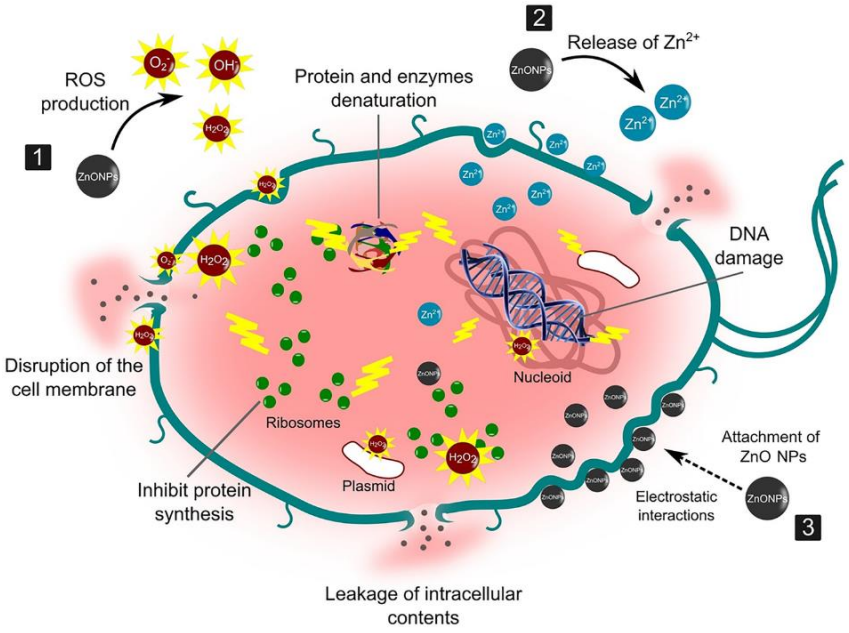

Figure 2-3 Schematic picture presenting the antimicrobial mechanism of ZnONPs against bacterial cells [47].

The antimicrobial performances of metal oxides in the aqueous medium have been reported in the literature. McCloskey et al. [48] checked the toxicity of different metal ions in water and then antimicrobial properties against *Marine bacteria*. They showed the ranking of bactericidal activity of metal ions based on Effective Concentration (EC50) value as Ag > Cu > Zn. Figure 2-4 presents the EC50 values of metal ions for several cases towards marine bacteria. Malachová et al. [49] has also observed the antibacterial ranking in investigations with *E. coli* as Ag > Cu ~ Zn.

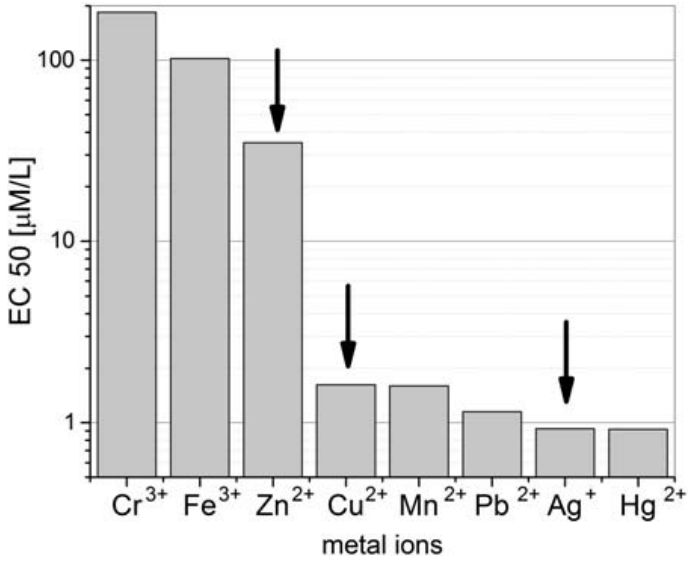

Figure 2-4 EC50 values of metal ions for several cases towards *Marine bacteria* [49].

ZnONPs have been employed as an active agent for the production of antimicrobial films in various studies. Nonetheless, a few of them have been developed by the melt blending method.

Emamifar et al. [50] produced low-density-polyethylene (LDPE) containing different ZnONPs and polyethylene-grafted maleic anhydride (PE-g-MA) content using melt compounding. It was reported that the antimicrobial activity increased by increasing ZnONPs content and showed 5 log reduction for fresh strawberries. Polat et al. [51] developed antimicrobial film with extrusion blending PP and ZnONPs The results also showed that the PP film with 5 wt% ZnONPs improved the shelf life of juice lemon. ZnONPs is adding antimicrobial activity, mechanical, barrier, and chemical stability in polymeric film packaging [41]. However, their potential migration into foodstuffs has been the major concern of any food industry [52].

The migration of substances from food packaging may have an effect on the safety of the product, and then substances on the food contact ares should not be transferred to food in unacceptable amounts. The articles No (EU) 10/2011 from Plastics Regulation and No (EU) 2016/1416 from European Commission published Commission Regulation has determined migration limits for some materials from plastic packages into food to guarantee the safety of consumers.

2.1.2 Essential oils

The antimicrobial performance of essential oils (EOs) as a natural source of bioactive agents for managing harmful microbial invasions on food products have been known and used for many years

[53]. EOs are categorized as GRAS under the conditions of intended use [54]. The great majority of antimicrobial substances are obtained by synthetic processes, but possible side effects are the main concern. Thus, naturally derived antimicrobial agents are used due to being mostly harmless than chemically-based substances. For this reason, many studies are recently focused on the packaging systems with antimicrobial properties based on using essential oils and plant extracts [55-59].

The EOs usually contain about 20–60 components in different proportions. These components are mixtures of aromatic oily liquids synthesized and obtained from plant organisms as secondary metabolites [60]. As presented in Figure 2-5, terpenes, terpenoids, and phenols are the primary constituents of essential oils.

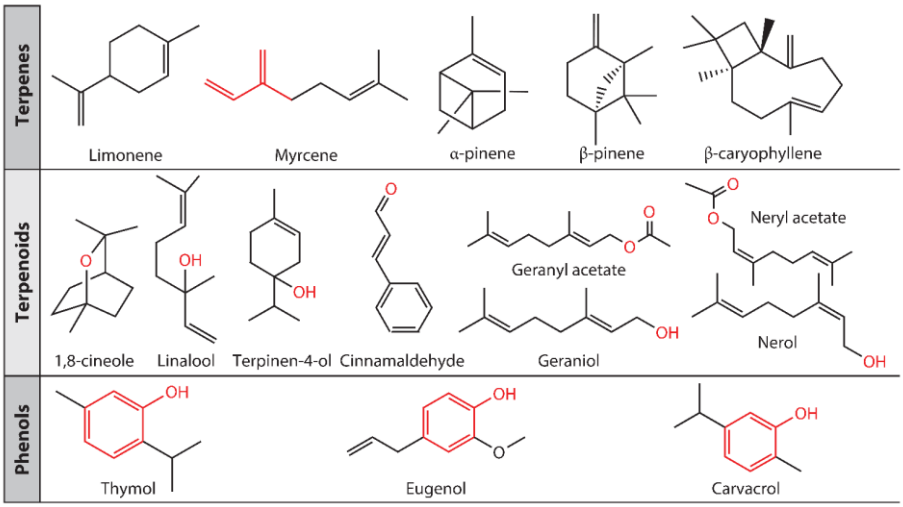

Figure 2-5 Chemical structure of major components in essential oils [61].

EOs show antimicrobial activity against gram-positive bacteria and gram-negative bacteria, viruses, fungi, and protozoa [62]. It is important to find how different EOs exert antimicrobial properties against different microorganisms. However, since different components are present in EOs, a clear mechanism of action has not been reported yet [63].

Generally, EOs and their components exert their antimicrobial effect by (1) the cell wall disruption [64-66], (2) disruption of the cytoplasmic membrane, resulting in cell contents leakage [67-69], (3)

coagulation of the cytoplasm [70, 71], (4) disruption of DNA [72] (5) depletion of the proton motive force [73], and (6) hydrolysis of ATP and decreasing in the synthesis of ATP, leading to reduction of intracellular ATP pool [73]. Figure 2-6 represents the different actions of EOs on microorganisms.

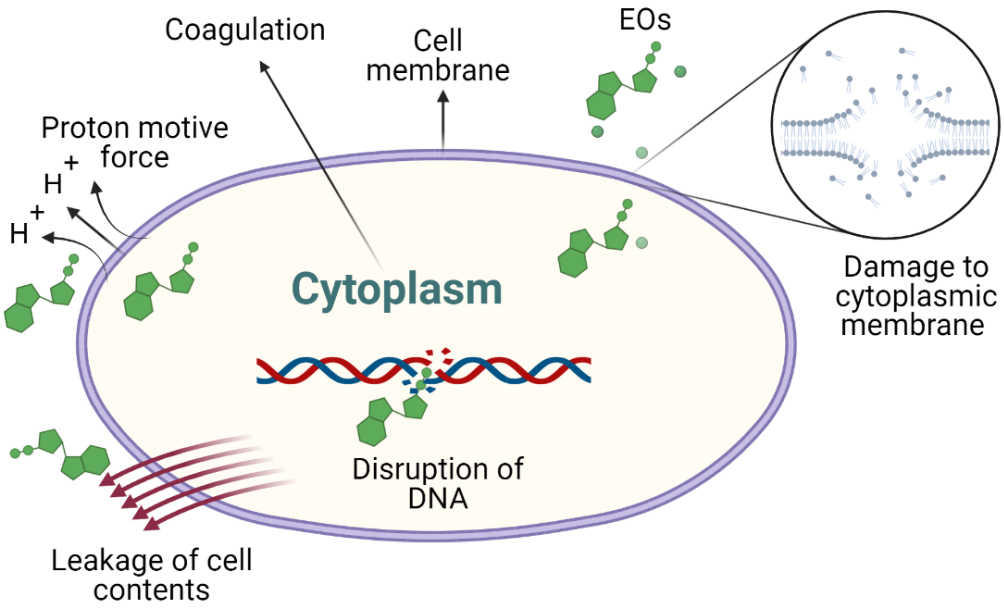

Figure 2-6 General mechanism of action using EOs against microorganisms [74]

Several studies have been carried out mainly on incorporating EOs, such as thymol, carvacrol, and cinnamaldehyde through harsh processing conditions in melt compounding processes. Nevertheless, it was reported complete losses [75] or losses reaching up 73% [76-78]. The antimicrobial performance of the majority of the EOs is the result of the presence of different phenolic compounds. These antimicrobial agents have been presented to be the most important candidates for preventing the growth of foodborne pathogens [79]. It has been reported that they are bacteriostatic as well as bactericidal against both gram-negative and gram-positive bacteria [80].

Thymol and carvacrol, each has a phenolic group, are active antimicrobial agents despite their low water solubility. Their activity is related to the aromatic ring of the phenol and the hydroxyl group of the phenolic structure, while the OH group can form hydrogen bonds with enzymes. In addition,

the number and the position of these groups at the level of the phenolic structure are the factors, which govern their activities, the increased rate of hydroxylation results in an increase in the toxicity. It has been shown that thymol and carvacrol, are the best components with inhibitory effects on the gram-negative and gram-positive bacteria [81].

As a model terpene, d-limonene ((+)-limonene) is a colourless liquid prepared by the cold pressing of citrus peels and pulps, where it can be found in concentrations of up to 90% [82]. Limonene principally found in Citrus spp. (30–98%) [83, 84], has been proven as an antimicrobial substance in food packaging. Because of its citrus-like flavor, the major use of limonene in consumer products is as a flavoring and/or fragrance agent in food such as fruit beverages and ice creams, pharmaceuticals, and cosmetic products [72]. Limonene EOs also as a food preservative exhibits very strong inhibitory activity against fungi [85-87].

There are some limitations for using essential oils on food products, which may cause off-flavors and odors within the packaged food product due to the characteristics of the EOs such as strong hydrophobicity and volatility [88, 89]. The level of these interactions depend on the properties of the flavors and the physical and chemical properties of the components in food (such as fat, starch, and proteins) [90]. However, such limitations can be successfully overcome through antimicrobial packaging, in which, incorporation of limonene into carriers or encapsulation will control the release rate.

The amount of limonene in the final produced foods can be in the range of 1-70% [91]. The pure limonene essential oil used for food purposes can be incorporated into flavoring agents at a lower content or directly mixed into food preparations [91]; The limonene intake in the USA from food was estimated to be 0.27 mg/kg/day [91, 92].

2.1.3 Ethyl lauroyl arginate (LAE)

Ethyl lauroyl arginate (LAE) is listed as “generally recognized as safe” (GRAS) for certain food applications by the FDA in 2005 [93-95]. LAE was also got approval for use as food preservative at a concentration of up to 225 ppm by the European Food Safety Agency (EFSA) in 2007 [96]. LAE has a cationic head group and a nonpolar tail that can adsorb to oil-water interfaces and biological membranes plasma of microorganisms. Figure 2-7 shows the structure formula of LAE.

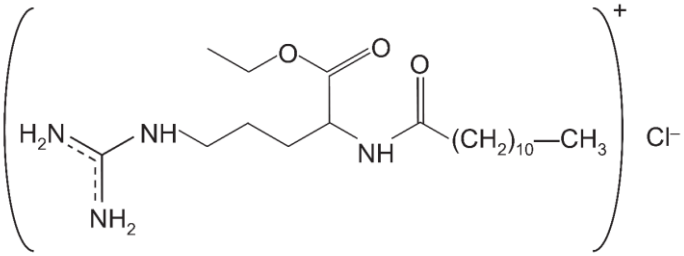

Figure 2-7 Structure formula of LAE

LAE exhibits antiviral and antimicrobial activities toward an extensive range microorganisms, including *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* [97], *Aspergillus flavus* [98], *Herpes virus type 1*, *Vaccinia virus*, and *Bovine parainfluenzae 3* [99, 100].

As shown in Figure 2-8, the cationic surfactant segment of LAE allows electrostatically interactions with negatively charged proteins present on the cell surface of microorganisms, leading to the depolarization of the cytoplasmic membrane, and consequently causing cell death [22, 101].

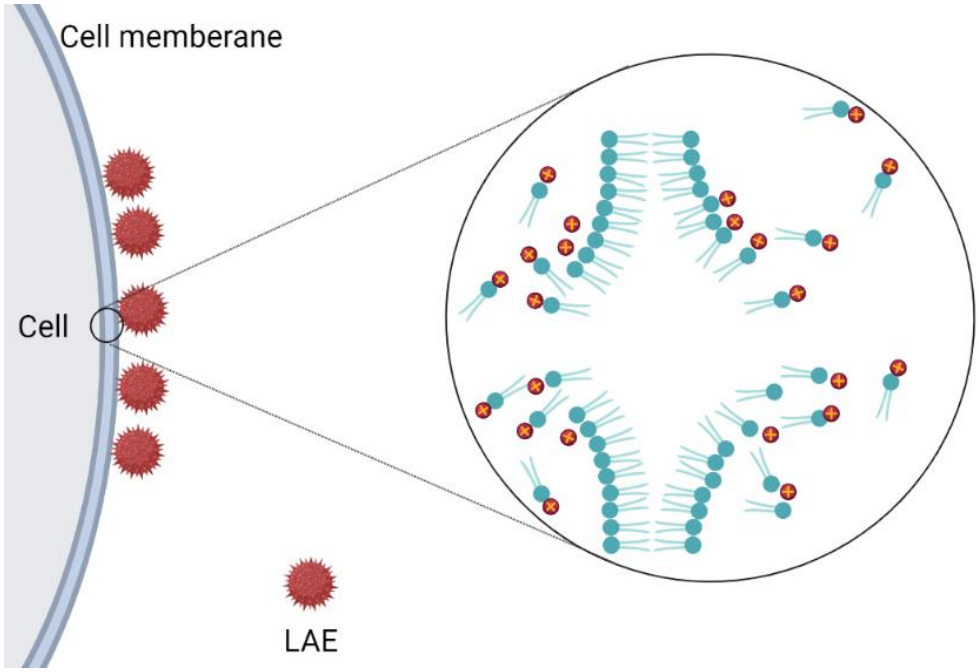

Figure 2-8 Antimicrobial action mechanism of LAE: disruption of the plasma membrane [22]

LAE as a potential alternative for essential oils has proven to be an hopeful candidate for active packaging due to its strong antimicrobial, colorless, and nontoxic properties [26]. Manso et al. [102] incorporated the LAE into a biofilm matrix (INZEA F19) by extrusion method. The results demonstrated that the matrix enriched with LAE removed the *Salmonella enterica* but did not inhibit *Aspergillus flavus*. In this study, LAE was added to polystyrene (PS) pads spraying on the PS pads as well as immersion of the PS pads into LAE solution and 4 log CFU/mL, reduction was reported against *Pseudomonas putida*.

2.1.4 Metal-organic frameworks (MOFs)

Metal-organic frameworks (MOFs) are porous crystalline materials that contain polydentate ligands and metal ions or metal clusters [103] and are used in different applications ranging from sensors, catalysis, biomedical and antimicrobial fields due to their constant release behavior, highly porous structure, large surface area, and structural flexibility [104].

Preparation methods of MOFs have been evolved with increasing speed due to their extensive application potential. Various methods for preparing MOF-based materials have been presented [105] from encapsulation, impregnation, infiltration, solid grinding, coprecipitation, and so on.

The main antimicrobial mechanisms of MOFs include physical interaction with the cell membrane, the metal ions actions , ligands, or a combination of them, oxidative stress such as generation of toxic ROS, photothermal effect, and synergistic effect, etc. [106]. Figure 2-9 presents a schematic view of mechanisms of action.

The antimicrobial mechanisms of MOFs are similar to the antimicrobial mechanisms of metal ions in some aspects. However, MOFs have further antimicrobial efficiency than metal ions, which might attribute to the constant release behavior of metal ions, crystalline nature, components, and frameworks of coordination materials [107].

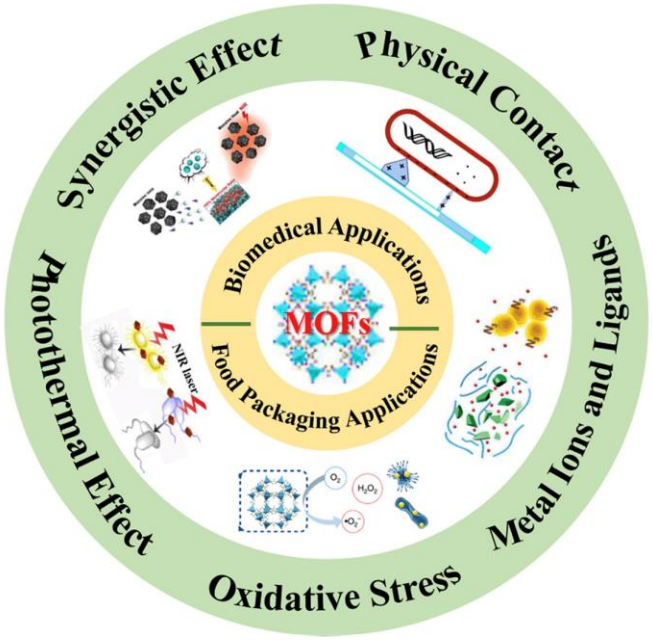

Figure 2-9 Antimicrobial mechanisms of MOFs [106]

2.2 Antimicrobial packaging systems

Antimicrobial active packaging contains antimicrobial additives into the matrix and delay microbial growth and improve the safety of products during transportation and storage. When designing antimicrobial packaging systems, several factors need to be considered such as the chemical nature and the targeted microorganisms, property of the antimicrobial additives like

thermal stability and polarity, storage temperature, and the residual antimicrobial activity after manufacturing [108, 109].

Adding directly the antimicrobial substances into the food will lead to losing the activity as well as undesirable reaction with food due to leaching into the food products. Then, antimicrobial packaging systems enable the slow migration of the antimicrobial additive as well as provide a continuous antimicrobial performance during transport and storage [110, 111].

The antimicrobial film packaging can be achieved by five different approaches: (1) antimicrobial sachets located in packaged food; (2) adding the antimicrobial additives in the polymers; (3) surface coating of antimicrobial agents; (4) immobilization of antimicrobial agents on polymers, and (5) using polymers already having intrinsic antibacterial and film-forming properties [5, 112-114].

Generally, antimicrobial packaging systems with regards to interactions of antimicrobial agents with the packaging and food matrix can be categorized in two ways as migratory and non-migratory agents. Figure 2-10 shows the basic releasing methods of the packaging systems with antimicrobial properties.

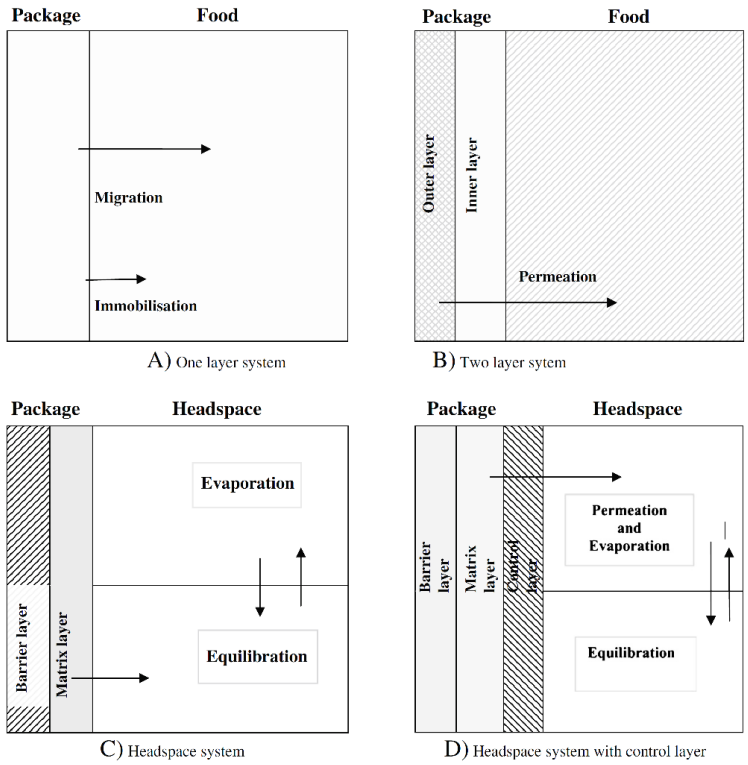

Figure 2-10 Basic releasing methods for antimicrobial packaging systems [30, 115].

In the packaging systems for food applications (A) and (B), antimicrobial agent migrates into the food through diffusion and in the (C) and (D); evaporation of volatile components is the main concept of migration. In (D), the volatile antimicrobial compound is regulated by a control layer [30].

Migratory packaging is designed to release antimicrobial agents in the space between the food and the packaging or even directly in the packaging. For such applications, antimicrobial agents may be volatile or not. Non-migratory type packaging may include polymers already having intrinsic antibacterial properties such as chitosan, or even structures containing immobilized antibacterial agents. In this type of packaging, antimicrobial agents must touch the food surface to ensure its complete effectiveness [12, 116, 117]. So parameters like surface properties and diffusion are significant [5]. The modes of antimicrobial packaging are summarized in Table 2-1.

Table 2-1 Action modes of antimicrobial packaging based on migration/contact [118].

	Migratory	**Non-migratory**
Direct contact	Diffusion and dissolution	Effect of surface on microorganisms reaching it
Noncontact	Adsorption of volatile active component onto food surface	None

2.2.1 Sachets or pads

Sachets for reducing microbial spoilage can be categorized into 1) sachets that produce and release the antimicrobial substances 2) and sachets that are used as a carrier for emitting the antibacterial agents into packaged food. LDPE is widely used for producing various sachets/pads, however, silica components, calcium alginate, gels, starch, are also used as a carrier/emitter [119, 120]. Generally, using volatile antibacterial additives like ethanol, organic acids, essential oils, chlorine dioxide, into sachets or pads is the most prosperous industrial approach for antimicrobial packaging [121, 122].

2.2.2 Direct incorporation

The direct addition of antimicrobial ingredients in the films through melt extrusion is preferred for industry because of its fast production capability [123]. However, direct incorporation is not feasible using volatile and thermosensitive antimicrobial additives as high thermal and pressure processes can damage the active additives [124]. Metal and metal oxides NPs are particularly good candidates for direct incorporation into the films due to certain functions like strong antimicrobial activity and high thermal stability under harsh production conditions [125].

2.2.3 Coating

Surface coating presents a nonthermal alternative method for making the film. The coating can be applied as post-processing, which minimizes the exposure to contamination due to mishandling during processing. In addition, the antimicrobials coating assists in the maintenance of high contents of the antimicrobial compound on the food surface. Bioactive property may be based on release in the headspace of volatile additives. However, there are several intrinsic drawbacks need to be consider.

First, antimicrobial substances embedded in a coated surface are subjected to migration into the food due to the improved active agents to food [126]. In this sense, high costs accrued during the health-risk regulatory compliance assessments (e.g., data collection, analysis and, recommendations), hamper the commercialization of fabricated coated films, especially films coated with metal or metal oxide NPs [127].

Second, uneven coating qualities are common issues for this method. A lack of evenness of coatings on film substrate leads to the irregular distribution of the active agents, further resulting in a sublethal concentration of antimicrobial agents on some parts of the packaged food surface [128].

Furthermore, the location of the antimicrobial agents at the surface makes them susceptible to inactivation due to potential interaction as well as additional stress factors present. Castro et al. [129] reported that nisin and essential oils are subjected to degradation when placed next to the lipids, or enzymes.

As shown in Figure 2-11, coating of surfaces with antimicrobial properties can be achieved by two different strategies, including antiadhesive approach that prevents microorganisms to adhere to the modified surface and/or killing microorganisms (either release or direct contact) [130].

Anti-adhesion coatings aim to prevent microorganism attachment and to delay the formation of biofilms on product contact surfaces by using approaches such as repelling or attachment of the bacteria. These kind of biopassive surfaces can be obtained through making specific surface topography that is not favorable to the microorganisms. Accordingly, repelling systems can be categorized i) steric impediment, ii) electrostatic interactions and iii) low surface energy, that will be found in the surface with superhydrophilic, charged and superhydrophobic properties, respectively [131]. A wide variety of polymers, including poly(ethylene glycol) (PEG) and zwitterionic structures, have been reported as biopassive surfaces [132].

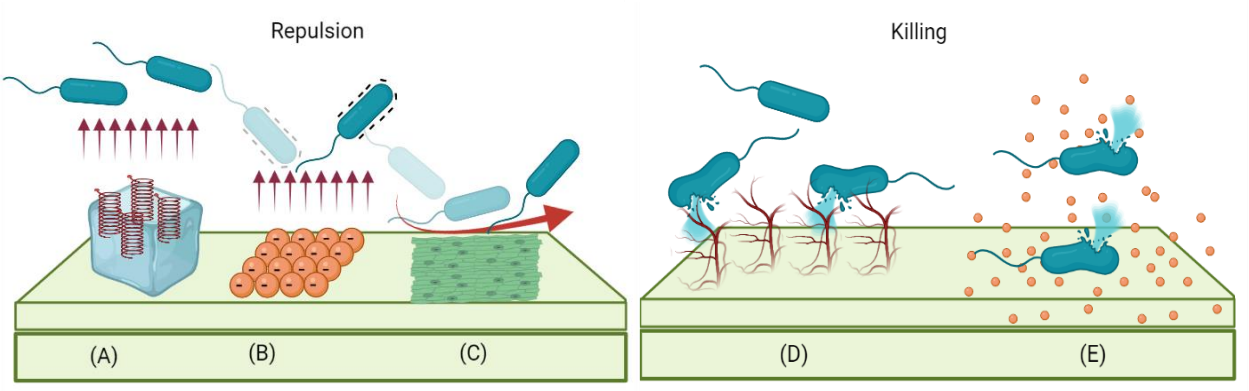

Figure 2-11 Some strategies for antimicrobial coatings classified by their functional principle: (A) steric repulsion, (B) electrostatic repulsion, (C) low energy surfaces, (D) contact killing and, (E) biocide releasing effects [130].

2.2.3.1 Superhydrophilic surfaces

Superhydrophilicity is unfavorable for the adhesion of microorganisms to the surfaces. Highly hydrophilic coatings provide surfaces that a layer of water can be coated on it and then microorganisms cannot be attached. This energetic water barrier layer is strongly linked by H-

bonding to the hydrophilic polymer like PEG that is an additional hurdle for cells or bacteria to overcome [133].

2.2.3.2 Charged surfaces

Negatively charged surfaces can repel bacterial due to electrostatic repulsion between microbial cell membranes and polymer surfaces [134]. Strong electrostatic forces can cause a disturbance of the membranes structures of adhered bacteria leading to cell shape change [135].

Bacteria will initially have the long-domain Van der Waals forces with the polymer surface, but then, short-doamin repulsive electrostatic forces at a separation distance of about 10-20 nm, due to an overlap of the electron clouds of both bacteria and surface may prevent contact to the surface [136, 137]. Surfaces functionalized zwitterionic polymers could be classified as antiadhesive materials with electrostatic and steric repulsion characteristics [138].

Gottenbos et al. [139] presented the *in vivo* and *in vitro* antimicrobial potential of positively charged surfaces against both gram-positive (*S. epidermidis* and *S. aureus*) and gram-negative bacteria (*P. aeruginosa* and *E. coli*). It was derived that positively charged surfaces may attract more bacteria. Further, it was concluded that food proteins can interact with functional groups of the surface, which may result in decreased cell adhesion.

2.2.3.3 Superhydrophobic surfaces

Materials with high surface energy coating surfaces show lower interaction with bacteria than coating with lower surface free energy. Superhydrophobic surfaces with low surface energy are characterized by a water contact angle higher than 150°. Reducing bacterial adhesion via superhydrophopic surface is a relatively novel approach and needs to be investigated thoroughly and systematically [140]. The methods for developing superhydrophobic antiadhesive coatings include photolithography [133], etching [141], chemical vapor deposition [142, 143], layer-by-layer deposition [144, 145], sol−gel chemistry [146], solution deposition (immersion) [147, 148], electrospinning [149], and/or a combination of them.

The main limitation of these surfaces that makes them a challenging issue is that the surface features are not consistently stable and need to be considered for the production of high film surface quality [150].

2.2.3.4 Contact-active surfaces

Contact-killing surfaces rely upon those antimicrobial substances that inactivate microorganisms on contact while being bound on surfaces. These active agents can be attached to the solid-support surface through long tethering polymer chains, allowing the biocide to reach and disrupt plasma membrane of the bacteria, and leading to microbial death [151, 152].

Contact-active surface technologies can employ quaternary ammonium compounds (QACs), antimicrobial proteins peptides (AMPs), Polymer brushes, bacteriophages, and Enzymes[153]. However quaternary ammonium compounds (QACs) are most commonly reported as contact active antimicrobial additives that can be attached to surfaces by covalent bonding [154].

2.2.3.5 Biocide-releasing surfaces

Another approach for developing release-based antimicrobial surfaces is the incorporation of antimicrobial agents into a coating that gradually releases biocide molecules for a long period of time. The majority of antimicrobial substances used in preparing of biocide-releasing surfaces includes plant extract, metals (in particular, silver and copper), metal oxide nanoparticles (e.g., TiO_2, ZnO, and CuO, etc.), and graphene oxides (GOs) [153].

The release of incorporated active agents is achieved by diffusion, erosion/degradation, or hydrolysis of covalent bonds [155]. The release profile in this system offers the possibility to deliver a high antimicrobial agent concentration locally, without exceeding systemic toxicity due to strong interaction between the coating and antimicrobial substances. Whereas the antibacterial agent contained in the reservoir coating surfaces is limited, their action is temporary and their commercial level remains limited [156].

However, this can be solved up to some extent by employing larger pore size carriers such as hydrogels, ceramics or nanostructured polymeric systems as well as using layer by layer (LbL) methods to retain the antimicrobial substance in the coating surface over a longer time [157].

2.2.4 Immobilization

Although immobilization is treated as a different antimicrobial packaging system, it is sometimes considered as a coating system due to a similar concept of active agent incorporated into or onto the film surface [158].

The immobilization process of an antimicrobial agent in/on the polymer material prevents the growth of bacteria on the surface of packaged products [159]. In this approach, the migration of active substances is limited due to a strong interaction between active substances and film substrate, thus reducing potential health risks for consumers ingesting active compounds compared to other types of packaging systems [160].

The different strategies for the immobilization of antimicrobial agents to substrates include covalent bonding [161, 162], adsorption [163, 164], and encapsulation [165, 166]. Covalent linkage contains the generation of a chemical coupling between antimicrobial substance and the solid-support surface, whereas adsorption involves noncovalent but strong interactions (e.g., electrostatic, ionic and hydrophobic, Van der Waals forces, and hydrogen bonding interactions) between antimicrobial agent and the immobilization support. Furthermore, in encapsulation method, active agent is entrapped into the carrier and leaches out over time microbial growth.

Functional groups present in the polymer surface such as amide, ether, ester, can be covalently attached with the amine, carboxylic acid, and hydroxyl of active compounds resulting in the antimicrobial substance becoming a part of the packaged product [167, 168].

An extensive range of active compounds have been immobilized on a host of surfaces and tested as antimicrobial substances, including quaternary ammonium polymers, polyamines, guanides, enzymes, chitosan, peptides, and other peptide mimetics. The activity of antimicrobial additives is essential while attached to the surface in order to eradicate the microorganism [169] .

2.2.5 Polymers with intrinsic antimicrobial properties

Some polymers have natural antimicrobial properties and have a good potential to be employed in antimicrobial films packaging. Polymers like chitosan and its copolymeric derivatives and poly(ε-lysine) have been reported for their inherent antimicrobial activity [170, 171]. Chitosan is a natural linear polysaccharide comprised of the alkaline deacetylation of chitin [172, 173]. The degree of de-acetylation and degree of polymerization are two decisive factors for the use of chitosan in various applications [174]. Chitosan is characterized by excellent antibacterial activity against bacteria which is influenced by the type of chitosan, its degree of polymerization, the pH [175, 176] the composition of the natural or chemical nutrient substrate, and environmental conditions. However, chitosan can act as an antibacterial agent in acidic environments because it

is poorly soluble in neutral or basic media [177]. Figure 2-12 presents the chemical formula of chitosan and chitin. The solution casting/solvent evaporation method [178, 179] and melt blending using twin-screw extrusion [180, 181] are mainly processes to prepare chitosan film.

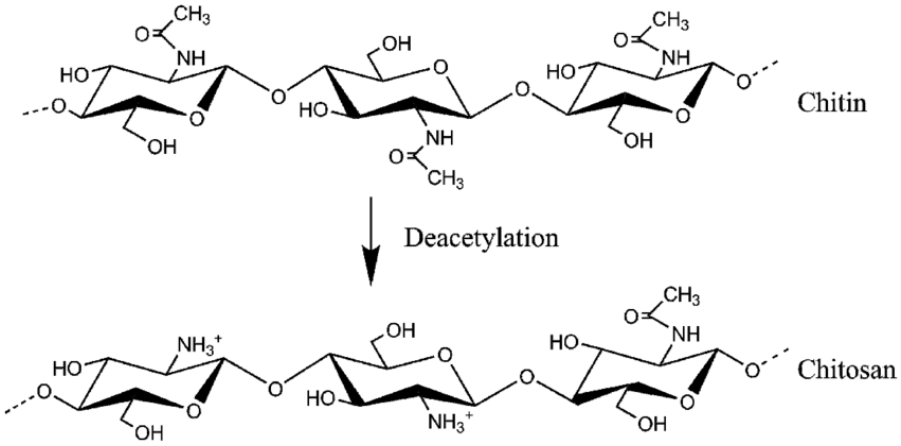

Figure 2-12 Chemical formula of chitin and chitosan [177].

2.3 Migration of active agents

The “migration” refers to the transfer of low-molecular-weight compounds from the surface packaging to the food [182]. It is important to emphasize that the migration of active substances from the packaging plays a major role in antimicrobial properties. The release of active agents required the process to apply a antimicrobial properties against the food contaminants, and then enhance the food quality [183, 184]. It means that the addition of antimicrobial materials into film packaging may be more efficient if the release of antimicrobial substances onto the food surface can be controlled for an extended period [185].

Antimicrobial agents can be released by evaporation from the polymer film surface or diffusion from the bulk polymer phase. In general, the mechanism of migration are 1) diffusion of active compounds in the bulk of the polymers, 2) desorption from the surface, 3) sorption of the additives in the interface, and then 4) desorption into the food [186].

The studies reviewed previously demonstrate that the migration process is mainly governed by both kinetic and thermodynamic properties [187, 188]. The diffusion coefficient is a characteristic constant in the specific matrix system and consists of the mass transfer due to random motion of individual molecules from high concentration (packaging system) to low concentration regions

(contained food) [189]. The partition coefficient, which is associated with the distribution of active migrants in the different phases, is the ratio of concentrations of the active migrants in the film and the food when the two concentrations are at equilibrium. By experimental estimation of both parameters, the concentration of transferring in any position of the package and within a certain time can be predicted.

As presented in Figure 2-13, the migration process various depending upon many factors, including the polymer material itself, the presence of additives in the films (for instance plasticizers, lubricants, impact modifiers, stabilizers, fillers, and viscosity modifiers); the antimicrobial substance properties (like polarity and volatility); the interaction between the active substance and the polymer matrix; possible changes in the polymer produced by the active compound; food characteristics (composition, pH and water activity); and finally environment factors, mainly storage conditions such as relative humidity and temperature [117, 190]. Furthermore, the migration testing method may significantly affect the sustained release properties of the active additives from polymer films.

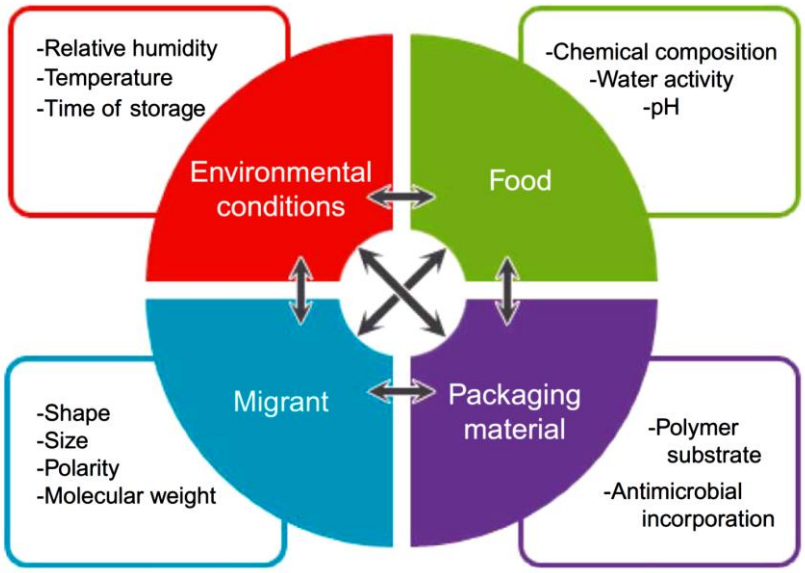

Figure 2-13 Major parameters that affect the interconnection and migration between them [22]

To date, several strategies to develop the packaging material for a more controlled diffusion of the active additive have been proposed. These approaches include modification of packaging material

by the irradiation [191-196], cross-linking agents [195, 197] and multilayer films (coextrusion method and layer-by-layer deposition) [198-201]. In addition, micro- or nano-encapsulation into the polymer [202-205], or using encapsulating materials such as nanoparticles and nanotubes which act as reservoir of active compounds [206-208], may promote the properties of active compounds.

An example of a typical antimicrobial multi-layer film that can be prepared using coextrusion is a structure composed of four layers, including the outer layer (optional), a barrier layer (optional), an antimicrobial-containing matrix layer, and a release-control layer (Figure 2-14) [209]. The matrix layer contains active substances and has a very fast diffusion of the antimicrobials. The release rate of the active additives from the matrix layer is controlled by the control layer next to the matrix layer. In other words, the initial lag period and penetration of the antimicrobials to the food are controlled by the control layer. By changing the thickness and the diffusivity, various custom-made multilayer products can be designed.

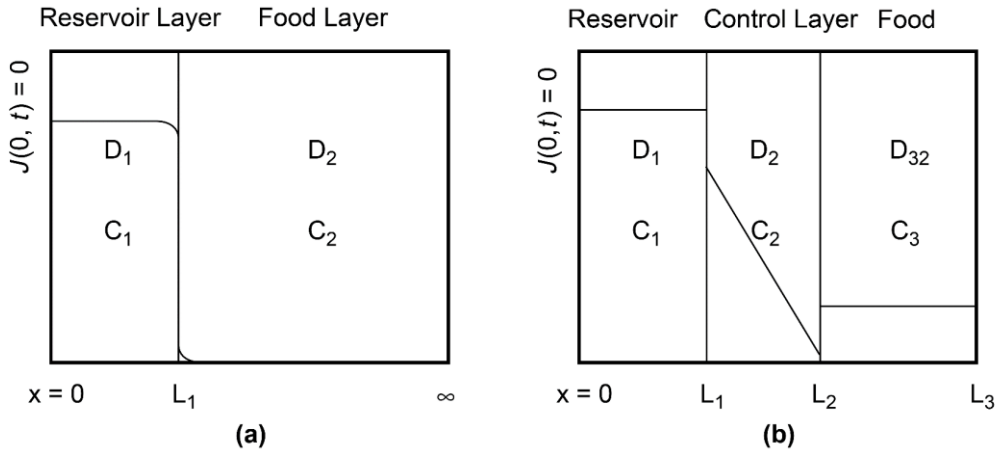

Figure 2-14 Mass transfer models for single and multilayer film with antimicrobial properties: (a) without release system and (b) with release system [15].

2.4 Particle migration

The success of particles migration and subsequent localization in polymeric systems is in many ways related to thermodynamics (the surface energy and wettability of the nanoparticles), and kinetics-driven approaches. As reported in the literature, Brownian motion and diffusion are not the right mechanisms regarding the migration of particles in viscous polymer matrices [210-212].

Some works explained the particle migration to kinetic factors, but mostly considered thermodynamic where the component with lower surface tension (Solids such as metals and metal oxides are usually classified as high-energy surfaces) tend to lower the interfacial energy. In other words, the system's tendency to minimize its free energy can explain nanoparticle localization in one polymer or at the interface [213-215]. Concerning the thermodynamic factors, surface properties, surface energy, or hydrophobicity, the polarity of nanoparticles, and particle interactions with the host polymer matrix play a key role in particle migration toward the interface and subsequent particle localization [216]. The wetting coefficient is a possible way to express this generally valid principle that is deduced from Young's equation. Many researchers have controlled the localization of particles by controlling their surface chemistry. Under quasi-equilibrium or quiescent conditions, the particles should be localized according to the wetting arguments laid out in Young's equation [217]. Moreover, the interaction between polymer and nanoparticles can be helpful in analyzing particle localization in polymeric systems. Entropy can also play a significant role in the prediction of the location of particles in polymeric systems. The migration of the nanoparticles to the substrate results from an overall gain in entropy of the system that also changes the overall system energy. Since entropy plays a crucial role in pushing the nanoparticles to the surface, it is obvious that the two most important length scales viz. size of the particle and linear polymer radius of gyration would also be crucial in determining the ultimate localization of nanoparticles [218].

Different processing parameters during melt compounding like particle size and shape, particle aspect ratio, mixing time, melt viscosity of polymer components, molecular weight, shear rate, and dispersion of nanoparticles is significant to achieve desirable localization and migration of particles, while those are part of kinetic factors [219].

2.5 Antiviral packaging films

Human infectious diseases can be transmitted by various contaminated surfaces by viruses including *SARS-CoV-2*, which causes severe acute respiratory syndrome (SARS) [220]. Food products may be contaminated by and cause the spread of disease. The viruses are stable for several hours to days on different surfaces. Therefore, there is the possibility that viruses can also be transmitted through the contaminated surfaces of packaging materials, especially during the *COVID-19* epidemic. In order to address customer concerns and

assure their safety, active packaging with antiviral properties may offer the opportunity to limit its transmission. This antimicrobial packaging is expected to provide an internal active layer to protect food products and an external active layer with antiviral properties to prevent its spread and protect customers (Figure 2-15) [221-223].

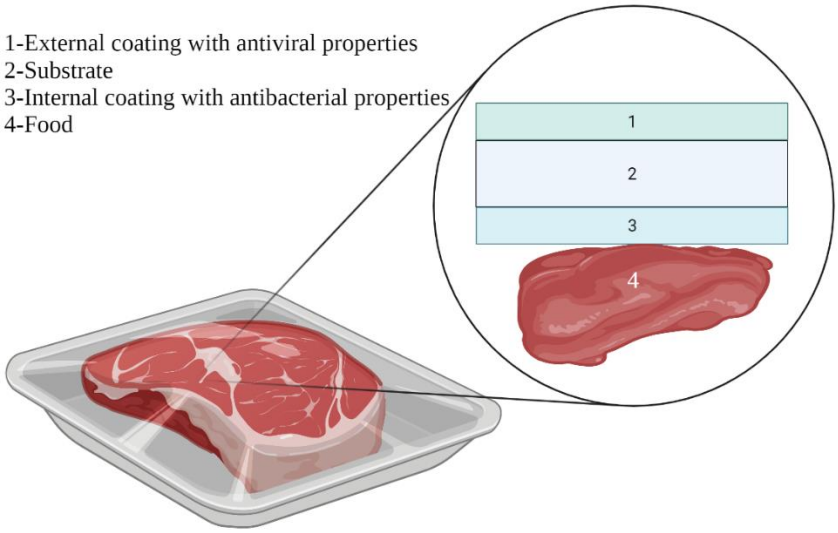

Figure 2-15 Multilayer film with dual antiviral and antibacterial functionalities [221]

Polymeric systems of antimicrobial surfaces to work against bacteria, viruses, and fungi are reported in the literature. Here are some of the latest developments and trends in the antiviral active packaging field. In addition, Mizielinska et al. [224], antiviral surface coating systems based on geraniol or carvacrol and a very small amount of ZnONPs on the polyethylene (PE) films were developed via Unicoater 409 film applicator. These films showed moderate activity against phi 6 phage *Influenza viruses* and *Coronaviruses*. Sharif et al. [225] developed and characterized edible coatings with antiviral properties based on gelatin and Persian gum in a 50:50 ratio. The prepared coating showed high antiviral performance against *Murine norovirus (MNV)*, a *Human norovirus* surrogate. Amankwaah et al. [226] incorporated green tea extract (GTE) into the chitosan by solution casting in order to produce edible films with antiviral and antibacterial properties. The developed films effectively inactivated and reduced infectivity of *Murine norovirus (MNV-1)* and *E. coli*. Similarly, Falcó et al. [227] worked on the antiviral activities of GTE in a coating solution

system against the MNV-1 virus. Moreover, Castro-Mayorga et al. [228] developed active silver nanoparticles-based (AgNPs) systems performing antiviral activity against *Human noroviruses* (*HuNoV)* surrogates, *Feline calicivirus (FCV),* and *Murine norovirus (MNV).* In their study, the antiviral performance of biodegradable poly (3-hydroxybutyrate-co-3- hydroxyvalerate) PHBV enriched with AgNPs reported total removing for *FCV* only, for 24 hr contact time at high humidity/temperature conditions.

2.6 Regulatory Aspects

It is important to consider the nontoxicity of the film during developing the antimicrobial food package. The migration capability of antimicrobial substances into food is a major challenge for food contact packaging and needs to be limited to a certain acceptable value. For example, ingested ZnONPs that have migrated into food may transfer to the surrounding tissues cause potential food safety issues. The toxicity of ZnONPs to human bronchial epithelial cells, human lung epithelial cells, and human kidney cells has been demonstrated in a number of studies [229-232]. Thus, an assessment of potential hazards from dermal, inhalation, or oral exposure to substances in food packaging must be made to ensure consumer protection [5].

General requirements have been integrated by regulation 450/2009/EC, which establishes the requirements for the use of active substances, including antimicrobial food packaging and materials for food contact [22]. Further, antimicrobial substances used for antimicrobial packaging need to be considered as food grades by the European Food Safety Authority (EFSA) or GRAS substances by the FDA for commercial purposes.

European Union established new legislation for use of migratory active additives. The Overall Migration Limit (OML) is the total quantity of all non-volatile compounds that can migrate into the food. The OML value for plastic food packaging is considered to be 60 mg/kg of food (or food simulants) or 10 mg/dm^2 on a contact area. Moreover, the Specific Migration Limit (SML) is the maximum permitted quantity of a certain compound released from plastic packaged into food. The SML of 5 to 25 mg zinc per kg food has been recommended for ZnONPs for food contact products.

Bumbudsanpharoke et al. [233] investigated the migration behavior of ZnONPs from LDPE-ZnONPs nanocomposite films into four types of food simulants. It was reported that the highest

amount of migrated Zn^{2+} measured (3.5 mg/L) was lower than the specific migration level suggested by European Plastics Regulation (EU) 10/2011 and related amendments.

Although FDA considers kaolin clay minerals as GRAS for indirect food contact, HNTs as a nanomaterial component of the mineral kaolin are not yet approved for any type of intake. Indeed, efforts are being made to regularize HNTs in preparation for future uses. HNTs are used in the passive part of an active system and must comply with the European Plastics Regulation (EU) No. 10/2011. Because the HNTs are immobilized within the polymer and are not expected to migrate into food in amounts higher than the specific migration limit (SML), the nanostructure of the HNTs is not considered an obstacle to approval.

Migration is affected by the interaction of food and polymer surfaces and can be analyzed by complex methods. The migration of undesirable molecules may cause some health risks for the consumer. Regulation 1935/2004/EC and, more specifically, Regulation 450/2009/EC set a new legal basis for the correct use, safety, and marketing of antimicrobial packaging.

These regulations includes the following provisions [234] [235]:

- Any changes due to active additives in the organoleptic profiles of foods must be consistent with the food legislation.
- The migration limitations and the type of materials intentionally added to active materials must be consistent with the relevant EU provisions applicable to food.
- Active substances shall not mask the spoilage of food and mislead the consumers.
- Active additives must be in accordance with both regulation 1935/2004/EC and 450/2009/EC
- The risk assessment by EFSA is mandatory for all materials before using as additives.
- Since the data for toxicity of nanoparticles is limited, these additives must be assessed on a case-by-case basis.

2.7 Summary

Based on the above literature review, zinc oxide nanoparticles (ZnONPs) are a promising candidate for using in food packaging by melt compounding due to their non-toxicity, thermal stability, and

considerable antimicrobial properties towards diverse microorganisms. Up to now, main reports in the literature was about using ZnONPs in suspensions or in nanocomposites (films, coating, nanofibers). However, no study has explored the effect of ZnONPs positions in the film on the antimicrobial performance of developed film.

Incorporated ZnONPs may inhibit the growth of microorganisms by a surface action or by diffusion and dissolution onto the food surface result in migration. Hence, in this system, antimicrobial agents must touch the food surface to ensure its complete activity against microorganisms.

Depending upon the required shelf life, regulatory aspects, consumer demand, and required spoilage properties, further modes of action can be applied in developing antimicrobial packaging systems. Unlike antimicrobial packaging containing ZnONPs, limonene (LEO) as a natural antimicrobial component can be used for noncontact antimicrobial systems. LEO can penetrate the food matrix, and direct contact with food is not necessary. In this system, the LEO can be evaporated from the active film to the headspace where it is absorbed onto the food surface. Therefore, using this noncontact system can protect the packaged food or/and to the food headspace. Several studies reported the efficacy of EOs for applications in food packaging with antimicrobial properties. However, no experimental study has investigated the synergistic effect of the volatile limonene with different mineral carriers in PE-based films produced by melt compounding.

Although the volatility of limonene is the big advantage for using in release systems, high volatility is the main obstacle for its industrial applications. Thermal processing may cause the evaporation of this additive, leaving a profound impact on the developed antimicrobial films. Hence, it is still needed to establish a more robust antimicrobial packaging with few regulatory and operational obstacles for industrial application.

LAE can also be directly added to the packaging as migratory antimicrobial packaging. LAE in solution form has been extensively explored in the literature; however, it is impracticable for industrial use. Hence, there is still the opportunity to investigate the antimicrobial performance of films containing LAE made via melt compounding, which could be of industrial relevance given the wide range of antimicrobial performance against yeasts, fungi, and bacteria. Moreover, the understanding of the LAE capacity to make plastics virucidal is an open area of research.

CHAPTER 3 OBJECTIVES AND ORGANIZATION OF THE ARTICLES

Referring to the literature review presented above, different active packaging systems encompassing antimicrobial substances have been developed for the preventing of microorganisms. However, there is a large gap between currently developed systems and commercial products that can be employed on an industrial scale. Inactivation, evaporation, and fast release of active compounds are among those limitations that should be taken into account in developing antimicrobial packaging systems. As a result, a proper system must be designed to overcome these restrictions and meet client requests. Antimicrobial performance, availability, nontoxicity, and variety in modes of action were the main reason to consider zinc oxide nanoparticles (ZnONPs), limonene essential oil (LEO), and ethyl lauroyl arginate (LAE), among the known sources of natural and synthetic antimicrobials. The key reasons for considering zinc oxide nanoparticles (ZnONPs), limonene essential oil (LEO), and ethyl lauroyl arginate (LAE) among the known sources of natural and synthetic antimicrobials were their antimicrobial performance, availability, nontoxicity, and variety of modes of action.

3.1 Objectives

The major objective of this work is to:

Develop an Antimicrobial Film for Food Packaging Using Melt Compounding

3.1.1 Specific objectives

The following specific objectives must be addressed in order to carry out the main objective:

1. To develop well dispersed Zinc oxide nanoparticles in PE and close to the surface,
2. To enhance the antimicrobial performance of Limonene/PE film by using mineral carriers in the PE matrix,
3. To investigate the use of LAE compounds as an antibacterial and antiviral agent in the PE film.

3.2 Organization of Articles

This portion is devoted to the thesis's scientific contribution, which is represented by three original articles. In Chapter 4, the initial step of this thesis's findings are presented as the first article entitled "Effect of polyethylene film thickness on the antimicrobial activity of embedded zinc oxide nanoparticles". The effect of thickness on antimicrobial performance of linear low-density polyethylene (LLDPE) skin layer film embedded with ZnONPs generated by coextrusion and dip-coating processes was investigated in this paper. The thickness of the skin layer of the generated films was measured using a variety of techniques, including SEM and TEM. Sandblasting treatment was carried out to the surface of multilayer film using fine sandpaper and the antimicrobial performances of treated films towards both *S. aureus* and *E. coli* regarding the new position of ZnONPs in the active films were studied and, finally, the optical properties were determined. This paper has been published in "ACS Omega" journal.

In the second part of this project, the potential synergistic effects of different mineral carriers with volatile limonene in LDPE films produced by melt compounding were investigated. The findings of this section are presented in Chapter 5 as an article entitled "Synergistic antimicrobial activities of limonene with mineral carriers in LDPE films for active packaging application". Five different carrier-limonene hybrids were fabricated and added into LDPE films using melt compounding and thermal, mechanical, optical, barrier, and antibacterial properties were reported. The short release behavior of all active films was analyzed and, finally, the antimicrobial efficiency of films was evaluated towards *E. coli* as a function of storage time. This article has been submitted to the journal of "Applied Polymer Science".

Finally, the use of LAE compounds as an excellent antibacterial and antiviral agent in the PE film matrix by an extrusion method was reported. The findings were given in the third piece, which was entitled "The antibacterial and antiviral potential of PE-based films enriched with LAE towards *E. coli* and *Human Coronavirus*" in Chapter 6. In this stage, different concentrations of LAE in its commercial form as LAM (10% LAE, 90% maltodextrin) were incorporated in the LLDPE matrix by an extrusion method. The compatibility of LAE with polymer matrix will control the amount and rate of LAE released from the film surface, thus the ability of LAE migration to the film surface was investigated as a function of storage time. The antibacterial and antiviral activities were determined and it was further found that developed films showed acceptable antibacterial and

antiviral performance towards *E. coli* and cytopathogenic *Human Coronavirus (HCoV-229E)*, respectively. This paper was submitted to the “Food Control journal”.

CHAPTER 4 ARTICLE 1: EFFECT OF POLYETHYLENE FILM THICKNESS ON THE ANTIMICROBIAL ACTIVITY OF EMBEDDED ZINC OXIDE NANOPARTICLES

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4.1 Abstract

Microbial contamination of most foods occurs primarily at the surface during post-processing and handling; therefore, preventing cross-contamination by incorporation of antimicrobial substances in contact with the surface of the product is an efficient strategy in reducing food contamination risks. Zinc Oxide nanoparticles (ZnONPs) have been used widely to achieve antimicrobial films in various applications including in the food industry. This work describes the fabrication of antimicrobial polymeric films containing ZnONPs produced by the coextrusion and dip-coating techniques. Effects of skin layer thicknesses containing ZnONPs on the antimicrobial effectiveness of the film by their capability to inactivate gram-positive and gram-negative bacteria were studied for both methods. The antimicrobial properties of the coextruded multilayer LLDPE/ZnONPs nanocomposite films evidenced antimicrobial activity in the range 0.5-1.5 log reductions, while in case of sandblasted multilayer film, showed high antimicrobial properties as around 99.99%. The optical properties of the coextruded multilayer films were measured and discussed. Furthermore, to achieve a thinner LLDPE thickness, ZnONPs were coated with different concentrations of LLDPE solution by dip-coating method. TEM confirmed a homogeneous layer is formed on the surface of the ZnONPs. The thickness LLDPE layer estimated by TEM was about 2 nm and film produced 3 log and 4 log reductions for *E.coli* and *S. aureus* respectively. The results show that developed films have the potential to be used as food packaging films and can extend shelf life, maintain quality, and assure the safety of food. The antimicrobial mechanisms of ZnONPs were also investigated. It was found that direct contact of the particles with products is necessary to assure high antibacterial activity of the films.

4.2 Introduction

Foodborne diseases are occasionally a major cause of death. Thus, protecting the health of the consumer by providing food safety and maintaining food quality are a concern for the food packaging industry. To achieve these goals, the food packaging industry is driven to search for inventive technologies to reduce the risk of pathogen contamination and development in raw and processed foods [1-4].

ZnONPs are inorganic, non-toxic and stable compounds widely used in drug delivery, cosmetics, dentistry, medicines and food packaging materials [5-9]. Additionally, ZnO has been considered

as a “generally recognized as safe” (GRAS) material by the US Food and Drug Administration (21 CFR 182.8991) [10, 11]. ZnONPs have emerged as appropriate candidates for food packaging due to their excellent antimicrobial activity and can provide a perfect dynamic role for preserving the food against food-borne pathogens when they are used in polymeric film packaging [12].

There are several studies in literature dealing with the influence of the shape [13-17], size [18-20], concentration [19, 21], surface modification [22, 23] and UV illumination [24] on the antibacterial activity of ZnONPs. Also, ZnONPs [25-30] have been widely introduced in different polymeric matrices including low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene (PP), poly(butylene succinate) , polyurethane (PU), paper and chitosan to develop antimicrobial packaging. Previous researches have shown that the antibacterial activities of ZnONPs are greatly reduced after incorporation in polymer matrices. Rojas et al. [31] successfully prepared low density polyethylene (LDPE)/ZnONPs nanocomposite film by melt compounding and studied the influence of modification and proportion of ZnONPs (3, 5 and 8 wt%) as well as white/UV lights irradiation on the antibacterial and mechanical characteristics of the films. As ZnONPs content increased, the antibacterial rate was improved against *E. coli*. At low ZnONPs concentration (3 wt%), the surface modification of ZnONPs with oleic acid enhanced the antimicrobial properties, as revealed by the bacterial reduction from ~33.87% to ~82.26%. All the LDPE/ZnONPs nanocomposite films after UV irradiation were effective against *E. coli* with a bacterial reduction of ~ 99.99% regardless of the nanoparticle concentration and surface modification. In addition, nanocomposites irradiated with white light showed increased effectiveness with nanoparticle incorporation. High antibacterial properties ~ 99% against *E. coli* were achieved for high ZnONPs concentration (8 wt%) regardless of the nanoparticle modification.

Li et al. [32] tested the antimicrobial activity of nanocomposites of high-density polyethylene (HDPE) and different content of modified ZnONPs (0.2, 0.5, 1 and 2 wt%). The ZnONPs surface was modified by γ-aminopropyltriethoxy silane. When the concentration of modified ZnONPs was only 2 wt%, a strong antimicrobial effect of HDPE/ZnONPs nanocomposite films against *E. coli*, with microbial reduction up to 97.7%, was observed. Petchwattana et al. [33] produced poly(butylene succinate) films containing ZnONPs by using an extruder and a film blowing unit at five ZnONPs contents ranging from 2 to 10 wt%. The results showed that a minimum of 6 wt% ZnONPs content was required to inhibit the *E. coli* and *S. aureus* growths with the clear zone of 1.31 and 1.25 cm respectively.

The antimicrobial properties of ZnONPs [34] may be attributed to a direct interaction between ZnONPs and the cell wall [20, 35, 36], release of Zn^{2+} ions [36-38] and reactive oxygen species (ROS) formation [6, 39, 40]. The theory behind this investigation is that the position of ZnONPs inside the polymeric film plays a vital role in antimicrobial properties. The ZnONPs position can be defined by the distance of the particles from the surface of the film. Actually, it can be assumed that particles placed on the top of the film will cause a higher antimicrobial property of the film compared to particles that are completely embedded inside the film. In other words, the possible contact with microorganisms and the release of Zn^{2+} ions into the surrounding medium is definitely stronger, if the particles are directly present close to the film surface [41]. ROS lifetime and potential migration out of the film are expected to be higher when ZnONPs are closer to film surface.

Thus, in this work, we investigated the effect of LLDPE skin layer film thickness obtained by coextrusion and dip-coating techniques on the antimicrobial activity of incorporated ZnONPs. In the coextrusion method, the produced films are investigated for antimicrobial effects against *E. coli* and *S. aureus* by LLDPE skin layer film thickness reduction. In dip-coating technique, different concentrations of LLDPE solution (0.2–0.8 wt%) were used for coating of ZnONPs in order to find the maximum LLDPE film thickness with highest antimicrobial activity.

4.3 Materials and Methods

4.3.1 Materials

ZnONPs (20 nm particle size and the specific surface area of 30 to 50 m^2/g) were supplied by SkySpring Nanomaterials Inc. (Houston, TX, USA). Food grade FPs317-A Octene sLLDPE film resin purchased from Nova Chemicals (Calgary, Alberta, Canada) was used as matrix and coating polymer solution. Low molecular weight ethylene-propylene copolymer/acid modified type (HW 1105A) from MITSUI HI-WAX (Molecular weight 1500 and density 939 kg/m^3) was used as a dispersant. Xylene solvent was purchased from sigma-Aldrich. *E. coli (DH5α)* and *S. aureus (ATCC 6538P)* were obtained from the Department of Microbiology, Infectiology, and Immunology at University of Montreal. Luria-Bertani (LB) medium and all of other chemicals used for growing and maintaining bacteria were purchased from VWR International, LLC.

4.3.2 Masterbatch Preparation

A masterbatch of LLDPE/ZnONPs was compounded with concentrations of 10 wt%, in the form of pellets using a 18 mm diameter with L/D=40 twin-screw extruder (Leistritz ZSE 18 HP, Nuremberg, Germany). To keep the final concentration of ZnONPs in the active skin layer at 3 wt%, the masterbatch was diluted during film extrusion. A temperature profile of 195-220 ^{o}C was used from the melting to die zones in the twin-screw extruder.

4.3.3 Film Preparation

Multilayer films prepared with a coextrusion cast process had four layers:

A) LLDPE/ZnONPs layer with concentrations of 3 wt%, (Diluted masterbatch as a skin on two film sides)

B) LLDPE as core layer

The film samples were prepared using a Labtech Engineering Coextrusion Multilayer cast film line with two single-screw extruders (type LE20-30 C). The extruders had a 20-mm diameter screw with L/D=30. The feedblock with (A/B/B/A) design for four-layers and a flat die with 30-cm width were used to process the films. The temperature profile for both extruders along the barrel was 200/210/220 ^{o}C and the die and feed-block temperature was 235 ^{o}C. The coextrusion line was equipped with a slit die with 2 mm opening. The prepared film was cooled at the die exit using an air knife operating at 13 psi. Chill rolls set at 77 ^{o}C were used to stretch the films to obtain different thicknesses in skin layer. Figure 4-1 shows a schematic view of four-layer films preparation with a coextrusion cast process.

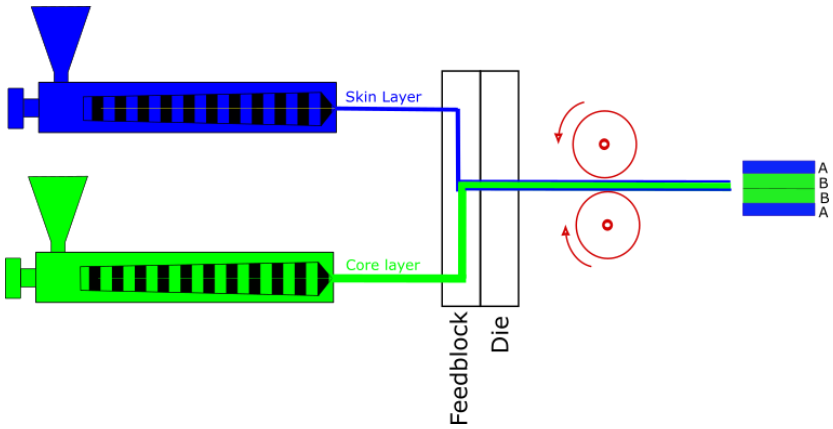

Figure 4-1 Four-layer films preparation with a coextrusion cast process

4.3.4 Preparation of Dip-coated ZnONPs

In ZnONPs dip-coating process, 0.8, 0.4 and 0.2 g LLDPE was dissolved in 100 mL xylene in a conical flask for 60 min at 100 °C. The final suspension was prepared by placing 0.24 g ZnONPs in the all LLDPE solutions and stirring for a 24 hr to obtain coated ZnONPs. Prior to antimicrobial testing, vacuum filtration was used to collect coated ZnONPs, which were then dried fully in a vacuum oven at 65 °C for 4 hours to eliminate xylene solvent residue. To assess the effect of xylene residue on the antimicrobial properties of coated ZnONPs, a positive control was also prepared. Table 4-1 shows the ZnONPs coating parameters in detail.

Table 4-1 Composition of the LLDPE Solution for ZnONPs Coating

Sample code	LLDPE concentration (wt/vol xylene %)	mass ratio LLDPE to ZnONPs	estimated coating thickness (nm)
LLDPE 0.8%	0.8	1:2	8 ± 0.2
LLDPE 0.4%	0.4	1:4	4 ± 0.2
LLDPE 0.2%	0.2	1:8	2 ± 0.1

The numbers after ± sign are standard deviations.

4.3.5 Morphology

The ZnONPs distribution in the surface and cross-section of the LLDPE/ZnONPs films was assessed using a tabletop SEM (TM3030plus, Hitachi,) operating at 15 kV acceleration voltage. Skin layer thicknesses were determined using SEM software. The reported layer thicknesses were averaged over 10 different measurements. A transmission electron microscopy (TEM) from JEOL JEM-2100F, Japan, operating at 200 kV was used to examine the surface condition of coated and noncoated ZnONPs.

4.3.6 Optical Properties

Optical properties of the films in terms of haze values were evaluated in accordance with the procedure specified in ASTM D 1003 using a LAMBDA 1050 spectrophotometers from PerkinElmer.

4.3.7 Thickness of Films

The film thickness was measured at six random positions using a digital micrometer (ProGage Thickness Tester, Thwing-Albert Instrument Company, NJ, USA), and the mean value was determined.

4.3.8 Antimicrobial Test

All samples with different skin layer thickness were tested against *E. coli* and *S. aureus*. These bacteria were grown in LB broth for 24 h at 37 ºC, to reach a density of 10^8 colony forming units (CFU)/mL. The bacteria culture was diluted in the same medium after 24 h to 10^5 CFU/mL. Antimicrobial tests were conducted according to the standard ISO 22196:2011 and as described by Abdali and Ajji [42]. Both active and control samples were cut out into 50 mm x 50 mm coupons. All sample coupons were disinfected by placing under a UV light in a class II biosafety cabinet for 20 minutes. Four hundred microliters of the diluted bacteria culture were deposited on each sample coupon placed in a sterile Petri dish and overlaid with a sterilized plastic thin film [40 mm x 40 mm] for spreading. All Petri dishes containing inoculated sample coupons were incubated at 37 ºC for 24 h. The next day, the bacterial inoculums thus placed on the surfaces were washed off with 2 mL PBS solution. Then, six serial PBS dilutions (10^{-1} to 10^{-6}) were conducted. Three samples were taken from each of the six dilutions and 10 µL droplets were applied onto the LB agar plates which were incubated overnight at 37 ºC for counting the surviving bacteria (CFU/mL). Each sample is made in triplicate, along with the negative control.

4.3.9 Statistical Analysis

Data are the mean ± SD of three replications. One way analysis of variance (ANOVA) and Tukey tests were performed to determine significant group differences and means were considered as statistically significant if $p < 0.05$.

4.4 Result and Discussion

4.4.1 ZnONPs Characterization

The size effect of ZnONPs in biological interactions and maximizing the generation of reactive oxygen species (ROS) has been thoroughly discussed in literature [43]. In other words, the antibacterial activity depends on the concentration and size of ZnONPs [19, 39, 44, 45]. Figure 4-2 shows the TEM, SEM, and size distribution of the ZnONPs. ZnONPs were spherical in shape and are uniform in size as well as regular in shape as depicted by the TEM image (Figure 4-2a). The SEM figure (Figure 4-2b) exhibits a uniform structure and size for ZnONPs. However, in some places, the size of particles is bigger, and they are agglomerated by different groups. The size histogram of the ZnONPs in water is shown in Figure 4-2c. The histogram indicates that the mean particle size of ZnONPs was 20 nm, and the particle size distribution is relatively uniform.

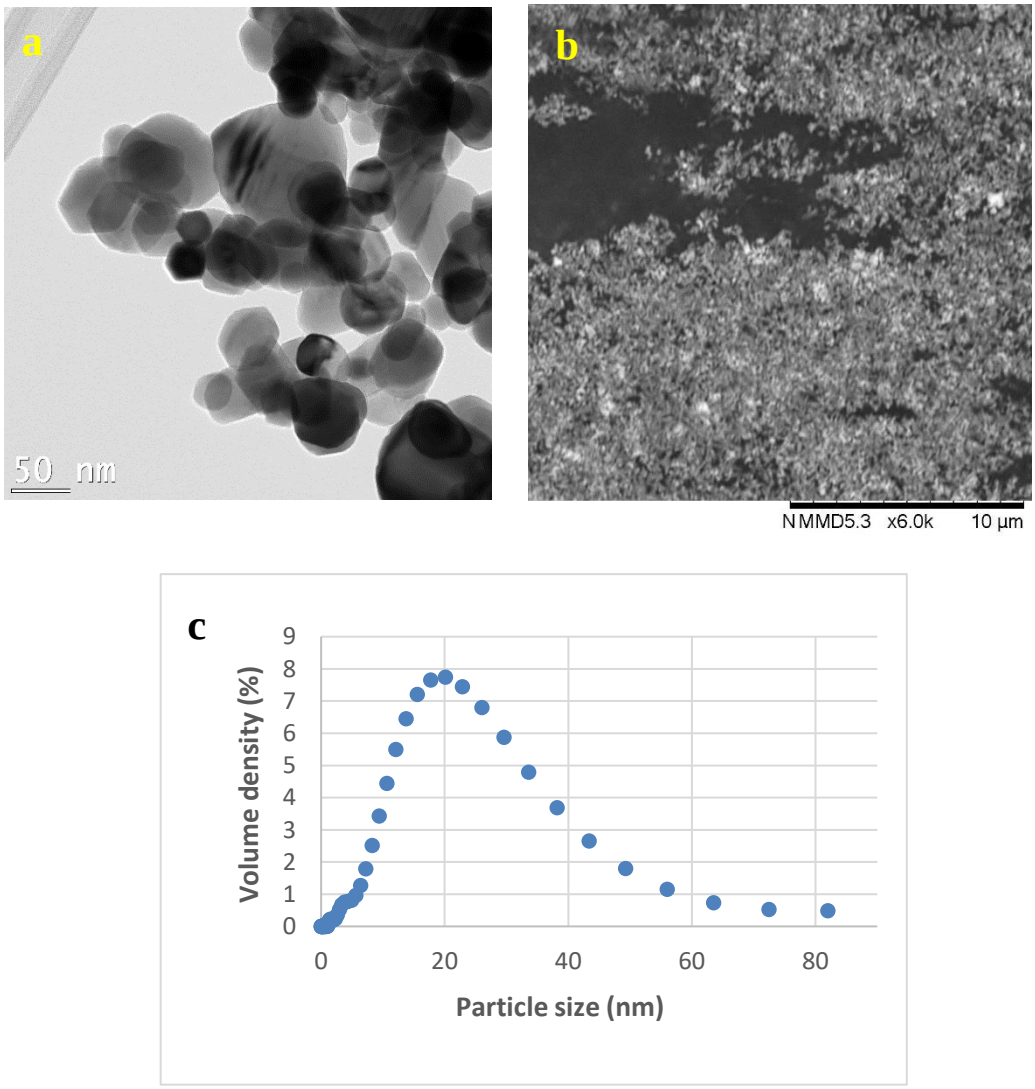

Figure 4-2 The TEM image of ZnONPs (a), the SEM of the ZnONPs (b), and the particle size distribution of the ZnONPs (c).

4.4.2 Thickness

To produce different film thicknesses with different skin layers thickness, the screw speeds were set at 20 rpm, 10 rpm and 5 rpm for the skin layer extruder and 60 rpm for the core layer extruder for all samples. In addition, draw-down ratio (ratio of the roll speed to the extrudate velocity at the die exit) was approximately set at 1.02 (Table 4-2). The first and last two digits of each sample

code refers to skin layer extruder speed (rpm) and master calender speed (ft/min) respectively. Thickness of the LLDPE/ZnONPs skin layer was determined by SEM analysis. It was found to be in the range of 1.5-12 μm. The thickness of the whole film specimens and thickness of the LLDPE/ZnONPs skin layers are shown in Table 4-2. The mentioned thicknesses were significantly affected by skin layer extruder screw speed.

Table 4-2 Sample codes for multilayer films with 3% (w/w) ZnONPs incorporation into the active skin layer

sample code	skin layer extruder screw speed (rpm)	film thickness (μm)	skin layer thickness (μm)
M2007	20	103 ± 2	12 ± 0.2
M2010	20	70 ± 3	5 ± 0.4
M2015	20	89 ± 1	4 ± 0.2
M1007	10	82 ± 3	6 ± 0.3
M0507	5	117 ± 1	2 ± 0.1
M0510	5	66 ± 2	1.5 ± 1

Draw down ratio for all samples was set at 1.02 approximately

The numbers after ± sign are standard deviations

4.4.3 Characterization of LLDPE/ZnONPs Film

To check the dispersion of the 3% (w/w) ZnONPs into the LLDPE matrix, SEM analysis on the cross-section and surface of the LLDPE/ZnONPs films was carried out in five random locations around each film sample. SEM of cross-section and surface of LLDPE/ZnONPs films are illustrated in Figures 4-3 and 4-4, respectively. The ZnONPs can be detected on the surfaces of films in the dispersed state. The cross section of LLDPE/ZnONPs films showed nanoparticles were adequately distributed within the different thicknesses of the skin layer of the multilayer films. In general, the SEM images of film surface of all samples did not show any significant differences. All of the films showed homogeneous and smooth surface structures may be due to the presence of the ethylene-propylene copolymer/acid modified type. Since this compound have high affinity

to metal oxide nanoparticles, it is useful as dispersing assistant for ZnONPs [46]. Our study is good agreement with results from Emamifar et al. [26] who concluded that ZnONPs were well dispersion at low concentrations in LDPE films. Similarly, Polat et al. [47] found that ZnONPs generally displayed good dispersibility in the polypropylene (PP) matrix.

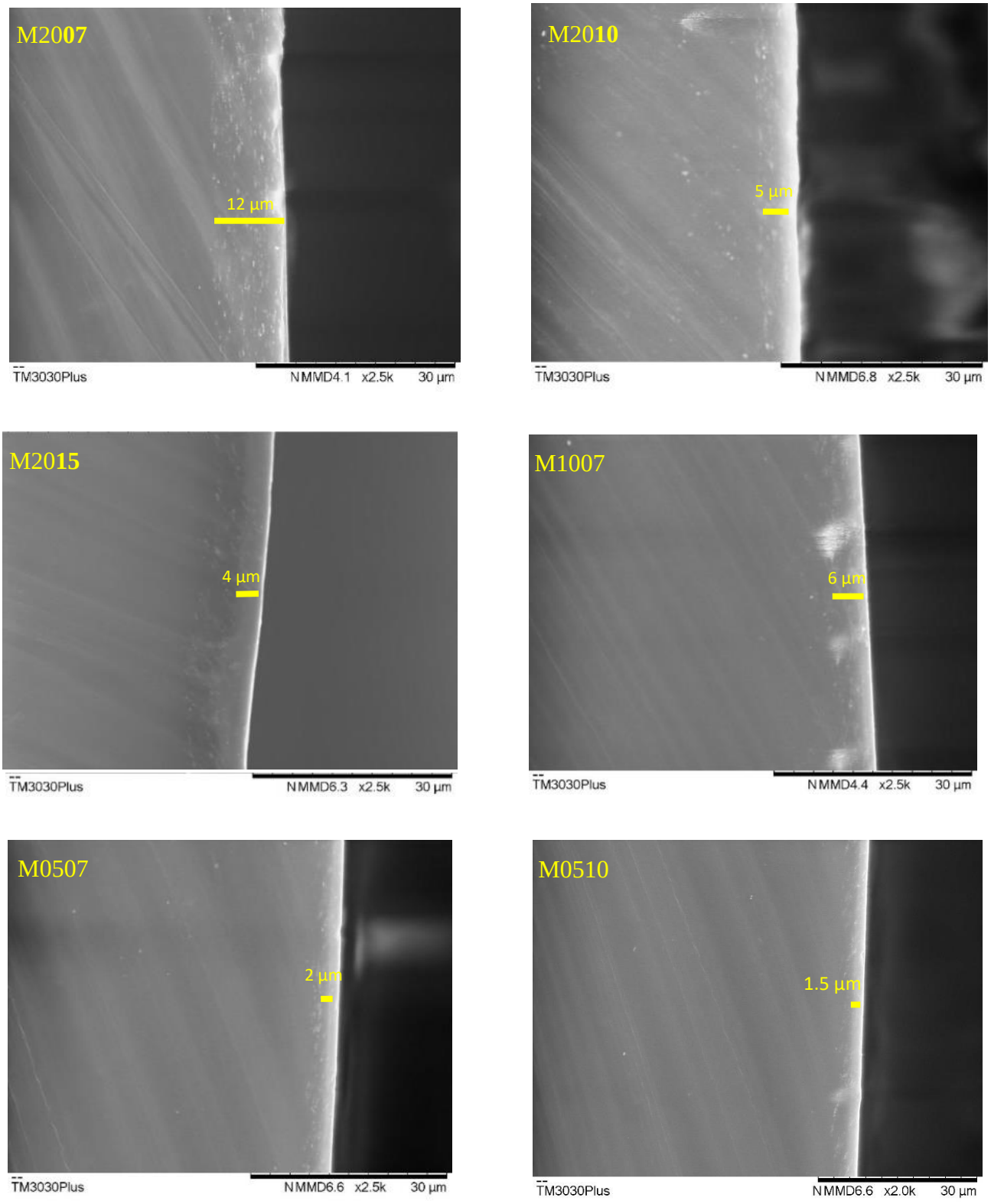

Figure 4-3 Film cross section SEM of LLDPE/ZnONPs with 3% (w/w) ZnONPs incorporation in the active skin layer

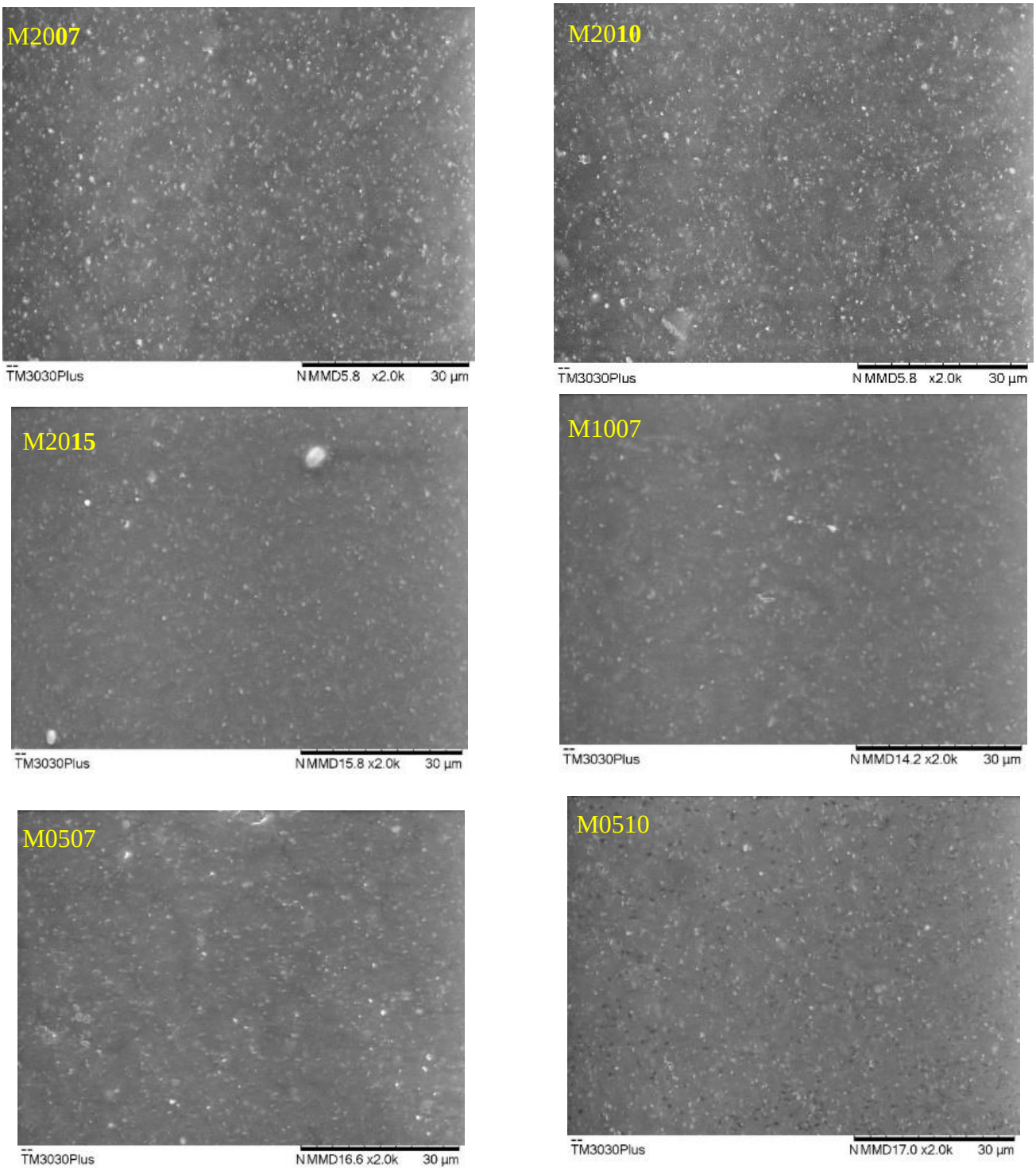

Figure 4-4 Film surface SEM of LLDPE/ZnONPs with 3% (w/w) ZnONPs incorporation in the skin layer

4.4.4 Optical Properties of LLDPE/ZnONPs Films

The presence of ZnONPs reduces the clarity and increases the haze of the films. The LLDPE/ZnONPs film samples (M0510 and M2007) showed a little higher haze compared to the neat LLDPE control film with thickness of 93µm due to low ZnONPs concentration of 3% (w/w) as illustrated in Figure 4-5. Similar to our results, Espejo et al. [48] reported that low density polyethylene filled with 2% (w/w) ZnONPs were prepared by extrusion and formed transparent nanocomposite film.

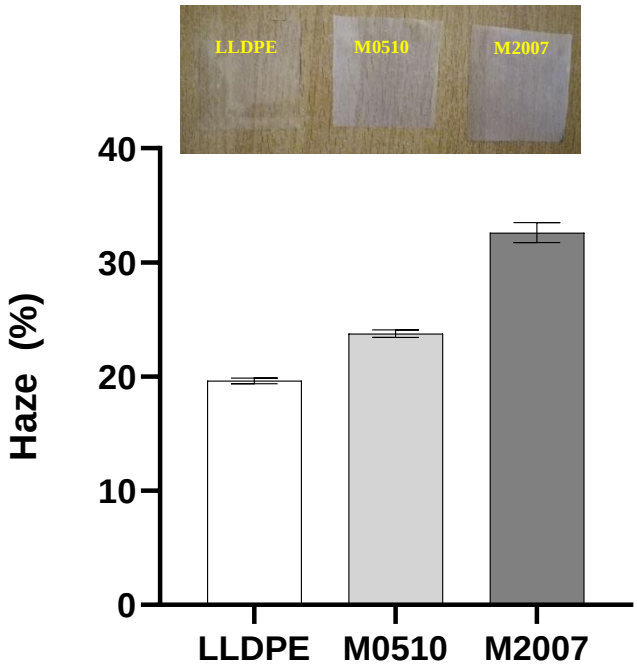

Figure 4-5 Haze of neat LLDPE and LLDPE/ ZnONPs film samples (M0510 and M2007) with 3% (w/w) ZnONPs

4.4.5 Antimicrobial Properties of LLDPE/ZnONPs Film

The antibacterial results of all the samples on the *E. coli* and *S. aureus* are presented in Figure 4-6. In Figure 4-6, it is shown that the number of both bacterial colonies dropped with the decreasing of skin layer thickness. It can also be observed that sample M2007 with skin layer thickness 12 µm present poor antibacterial properties. The thin skin layer films having thicknesses of 4–6 µm

(samples M2015, M2010 and M1007) decreased the *E. coli* growth by less than one log (CFU/mL). *S. aureus*, was reduced by 1 log (CFU/mL) for samples M2015, M2010 and M1007. Film samples having thicknesses of 1.5 and 2 µm (samples M0510, M0507) registered 1 and 1.5 logs (CFU/mL) reduction for *E. coli* and *S. aureus* respectively. These results demonstrated that the antibacterial activity of the film samples to inactivate *E. coli* and *S. aureus* was improved by decreasing the skin layer thickness. In the other word, a higher thickness of treated skin layer is a contact barrier between ZnONPs and bacterial cell wall.

In order to prove that this layer interrupt antibacterial activity of ZnONPs, a 8 µm ultra-fine sand paper was used to rub the surface of sample M2007. Sanding was performed by hand. Surface was rubbed in circular motion in two sandblast passages and dust was removed by vacuum cleaner. This treatment caused increased roughness of the film surface compared to intact sample M2007. This modified film reduced the *E. coli* and *S. aureus* density by approximately 4 logs (CFU/mL). The result for sandblasted film indicated that the antimicrobial activity of film should be attributed to the direct or very close contact of ZnONPs to the bacteria. The result also agreed with Shen et al. (2013) and Rokbani et al. (2019) when reported that ZnONPs contact to the cells are required to elicit cytotoxicity [49,50].

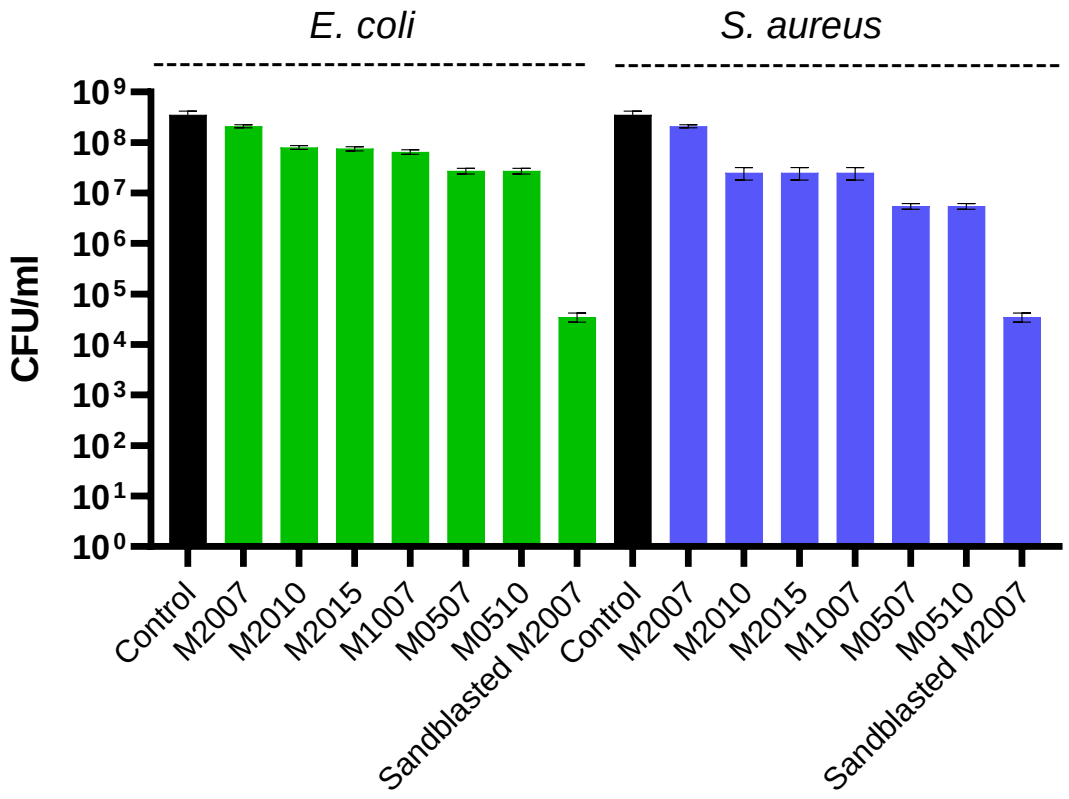

Figure 4-6 Inhibitory effects of different thickness of LLDPE/ZnONPs skin layer film towards *E. coli* and *S. aureus*

4.4.6 Antimicrobial Activity of Dip-coated ZnONPs

In an attempt to find the maximum thickness of polyethylene between ZnONPs and bacterial cell wall in order to have proper antimicrobial properties, ZnONPs were dipped into LLDPE solution with different concentrations. The coated ZnONPs were characterized using TEM. The TEM micrographs of coated ZnONPs before and after LLDPE coating can be seen in Figure 4-7 The most notable feature of Figure 4-7 (b) is the appearance of an irregular-shaped region in the middle of the figure, indicating that LLDPE formed a very thin layer thickness (2 nm) in embedded ZnONPs. Figure 4-8 demonstrates that the thickness of the LLDPE layer on the ZnONPs has a significant impact on the coated ZnONPs. For ZnONPs coated in a solution of LLDPE 0.2%, the *E. coli* population reduced from 11.6×10^8 CFU/mL to 2.3×10^5 CFU/mL due to the lower LLDPE concentration. *E. coli*, was reduced more than 1 log (CFU/mL) for both LLDPE 0.4% and LLDPE 0.8% samples. For the gram-positive strain (*S. aureus*), ZnONPs coated in a solution of LLDPE

0.2%, 0.4% and 0.8% decreased the *S. aureus* growth by approximately 4, 3 and 2 logs (CFU/mL) respectively. These results proved that decreasing the coating thickness of ZnONPs to 2 nm improved the antibacterial activity. In general, the results confirm that the antibacterial activity of ZnONPs is a consequence of its direct or very close contact with the microbial cell wall resulting in cell integrity disruption, generation of reactive oxygen species (ROS) and release of Zn^{2+} ions [43]. It can also be concluded that the thickness of the coating with different concentrations of LLDPE, modulates the antimicrobial activity of ZnONPs. Our results are in good agreement with Saekow et al. [51], who evaluated the antifungal activity of carboxymethyl cellulose (CMC) coating combined with ZnONPs on persimmon and tomato fruits.

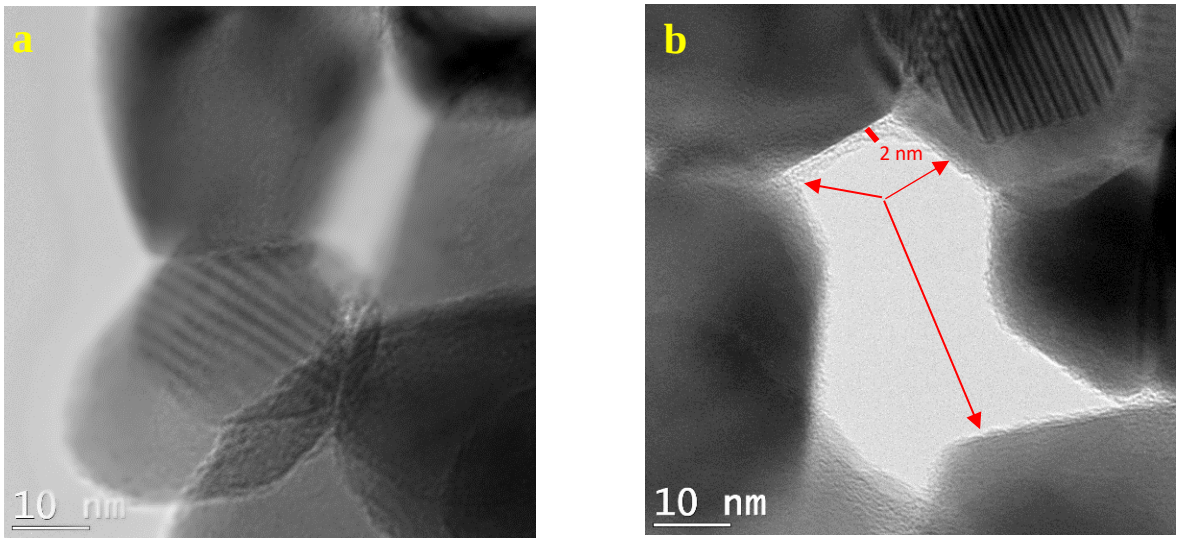

Figure 4-7 TEM of noncoated (a) and coated (b) ZnONPs with 0.2 wt% LLDPE solution

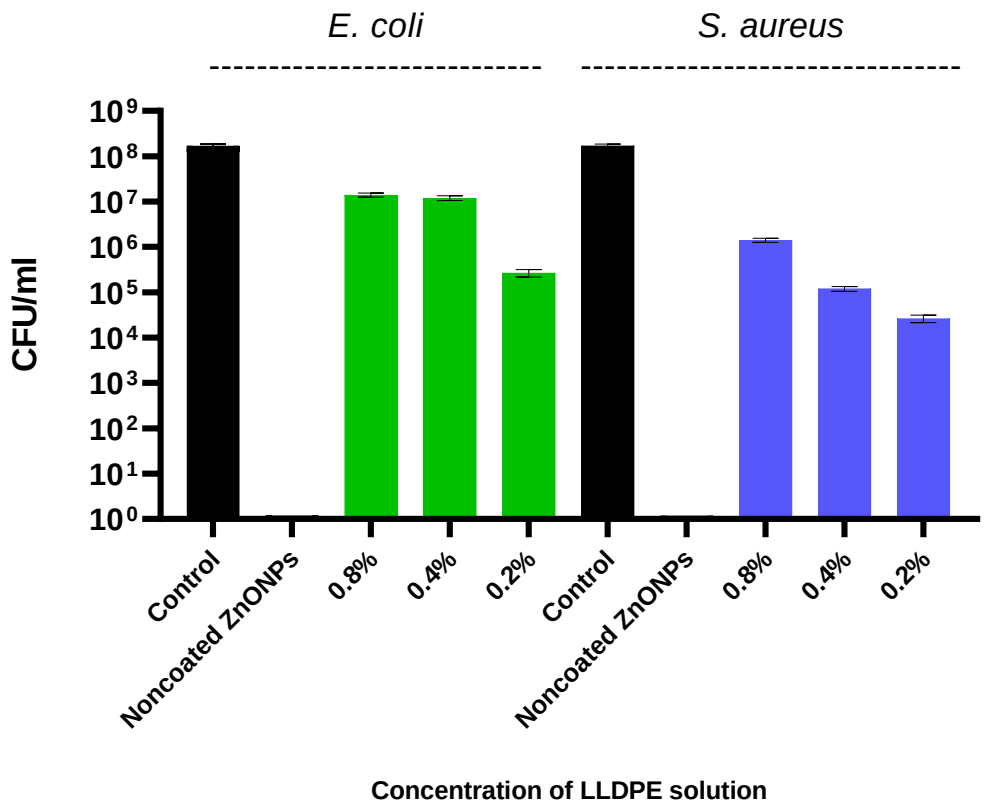

Figure 4-8 Inhibitory effects of coated ZnONPs in different concentrations of LLDPE solution towards *E. coli* and *S. aureus*

4.5 Conclusions

LLDPE/ZnONPs skin layer films with thickness varying from 1.5-12 µm were produced using the coextrusion technique. To better understand the effect of required LLDPE thickness to achieve a desired antimicrobial activity in the films, dip-coated ZnONPs in different LLDPE solution concentrations and thicknesses closed to 2 nm were also prepared. All the LLDPE/ZnONPs skin layer films and coated ZnONPs showed antibacterial activity. The decrease of LLDPE/ZnONPs skin layer thickness down to 1.5 to 2 µm enhances antibacterial activity of film whereas further decreasing the thickness of LLDPE coating on the ZnONPs down to 2 nm dramatically increases the antimicrobial activity of coated ZnONPs. Sandblasting treatment extremely improved the multilayer film antimicrobial properties against both *E. coli* and *S. aureus*. All of the films showed homogeneous distribution of ZnONPs. SEM images of LLDPE/ZnONPs films clearly revealed that

the ZnONPs distribution was uniform. Antibacterial properties of film samples and coated ZnONPs were slightly improved by LLDPE thickness reduction, which may be due to ZnONPs direct contact with the microbial cell wall or a better release of reactive oxygen species (ROS), and Zn^{2+} ions from the film surface. Therefore, the proposed LLDPE/ZnONPs film with a thin LLDPE inert layer thickness with suitable biocidal properties may be considered as good candidates to be exploited in food packaging.

4.6 Acknowledgements

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CHAPTER 5 ARTICLE 2: SYNERGISTIC ANTIMICROBIAL ACTIVITIES OF LIMONENE WITH MINERAL CARRIERS IN LDPE FILMS FOR ACTIVE PACKAGING APPLICATION

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5.1 Abstract

In this study, active films based on low-density polyethylene (LDPE) and limonene essential oil (LEO) were prepared and characterized. Before incorporation of LEO into LDPE, vacuum pulling method was used to load the LEO into five different mineral carriers. All Carrier-LEO complexes were added into LDPE using melt compounding. The goal is to analyze the potential use of these formulations to achieve prolonged antimicrobial film packaging. The halloysite nanotubes (HNTs), kaolinite (Kao), mesoporous silica nanoparticles (MSNs), zinc oxide nanoparticles (ZnONPs), and molecular sieve type 4A (Z4A) were used as mineral carriers for limonene. The functional characterizations including mechanical, thermal, optical, barrier, and antimicrobial properties as well as limonene release behavior from the LDPE composite films were investigated. As expected, free limonene molecules acted as a plasticizer in the LDPE matrix. Thermogravimetric analysis (TGA) showed 20-25% of the initial limonene content was retained against thermal degradation in compounding and film making steps and its release from the films was efficiently delayed. A decrease in optical and oxygen barrier properties, as well as elastic modulus and tensile strength, was obtained for all developed films compared with neat LDPE. Significant antibacterial activities of the films were observed against *E. coli* as a model gram-negative bacterial species. Moreover, the obtained results and the short-term and long-term release studies indicated that both HNTs and the MSNs due to their strong synergistic interactions with limonene exhibited sustained release profiles of limonene from LDPE films. Thus, these new active polymer composites present promising features in controlling microbial contamination, rendering them as excellent candidates in active packaging applications.

5.2 Introduction

A tremendous amount of attention has been directed towards polymeric materials with antimicrobial activity over the past decade. These polymers can greatly prevent the growth of most bacteria and fungi [1, 2]. Among these polymeric systems, polymers loaded with biocide materials or nanoparticles known as biocide-releasing polymers have been extensively studied [3, 4]. Natural active compounds like volatile essential oils are introduced as an interesting antimicrobial agent because of their diverse functionality, level of usage, and advantages in legislation and safety [5-7].

Limonene essential oil (LEO) is a volatile antimicrobial compound with an oily texture that widely exists in the citrus fruit peel and several plants [8, 9]. Limonene has major application prospects in antimicrobial packaging due to its wide-spectrum bactericidal and fungal properties [10, 11]. Additionally, it is “generally recognized as safe” by FDA [12].

One of the challenges in the processing of polymer with essential oils is the high volatility of the oil resulted in losing the volatile compounds during processing at high-temperature and storage of the packaging products [13]. To overcome this drawback, many works have explored developing the active packaging systems with considering the retaining of these active agents in the polymer media and having control over their release for a specified time [14-16]. Moreover, different processing techniques such as solvent casting [17-19], encapsulation [20, 21], electrospinning [22-24], and supercritical CO_2 impregnation [25, 26] have been studied which are not the techniques suitable for industrial scales. Melting extrusion and compression molding are the most popular used techniques for the incorporation of active agents in the wide range of commercial polymers [26]. However, these techniques are limited because the volatile agents can be evaporated or degraded during the film production process at high-temperature condition.

In this sense, mineral carriers capable of retaining bioactive molecules by physical and chemical adsorption or interaction with them are promising materials for techniques in which high-temperatures are needed [27, 28]. Different release behavior and mass transfer mechanisms can be obtained because of the interactions between the volatile additive, carrier, and polymer. Saucedo-Zuniga et al. [16] studied the incorporation of thymol and orange essential oils into porous HNTs and modified montmorillonite (MMT) for pesticide and antimicrobial applications. It was reported that MMT has a stronger potential to adsorb thyme than orange oil due to the high polarity of cymene. Following this line, the synergic antimicrobial effect of volatile vanillin and aminofunctionalized mesoporous silica incorporated in PCL polymer films via melt blending has been investigated by Stanzione et al. [29]. In this case, active polymer films containing the embedded vanillin in the aminofunctionalized mesoporous silica carrier showed a slower antimicrobial release due to interactions between amine groups of mesoporous silica nanoparticles and vanillin aldehyde. Shemesh et al. [30] investigated the advantages of HNTs as a nano-carrier for encapsulating carvacrol and incorporation of this HNTs-carvacrol complex into LDPE films using melt compounding for sustained release of carvacrol. It was further found that HNTs can be employed as a nano-carrier to improve the thermal properties of carvacrol. Following this trend,

Pajnik et al. [31] reported that the strong interaction between natural zeolite and phenolic groups of thymol led to significant retention of thymol in the polymer film. Ahmed et al. [32] evaluated the synergistic antimicrobial activity of cinnamon essential oil in combination with ZnONPs and synthesized silver–copper nanoparticles (Ag-Cu NPs) towards *S. typhimurium and L. monocytogenes*. The results demonstrated that cinnamon EOs and nano-carrier have no synergistic effects against the tested bacteria.

In this work, we aim to produce films based on LDPE with limonene-loaded in different mineral carriers, with the goal of developing sustainable antimicrobial packaging. For this purpose, first, LEO was loaded into carriers in a pre-compounding step via the vacuum pulling method. In the next step, the Carrier-LEO complexes were melt compounded with LDPE, followed by a hot press in order to produce active films. A comprehensive characterization including thermal, mechanical, optical, and barrier properties was performed. Finally, the antimicrobial performance of resulted films was investigated towards *E. coli* as a function of storage time.

5.3 Materials and methods

5.3.1 Materials

Nova Chemicals' low-density polyethylene (LDPE) resin with an MFI of 2.3 g/10min and a density of 0.918 g/cm3 was chosen as the matrix polymer. The limonene essential oil was supplied from Sigma–Aldrich (Darmstadt, Germany). *Escherichia coli DH5-Alpha* was obtained from the University of Montreal. VWR International, LLC provided the Luria-Bertani (LB) medium and all other reagents required in bacteria cultivation. Five commercial mineral carriers were selected. Five commercial mineral carriers were selected. Some characteristics of the selected carriers are summarized in Table 5.1 while provided by the carrier suppliers.

Table 5-1 Summary of the carriers used in this study with some of their properties

Nomenclature	Supplier	Density (g/cm^3)	Specific surface area (m^2/g)	Particle size
Halloysite Nanotubes (HNTs)	American Elements	2.2	50.8	OD <100 nm Length: 0.5-1.2 µm
Kaolinite (Kao)	BASF	2.58	11	2 µm
Mesoporous silica Nanoparticles (MSNs)	Sigma-Aldrich	2.2	80.7	200 nm
Zinc Oxide Nanoparticles (ZnONPs)	SkySpring Nanomaterials In.	5.6	30-35	10-30 nm
Molecular Sieve Type 4A (Z4A)	Sigma-Aldrich	0.4	800	2-3 µm

5.3.2 Preparation of limonene-loaded carrier

Vacuum pulling treatment was used to incorporate limonene into the different carriers according to a report by Abdullayev et al. [33]. Briefly, about 10.0 mL of limonene was fully mixed with 30.0 mL of ethanol by vortexing. Two grams of the carrier were added to limonene solution and dispersed by the ultrasonic cleaner. The suspension was evacuated using a vacuum rotary evaporator for 30 min at 30 °C, and then cycled back to atmospheric pressure. This procedure was repeated 3 times at 30 min intervals. The limonene-loaded carrier was then dried in a vacuum oven at 40 °C for 12 hours.

5.3.3 Preparation of the LDPE/Carrier-LEO composite film

LDPE/Carrier-LEO composite films were melt blended under N_2 atmosphere at 180 ^oC for 5 min at 50 rpm. After filling the internal batch mixer (Haake Rheocord 900, Germany) with LDPE pellets until they were completely melted (1 minute), the limonene-loaded carrier was introduced and mixed for 5 minutes. Then, for all compositions, a mixed compound was obtained, as presented in Figure 5-1. Neat LDPE also processed in the same conditions as a control sample. Finally, using a Carver hydraulic press, the various films were compression molded (USA). Mixed compounds were pressed for 5 minutes at 180 ^oC under a 500 kPa pressure before cooling to room temperature over 3 minutes under a 550 kPa pressure. The final appearance of the films as presented in Figure 5-1 was semitransparent. For all samples, the concentration of Limonene was set at 20 wt% to meet

the minimum inhibitory concentration (MIC) for pathogenic and spoilage microorganisms. The composition of the investigated blend nanocomposites and reference films is shown in Table 5.2.

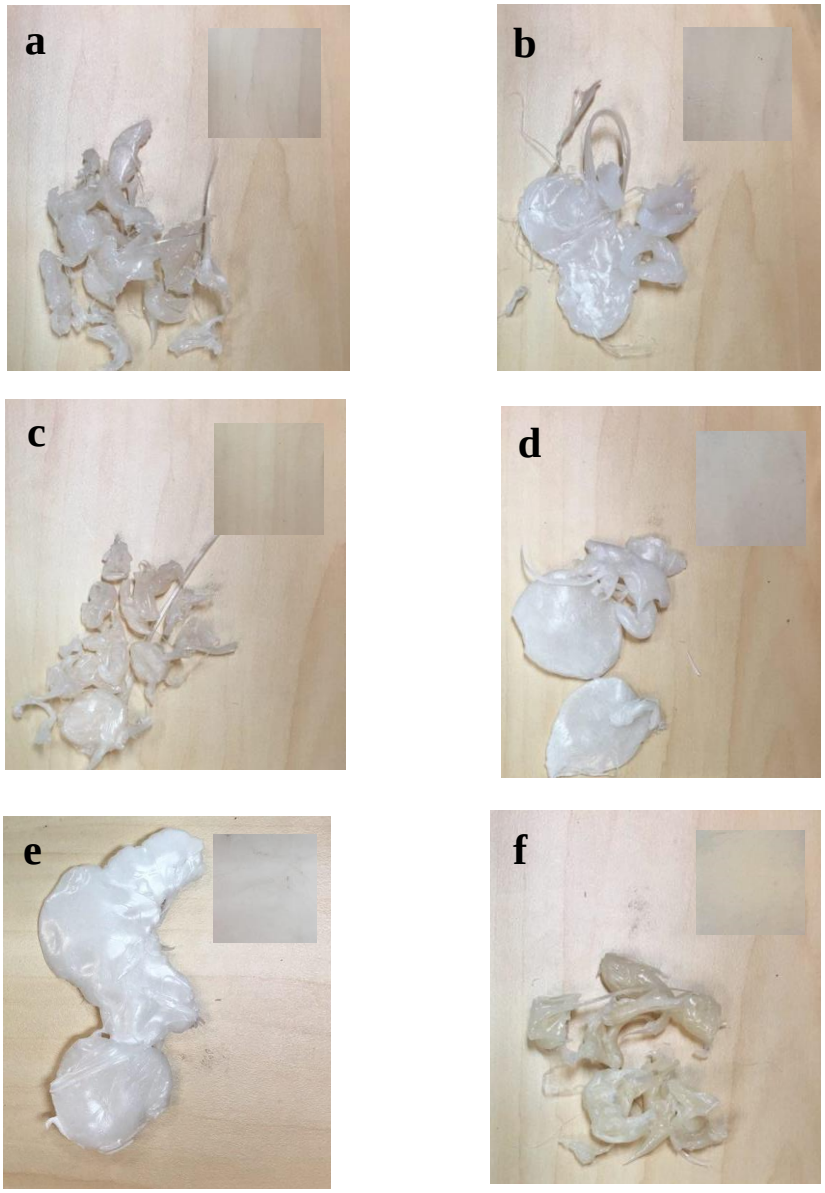

Figure 5-1 The visual appearance of mixed compounds and final films: a) LDPE/LEO, b) LDPE/HNTs-LEO, c) LDPE/Kao-LEO, d) LDPE/MSNs-LEO, e) LDPE/ZnONPs-LEO and f) LDPE/Z4A-LEO

Table 5-2 The composition of different blends

Films	Carrier (wt%)	Limonene (wt%)
LDPE	None	None
LDPE/LEO	None	20
LDPE/HNTs-LEO	5	20
LDPE/Kao-LEO	5	20
LDPE/MSNs-LEO	5	20
LDPE/ZnONPs-LEO	5	20
LDPE/Z4A-LEO	5	20

5.3.4 Thickness of films

The film thickness was measured with a digital micrometre (ProGage Thickness Tester, Thwing-Albert Instrument Company, NJ, USA) at six random positions, and the mean value was reported. The average thickness of the films was discovered to be around 150 μm.

5.3.5 Thermogravimetric analysis (TGA)

Thermal stability of the LDPE matrix and the additives content in the films were determined by TGA, using a Q500 (TA Instruments, New-Castle, DE, USA). Each LLDPE/LAM film (5–10 mg) was located in a platinum pan and heated under atmosphere containing pure nitrogen flow from 25 °C to 800 °C at a heating rate of 20 °C/min. Universal Analysis V4.5A build 4.5.0.5 software was used to analyze the results with triplicate films for each sample

5.3.6 Mechanical Characterization

Tensile tests were performed using an Instron 3365 (USA) tensile machine with a 5kN load cell at a cross-head speed of 50 mm/min, in accordance with ASTM D638 [34]. All film samples were conditioned for 24 hours at 23 °C and 50% relative humidity before being tested. Five replicates of each sample were used to test tensile strength, modulus, and elongation at break. Five replicates of each sample were used to test tensile strength, modulus, and elongation at break.

5.3.7 Optical properties

Optical characterization of the films in terms of haze values was carried out in accordance with the ASTM D 1003. Using a PerkinElmer LAMBDA 1050 spectrophotometer, three samples of each film were measured five times at slightly varied places on the surface.

5.3.8 Oxygen permeability

According to ASTM D 3985, the permeability to oxygen was evaluated using a MOCON OXTRAN 2/21 (Minneapolis, USA) at 25 ^{o}C, 0% relative humidity, and 1 atm pressure. The carrier gas for these experiments was a mixture of 2% hydrogen (H_2) and 98 percent nitrogen (N_2), and the test gas was 100% oxygen (O_2).

5.3.9 Short-term release studies

The accelerated migration of limonene from the films was studied by isothermal gravimetric analysis using a TGA-Q500 (TA Instruments, New-Castle, DE, USA) at a constant temperature of 60 ^{o}C under nitrogen atmosphere for a duration of 10 h, after reaching the plateau (equilibrium).

The release of limonene is calculated with the mass loss for each sample at this temperature. This method is performed to measure limonene release from the films to the atmosphere, simulating the developed film's mode of use in real applications e.g., food packaging. Several methods have been used to measure the diffusion of essential oils in polymer matrices [35-38]. In this work, the limonene diffusion coefficient $D(m^2S^{-1})$ was estimated using Eq. 1 [35, 39]

$$\frac{m_t}{m_\infty} = 4\left(\frac{Dt}{\pi l^2}\right)^{1/2} \qquad (1)$$

where, m_t and m_∞ are the amounts of limonene released from the film at time t and at equilibrium $t = \infty$, respectively, and l is the overall film thickness.

5.3.10 Antimicrobial assays

E. coli bacteria were tested on all developed LDPE/Carrier-LEO films. *E.coli* was cultured in LB broth at 37 ^{o}C for 24 hours with continual agitation to obtain a concentration of 10^8 colony forming

units (CFU)/mL. After 24 hours, the bacteria culture was diluted 1:1000 with sterile LB to achieve a density of 10^5 CFU/mL.

Antimicrobial test method based on ISO 22196:2011 explained by Abdali and Ajji [40] was used to investigate the performance of the active films.

In summary, both active and reference films were cut out into 50 mm × 50 mm coupons and disinfected for 20 minutes using UV light. Each sample coupon was placed in a sterile Petri dish and covered with a sterilized cover plastic thin film [40 mm x 40 mm] for distributing 400 µL of the diluted bacteria culture. Both the active and cover films are rubbed against each other, contaminating the surface. All Petri dishes holding inoculated sample coupons were placed in the incubator for 24 hours at 37 °C and 90% relative humidity. The next day, the bacterial inoculums thus placed on the surfaces were washed off with 1 mL PBS solution. Then, six serial PBS dilutions (10^{-1} to 10^{-6}) were carried out. Three samples were taken from each of the six dilutions and 10 µL droplets were applied onto the LB agar plates which were incubated overnight at 37 ^{o}C for counting the surviving bacteria (CFU/mL). Each sample is made in triplicate, along with the negative control.

5.3.11 Dynamics of antimicrobial activities

All produced films were wrapped in aluminum foil and placed at 25 ± 2 ^{o}C and 50 ± 2 % relative humidity. Every 2 weeks until the 8th week, samples were taken out to study the influence of storage time on the antimicrobial performance of the active films.

5.3.12 Statistical analysis

The OriginPro8 software was used to do an analysis of variance (ANOVA) and a multiple comparison test (Tukey) with a 95% significant threshold ($p \leq 0.05$). The data is presented as mean ± standard deviation.

5.4 Result and discussion

5.4.1 Thermogravimetric analysis

As the thermal processing for film production involves high-temperature treatments and long processing time, volatile compounds such as limonene may be degraded or evaporated [41].

Therefore, the limonene retention and distribution within the LDPE matrix plays a key role in the prolonged antimicrobial performance of the active films. Total limonene content in the developed films was determined using TGA, Table 5-3 summarizes the limonene content in active film samples before and after melt processing and film production.

The limonene concentration is found to fluctuate between 4.1-5.1 wt% in all generated films using Carrier-LEO, showing that 20-25% of the initial limonene content is kept during the high-temperature processing. Our findings are in line with those of Ramos et al. [42, 43], who used batch melt compounding and compression molding to obtain 25-40% carvacrol retention during PP/carvacrol film fabrication. It should be highlighted that limonene entrapment in both HNTs and MSNs was shown to have a considerable impact on the residual content of the films. Thus, it could be concluded that the HNTs and MSNs preserve the volatile limonene molecules during the harsh conditions of the compounding step.

HNTs with a tubular morphology by rolling-up of the layers can be employed as an ideal host material for loading of limonene as well as a thermal protector in case of degradation due to thermal stress. The limonene expelling from HNTs was slow because the limonene existed in narrow space between the layers. Compared to other carriers, MSNs exhibited also a higher loading capacity towards limonene due to its higher nonporous structure. Although Z4A has the highest porosity, the size and charge of the limonene as a bioactive compound are important variables in zeolite performance. These results showed that Z4A pore entrance size was not proper for limonene adsorption. The same results were derived by Shemesh et al. [30, 44] who reported that HNTs loaded carvacrol was necessary to achieve a high essential oil content in LDPE and polyamide films.

Table 5-3 Limonene film samples with various carriers measured by thermal gravimetric analysis (TGA)

Films	Pre-processing content of Limonene (wt%)	Post-processing content of Limonene by TGA (wt%)
LDPE	-	0.0 ± 0.0
LDPE/LEO	20	3.1 ± 0.1
LDPE/HNTs-LEO	20	5.1 ± 0.2
LDPE/Kao-LEO	20	4.3 ± 0.1
LDPE/MSNs-LEO	20	4.8 ± 0.1
LDPE/ZnONPs-LEO	20	4.2 ± 0.1
LDPE/Z4A-LEO	20	4.1 ± 0.1

5.4.2 Mechanical Characterization

Mechanical properties are important in packaging materials because they are linked to structural integrity, which is required to provide physical protection to the goods inside. The effect of Carrier-LEO incorporation on the mechanical properties of films was studied by measuring tensile strength (TS), elongation at break (% E), elastic modulus, and thickness and the results are summarized in Table 5-4. There were no remarkable deviation in the thicknesses of the films in these tests. The inclusion of additives has a significant impact on the mechanical properties of produced films; in particular, compared to the neat LDPE film, an overall drop in mechanical properties in terms of TS and elastic modulus values can be observed. There was a modest increase in elongation at break in these samples. The plasticizing impact of limonene and the complex role of mineral carriers in nanocomposites are responsible for this phenomenon. Other authors have reported results that are similar to ours. [45, 46]. Consequently, the mechanical performances of the produced films containing Carrier-LEO are improved by their synergistic effect with respect to LDPE/LEO films. In addition, the mechanical properties of the films containing HNTs-LEO and MSNs-LEO are

improved in comparison to the other LDPE/Carrier-LEO films. The plasticizing impact of free limonene dissolved in the amorphous phase of LDPE/Carrier-LEO films is responsible for this behavior. These results revealed that the limonene fraction which is entrapped within the HNTs and MSNs carriers is higher than that entrapped within other carriers viz. LDPE/HNTs-LEO and LDPE/MSNs-LEO films contain more essential oil but are less plasticized. This research supports the findings of Krepker et al. [37], who found that polypropylene nanocomposite films containing carvacrol-loaded HNTs have superior mechanical properties to PP/carvacrol blends.

Table 5-4 Mechanical properties of LDPE and LDPE/Carrier-LEO films

Films	Film thickness (μm)	Elastic modulus (MPa)	Tensile strength (MPa)	Elongation (%)
LDPE	150 ± 3	111.4 ± 15	15.1 ± 1.1	750 ± 50
LDPE/LEO	151 ± 1	90.3 ± 4	10.1 ± 1.2	800 ± 40
LDPE/HNTs-LEO	153 ± 3	104.2 ± 16	12.9 ± 0.7	780 ± 60
LDPE/Kao-LEO	150 ± 3	94.2 ± 8	11.8 ± 2.3	830 ± 80
LDPE/MSNs-LEO	152 ± 1	103.2 ± 17	12.1 ± 1.7	785 ± 20
LDPE/ZnONPs-LEO	153 ± 3	94.2 ± 5	11.1 ± 1.2	848 ± 90
LDPE/Z4A-LEO	151 ± 4	91.2 ± 6	10.8 ± 1.6	850 ± 70

5.4.3 Optical properties

Optical properties in nanocomposite films are of significant commercial importance. The addition of nanoparticles in a polymer matrix can negatively affect the haze value of the films, and this can affect the commercial potential of this class of materials for packaging applications [47, 48]. The haze values of all investigated films are reported in Figure 5-2. As it can be seen, the presence of additives raises the haze of the films. When making a comparison with the neat LDPE control film, the LDPE/LEO film sample had a little more haze as presented in Figure 5-2. On the contrary, both films containing Kao and Z4A showed a higher haze value than those containing other carriers and this is because of the presence of the larger particle size carries in films [49]. Moreover, films containing HNTs-LEO, MSNs-LEO, and ZnONPs-LEO showed approximately the same haze

value for the same reason. Similar to our results, Druffel et al. [50] concluded that the haze of the PMMA films reduces by decreasing nanoparticle diameter.

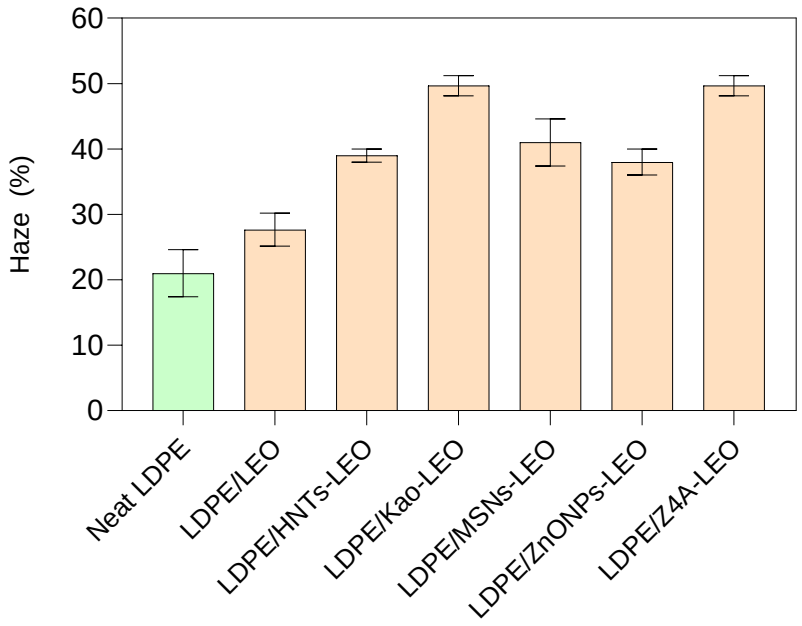

Figure 5-2 Haze values for the neat LDPE and LDPE/Carrier-LEO films

5.4.4 Oxygen barrier properties

Oxygen diffusion into food through a package could accelerate bacterial growth; thus, barrier properties are important for protecting packaged foods from the environment and ensuring that they maintain their desired quality. The chemical structure and morphology, which are related to the composition and dispersion of fillers in the polymer matrix, have a strong influence on the gas barrier properties [51]. The results of oxygen permeability for the various developed films are presented in Figure 5-3. All limonene-containing films have higher OTR values than the neat LDPE reference film in general. This is probably is because of the plasticizing effect of limonene in films. Same results were reported by other authors [37, 45]. The films containing Kao-LEO, ZnONPs-LEO, and Z4A-LEO showed similar oxygen barrier properties with only a slightly higher oxygen barrier for the film containing Z4A-LEO. Conversely, films containing HNTs-LEO and MSNs-LEO presented significantly lower oxygen permeability compared with the other LDPE/Carrier-LEO films. This is most likely due to the superior efficacy of HNTs and MSNs in entrapping

limonene molecules, which results in a decrease in free limonene content in films. These findings are consistent with TGA findings, which demonstrated that volatile limonene molecules are more efficiently retained into the HNTs and MSNs carriers instead of polymer matrix during the harsh conditions of film production steps. Our findings are in accordance with those of Solano et al. [46] who reported that a high concentration of oregano in the amorphous region significantly interfered with the polymer-polymer interactions, resulting in the increase in the OTR properties of the LDPE films.

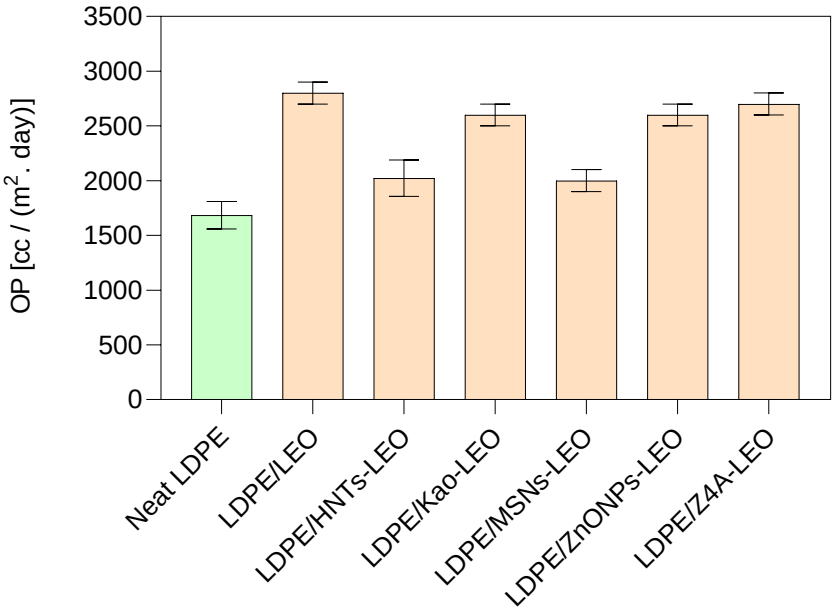

Figure 5-3 Oxygen permeability of neat LDPE and LDPE/Carrier-LEO films

5.4.5 Short-term release studies

Perhaps the residual amount was actually below the minimum inhibitory concentration (MIC), insufficient to remove the bacteria examined, due to the release of active volatile chemicals from the LDPE films over time. As a result, knowing the limonene release kinetics and controlling its release from the film is crucial for establishing its antimicrobial effectiveness and prospective application as a packaging material [6, 52, 53]. TGA was utilized to characterize the limonene release from the various manufactured films by measuring weight loss over time at a constant

temperature. The calculated diffusion coefficient values are presented in Table 5-5. The diffusivity of limonene in the LDPE/HNTs-LEO and LDPE/MSNs-LEO films is lower by 30% and 25% in comparison to LDPE/LEO, respectively. Thus, LDPE/HNTs-LEO and LDPE/MSNs-LEO films were observed to keep significantly higher limonene content than LDPE/LEO and the other LDPE/Carrier-LEO films. Results presented that the diffusivity of limonene in the LDPE/Carrier-LEO films is strongly influenced by the carrier type used to produce the films. The LDPE/LEO film had the highest effective limonene diffusivity, while the addition of Carrier-LEO reduced limonene out-diffusion from the developed films. These results are ascribed to the reduction of limonene desorption rate from the carrier's surface and forming a tortuous diffusion path in presence of mineral carriers in the LDPE matrix [54-56]. Krepker et al. [57] examined the effective diffusion coefficient of carvacrol in the presence of HNTs in the LDPE matrix and found similar results. Furthermore, the effective role of HNTs and MSNs as mineral nano-carriers, hindering the release of confined limonene molecules and reducing the effect of plasticizing, is attributed to the slower out-diffusion kinetics of limonene from the LDPE/HNTs-LEO and LDPE/MSNs-LEO.

Table 5-5 Estimated diffusivity of limonene from developed films at 60 ^{o}C.

Films	Limonene diffusivity $\times 10^4$ (m^2s^{-1})
LDPE/LEO	510 ± 63
LDPE/HNTs-LEO	360 ± 20
LDPE/Kao-LEO	430 ± 33
LDPE/MSNs-LEO	380 ±11
LDPE/ZnONPs-LEO	460 ± 25
LDPE/Z4A-LEO	470 ± 28

5.4.6 Antimicrobial property of films

Antimicrobial studies were initially carried out using the ISO 22196:2011 method to verify the limonene content within the films. The results obtained are summarized in Table 5-6. Fresh films

containing limonene had strong antimicrobial activity against *E. coli* bacteria, as expected. Because some strains of *E. coli* are widely spread foodborne pathogens, it was selected as a model bacterial species. All studied films reduced the *E. coli* density by approximately 5-6 logs (CFU/mL) in comparison with neat LDPE film. This output is consistent with the TGA results, which confirmed the presence of limonene in the studied films.

Table 5-6 Log reduction of LDPE/Carrier-LEO films towards *E. coli*.

Films	Log reduction by viable cell count (CFU/mL)
LDPE	None
LDPE/LEO	6.0 ± 0.20
LDPE/HNTs-LEO	6.0 ± 0.10
LDPE/Kao-LEO	5.0 ± 0.10
LDPE/MSNs-LEO	5.5 ± 0.10
LDPE/ZnONPs-LEO	5.0 ± 0.20
LDPE/Z4A-LEO	5.5 ± 0.30

5.4.7 Effect of storage time as long-term release study

These tests are being carried out to see how storage time affects the antimicrobial efficiency of the produced films and to evaluate the bio-functionality of limonene after processing. Figure 5-4 demonstrated the results of these experiments for studied films versus storage time. All fresh films registered 5-6 logs (CFU/mL) reduction for *E. coli*. Antimicrobial activity of LDPE/LEO, LDPE/Z4A-LEO, and LDPE/Kao-LEO films decreased by two logs (CFU/mL) after two weeks of storage, while LDPE/HNTs-LEO and LDPE/MSNs-LEO films remained unchanged. After 6-week storage, LDPE/LEO film has completely lost its antimicrobial activity; while, the films containing MSNs-LEO and HNTs-LEO have kept their activity by 2 and 3 logs (CFU/mL) reduction respectively, and the rest of the films showed less than 1 log reduction. Finally, all developed films lost their antimicrobial activity within 8-week of production, while LDPE/HNTs-LEO film has

preserved its antimicrobial activity. The film remained active for another 30 days, but with a 2 log reduction in *E. coli*. Thus, these results showed that the storage time has an extreme effect on the antimicrobial potency and further emphasize the advantages of using HNTs-LEO and MSNs-LEO synergistic mixtures rather than the individual limonene or other Carrier-LEO combinations to achieve a controlled release rate over a prolonged period. Similarly, In comparison to LDPE-based films containing the individual essential oils, Krepker et al. [58] found that LDPE films containing this composition exhibited excellent enduring antimicrobial activity against *E. coli* due to synergistic interactions between carvacrol and thymol essential oils entrapped within HNTs. These results are also evident that the entrapment of limonene into HNTs and MSNs enables slow release of limonene function over long periods of time and are consistent with the results from short-term release analysis of the studied films.

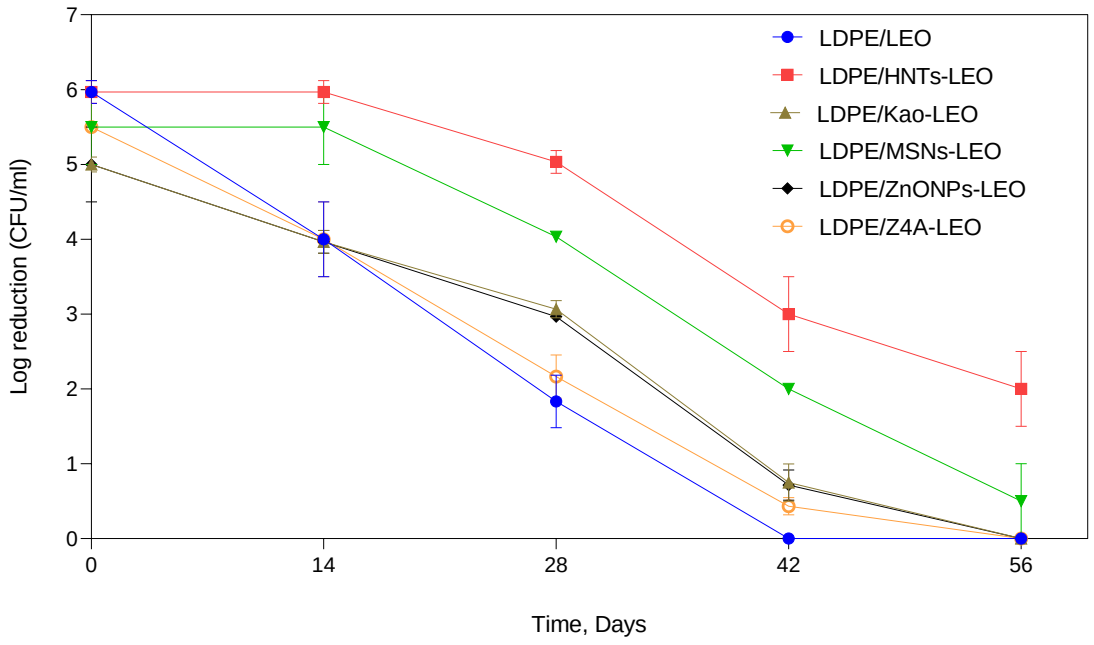

Figure 5-4 Antimicrobial properties of LDPE/Carrier-LEO film samples towards *E. coli* as a function of storage time

5.5 Conclusion

In the present study, LDPE/Carrier-LEO films were produced by incorporating limonene, loaded into the different mineral carriers, via batch melt compounding. To assess the effect of these

Carrier-LEO combinations in the LDPE matrix and their material stabilization performance during processing, different analytical techniques were used to characterize active films. The addition of these Carrier-LEO systems was found to affect the optical, oxygen barrier, mechanical, and long-term antimicrobial properties. The inclusion of free limonene in the LDPE matrix as a plasticizer causes poor mechanical and barrier properties, as well as rapid limonene release from the films. The high amount of limonene retained within HNTs and MSNs carriers produced superior prolonged antimicrobial films against *E. coli* in comparison with other carriers, revealed strong synergistic interactions between these carriers and limonene due to their morphological characteristics and high porosity. As a result of this synergistic interaction, desired antimicrobial performance can be achieved with lower active component concentrations and slower limonene molecule out-diffusion, thus leading to the potential use of these systems for numerous applications, such as antimicrobial active packaging, medical and hygiene.

5.6 Acknowledgements

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CHAPTER 6 ARTICLE 3: THE ANTIBACTERIAL AND ANTIVIRAL POTENTIAL OF PE-BASED FILMS ENRICHED WITH LAE AGAINST E. COLI AND HUMAN CORONAVIRUS

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6.1 Abstract

This work was performed to monitor the antibacterial and antiviral effects of ethyl lauroyl arginate (LAE) with the maltodextrin carrier incorporated into LLDPE films. LAE in its commercial form as LAM (10% LAE, 90% maltodextrin) was added into LLDPE films using melt compounding at 6.8, 8.7, 14, and 16.5 wt%. Antibacterial and antiviral performance of the produced films against *E.* coli bacteria and *Human Coronavirus (HCoV-229E)* were examined. The TGA results showed that the thermal stability of the LLDPE/LAM films diminished with the increment of concentrations of LAM. The films containing 14 and 16.5 wt% LAM showed significant antimicrobial properties, while films with low concentrations of LAM (6.8 and 8.7 wt%) exhibited poor antibacterial activity against *E. coli*. Furthermore, films with 14 and 16.5% LAM lost their antibacterial performance after 10 days of immersion in water. The antimicrobial activity of these tested films was recovered after 5 months of storage at room temperature with a 2 log (CFU/mL) reduction of *E. coli* due to LAE migration from bulk to the film surface. Antiviral activity of film with 14 wt% LAM against *Human Coronavirus (HCoV-229E)* was evaluated and reductions of 0.7 and 1.18 log $TCID_{50}$/mL were shown after 1 and 2 hr contact time, respectively. The results indicate that LLDPE/LAM films could be potentially used in antimicrobial packaging systems; in particular, it could provide a powerful measure against the spreading of *Coronaviruses* at the outer layer of food packaging.

6.2 Introduction

Antimicrobial packaging, as a promising and innovative form of active packaging, has received attention over the past two decades, since the high morbidity of food-associated illnesses, related to pathogenic species, represents a significant public health threat worldwide [1, 2]. Furthermore, food products may be contaminated by viruses along different steps of food processing, storage, and transportation, supplying products to customers, and so cause the spread of the foodborne disease. Recently and during the *SARS-COV-2* pandemic, some studies showed cross-contamination of surfaces as a source of indirect *Coronavirus* transmission [3]. Suman et al. [4] reported that viable *Coronavirus* could remain active on the plastic surface for up to 72 h. Therefore, there is the possibility that viruses can also be transmitted through the contaminated surfaces of packaging materials, especially during the *COVID-19* pandemic period, and customers

to be infected and so cause the spread of contagious diseases. In order to address customer concerns and assure their safety, antiviral packaging may offer the opportunity to limit its transmission.

Many previous studies have shown the interesting antimicrobial properties of ethyl lauroyl arginate (LAE) [5]. This cationic surfactant is synthesized through esterification reaction between lauric acid, L-arginine, and ethanol. [6, 7]. FDA categorized LAE as a GRAS at concentrations below 200 ppm for use in some foodstuffs such as meat, poultry, and cheese in 2005 [8]. LAE was also approved as a safe food preservative at a concentration of up to 225 ppm by the European Food Safety Agency (EFSA) in 2007 [8, 9]. LAE exhibited remarkable antimicrobial performance against a broad range of microbial pathogens and spoilage microorganisms that include gram-negative, gram-positive bacteria, molds, and viruses [10]. Its efficiency in the inhabitation of microorganisms growth such as *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes, L. innocua*, *B. thermosphacta*, *Staphylococcus aureus*, *Herpes virus type 1*, *Vaccinia virus*, *Bovine parainfluenza 3* has been proved [7, 10-12].

The most prevalent packaging material in the world, polyethylene (PE), was used as a polymer matrix in this study. PE is an excellent contender for a wide range of applications due to its inexpensive cost, excellent chemical resistance, ease of production, high elongation at break, and FDA/USDA approval. [13]. The addition of pure LAE into the LLDPE matrix can lead to unstable conditions during melt extrusion and film production. Thus, LAE in combination with maltodextrin as LAM (10% LAE, 90% maltodextrin) has been taken to prevent this issue and to improve the surface quality of films.

Maltodextrin as a commercially important uncharged water-soluble biopolymer is an easily digested, branched polysaccharide produced by hydrolysis of starch through chemical and enzymatic processing [14]. Maltodextrin is generally used in foods as a filler or thickener, texturizer [15] and is “generally recognized as safe” (GRAS) by the FDA [15].

LAE as a cationic surfactant can bind to the maltodextrin to form complexes that allow modifying release of LAE compound to enhance food quality by avoiding spoilage, and microbial contamination. Wangsakan et al. [16] reported that maltodextrin shows sufficient capacity to interact with dodecyl trimethylammonium bromide (DTAB) as a cationic surfactant via hydrophobic linkages.

The antimicrobial properties of LAE is attributed to the damage of plasma membrane and cell wall disruption, resulting in cell growth inhibition [17]. The electrostatic interactions of cationic surfactant segment of LAE with anionic charged proteins present on the cell membrane of microorganisms leads to the instability of the plasma membrane and consequently cause cell death [18]. Different studies have demonstrated that the antimicrobial mechanism of LAE is most likely due to disrupting plasma membrane integrity, leading to the morphological changes in the cell wall structure and the leakage of the cytosol components [7, 17, 19-21]. Higueras et al. [22] produced chitosan films containing 1, 5, and 10 wt% LAE by solvent casting. It was shown that the combination of LAE with chitosan dramatically enhanced the antimicrobial performance towards several microorganisms including *Colifoms, Lactic acid bacteria*, *Mesophiles, Pseudomonas spp.*, *Psychrophiles,* yeast, and fungi.

Melt compounding is the most widely used method for incorporating active additives into a wide range of polymer matrices in the plastic packaging sector, owing to its technical advantages, cheap cost, and greater flexibility [23]. Gaikwad et al. [24] incorporated different concentrations of LAE powder (1, 3, 5, and 10 wt%) into LDPE film using melt extrusion. The LDPE/LAE containing 5 and 10 wt% LAE showed the highest antimicrobial performance in comparison with other concentrations of LAE towards fungal growth in strawberries.

LAE plays an active role in the field of preservation for food and cosmetics [5, 25]. However, limited information concerning the incorporation of LAE with a carrier into polymer films via melt extrusion and its antiviral performance have been reported. This indicated the importance of antiviral packaging, which deactivated the virus upon contact to prevent cross-contamination through food packaging in the food distribution chain. Recently some studies evaluated the efficacy of antiviral packaging on *Human enteric viruses* [26-31], but despite the significant potential of LAE incorporated packaging, its antiviral activity against *Human Coronaviruses* has not been explored yet.

In light of the foregoing, the primary purpose of this research is to investigate the antibacterial and antiviral activity of polyethylene films containing various concentrations of LAM against *E. coli* and the *Human Coronavirus* in food packaging applications.

6.3 Materials and methods

6.3.1 Materials

A&B Ingredients, Inc. provided the LAE in its commercial form as LAM (10 percent LAE, 90% maltodextrin) (New Jersey, USA). Before the experiments, the powder was dried in a vacuum oven at 100°C and under vacuum for 38 hours. Nova Chemicals (Calgary, Alberta, Canada) provided food grade FPs317-A Octene sLLDPE, which was used as the polymeric matrix. The University of Montreal provided *Escherichia coli DH5-Alpha*. VWR International, LLC provided the Luria-Bertani (LB) medium and all other reagents required in bacteria cultivation. The American Type Culture Collection provided *human Coronavirus strain 229E* (ATCC #VR-740) and MRC-5 cells (human fetal lung fibroblast, ATCC #CCL-171) for this study (ATCC, Burlington, ON, Canada).

6.3.2 Morphology

The distribution of LAM powder on the surface of the LLDPE/LAM films was investigated using a tabletop SEM (TM3030plus, Hitachi) operating at 15 kV acceleration voltage.

6.3.3 Powder moisture content

The moisture content of LAM powder plays an important role in achieving excellent film quality. To minimize the moisture intake, the LAM powder needs to be dried by vacuum oven at 100 ^{o}C for 38 hours before the experiment. TGA Q500 from TA Instruments (New Castle, DE, USA) was used to evaluate the moisture content (Xw, in% wet basis) of LAM powder before and after drying. Under a clean nitrogen environment, 5 mg of LAM were heated at a rate of 20 ^{o}C min1 from 25 ^{o}C to 800 ^{o}C.

6.3.4 Film preparation

LLDPE/LAM cast film samples were prepared using a co-rotating twin-screw extruder (Leistritz ZSE 18 HP, Nuremberg, Germany) with an 18 mm screw diameter and an L/D ratio of 40. The extruder was operated using a flat temperature profile of 170 ^{o}C and die temperature of 180 ^{o}C. The melt mixing was done at a screw speed of 200 rpm. Single-layer cast films (LLDPE/LAM) were produced with different concentrations of the LAM including 6.8, 8.7, 14, and 16.5 wt%. The formulation of each LLDPE/LAM film is presented in Table 6-1. The extrusion line included a slit

die with a 2 mm opening, as well as an air knife cooling system. At room temperature, the films were stretched with chill rolls.

Table 6-1 Composition of LLDPE/LAM films

Sample Code	LAE (wt%)	Maltodextrin (wt%)	Total LAM (wt%)
S-6.8	0.68	6.12	6.8
S-8.7	0.87	7.83	8.7
S-14	1.4	12.60	14
S-16.5	1.65	14.85	16.5

6.3.5 Thickness of films

A digital micrometre (ProGage Thickness Tester, Thwing-Albert Instrument Company, NJ, USA) was used to measure the thickness of film samples at six random positions, and the mean value was calculated. The average thickness of the films was discovered to be around 160 μm.

6.3.6 Thermogravimetric analysis (TGA)

Thermal gravimetric analysis (TGA) was used to investigate the thermal stability of LLDPE/LAM films using a Q500 (TA Instruments, New-Castle, DE, USA). Each LLDPE/LAM film (5–10 mg) was located in a platinum pan and heated under atmosphere containing pure nitrogen flow from 25 °C to 800 °C at a heating rate of 20 °C/min. Universal Analysis V4.5A build 4.5.0.5 software was used to analyze the results with triplicate films for each sample.

6.3.7 FTIR spectroscopy

Transmission infrared analysis was performed on LLDPE film samples using a Spectrum 65 FTIR spectrometer from PerkinElmer (Waltham, MA) with a resolution of 4 cm^{-1} and a 32 scans accumulation within the wavenumber range of 4000–600 cm^{-1}.

6.3.8 Antimicrobial test

All LLDPE/LAM films were tested against *E. coli*. These bacteria were grown in LB broth for 24 h at 37 ºC, to reach a density of 10^8 colony forming units (CFU)/mL. The bacteria culture was diluted in the same medium after 24 h to 10^5 CFU/mL. Antimicrobial tests were conducted according to the standard ISO 22196:2011 and as described by Abdali and Ajji [32]. Both active and control samples were cut out into 50 mm x 50 mm coupons. All sample coupons were disinfected by placing them under a UV light in a Class II biosafety cabinet for 20 minutes. Four hundred microliters of the diluted bacteria culture were deposited on each sample coupon placed in a sterile Petri dish and overlaid with a sterilized plastic thin film [40 mm x 40 mm] for spreading. All Petri dishes containing inoculated sample coupons were incubated at 37 ºC for 24 hr. The next day, the bacterial inoculums thus placed on the surfaces were washed off with 2 mL PBS solution. Then, six serial PBS dilutions (10^{-1} to 10^{-6}) were conducted. Three samples were taken from each of the six dilutions and 10 µL droplets were applied onto the LB agar plates which were incubated overnight at 37 ºC for counting the surviving bacteria (CFU/mL). Each reported result is an average of three experimental measurements.

6.3.9 Determination of antiviral activity

MRC-5 cells (CCL-171) were used for the cultivation of the cytopathogenic *Human Coronavirus strain 229E* (ATCC #VR-740). After that, viral stocks were created by centrifuging infected cell lysates. As a general procedure used for antiviral packaging films, the evaluation considers a viral inoculation step onto the active films, a contact time while the active compound in the film exerts its expected antiviral activity, and, finally, a recovery step with a neutralizer reagent to stop the inhibiting action [28]. To measure the antiviral performance of LLDPE/LAM films, a version of ISO 22196:2011 was used to assess the antiviral performance of LLDPE/LAM films. Concisely, 1×1 inch film samples were cut and UV sterilized for 10 minutes on each side. Then, each film sample was fixed in a Petri dish and 0.100 ml inoculum of virus suspension diluted in PBS buffer (5.5-6.5 log $TCID_{50}$/mL) was placed on each test film. A plastic cover polyethylene film was placed gently over each sample to evenly spread the inoculum over the surface, and the Petri dish was closed with the lid. Samples were incubated at room temperature (23.7 – 24.1°C) and 40% RH for 1 and 2 hours. At the end of each contact time, the cover film was aseptically removed, and both the cover film and test sample were rinsed with an aliquot of test media. The resultant volume was

aseptically collected and diluted by serial 10-fold dilutions. Following neutralization of the viral suspensions for both reference and test film specimens, tissue culture infectious dose-50 ($TCID_{50}$) using the Spearman-Karber method for enumeration of infectious viruses was applied [33]. Assay plates were incubated for a period of 3-4 days. A control neat LLDPE film was used as the negative reference film. The amount of infectious viruses on reference films and LLDPE/LAM films were compared to determine antiviral capabilities. Each reported result is an average of three experimental measurements.

6.4 Result and discussion

6.4.1 LAM powder characterization

6.4.1.1 Morphology

SEM images of LAM powder with average particle size ranges of 20 to 60 µm are shown in Figure 6-1(a). It can be derived that the LAM powder is comprised of highly packed irregular shape particles. The shape and size of a typical LAM particle are shown in Figure 6-1(b).

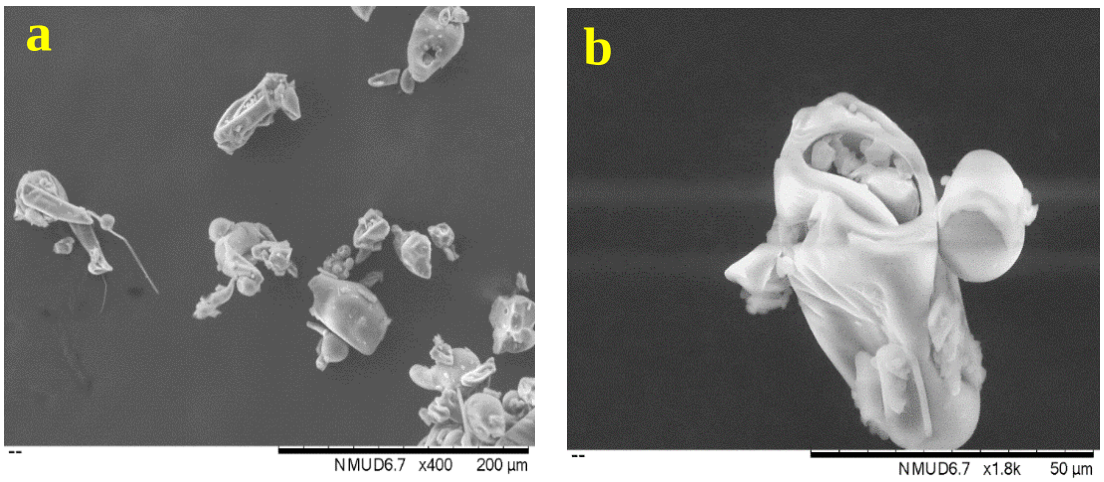

Figure 6-1 SEM of LAM powder showing (a) the irregular morphology (b) the shape and size of a typical particle

6.4.1.2 Moisture content

The moisture content (Xw, in% wet basis) of LAM powder before and after drying was determined by a thermogravimetric analyzer (TGA). Figure 6-2 shows the obtained results. On average, LAM powder before and after drying contains 6 and 0.5 wt% respectively.

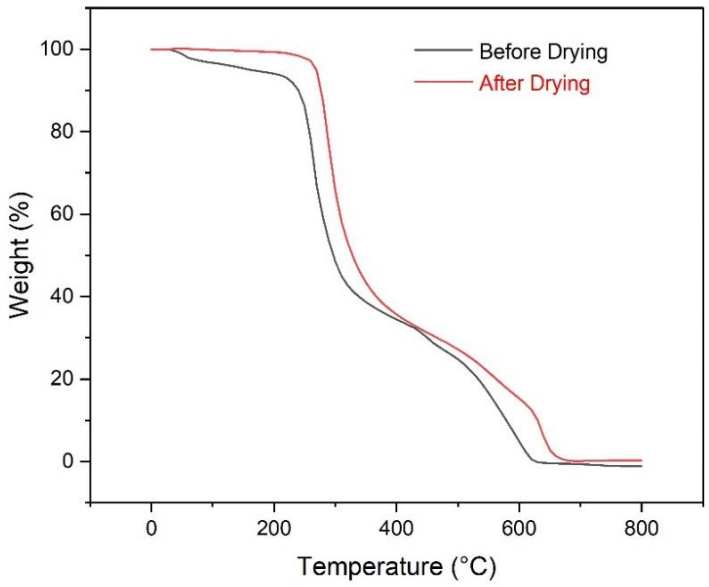

Figure 6-2 TGA of LAM powder before and after drying

6.4.2 Characterization of LLDPE/LAM films

6.4.2.1 Dispersion of LAM in LLDPE matrix

SEM was employed to evaluate the surface morphology of generated samples since the control of food spoilage microflora is often a surface-associated process. SEM examination was performed on the surface of LLDPE/LAM films with various LAM contents to evaluate the level of LAM dispersion in the LLDPE films prepared by melt extrusion. Figure 6-3 (a, b, c, and d) shows the surface morphology of the LLDPE/LAM with 6.8, 8.7, 14, and 16.5 wt% of LAM, respectively.

The film samples had a very coarse phase morphology with large particles. This is because of the different polarity, and the high surface tension between the two blend components. Therefore, better dispersion of the LAM particles may be expected with thinner films using a coupling agent such as polyethylene-grafted maleic anhydride (PE-g-MA) [34].

All samples had a rough surface morphology. However, the films with 6.8 and 8.7 wt% LAM content were comparatively smoother and had fewer aggregates as shown in Figure 6-3 (a, b). Both films with 14 and 16.5 wt% LAM were rough with microscale protrusions and aggregates as shown in Figure 6-3 (c, d). The surface microstructure of film with the highest LAM content (16.5 wt%) showed a very rough surface due to the incorporation of a large amount of antimicrobial LAM powder into the polymer.

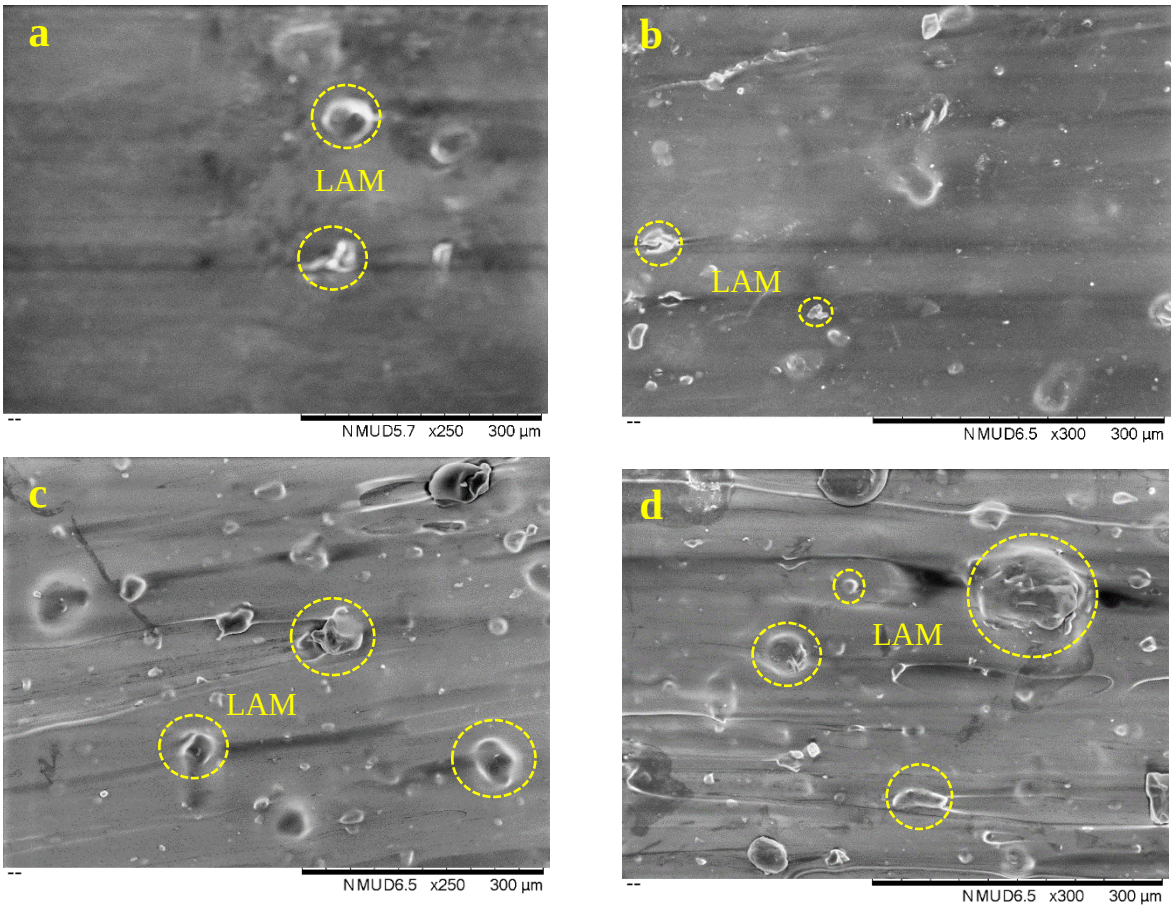

Figure 6-3 SEM images of developed films showing dispersion of LAM particles in the film surface: (a) S-6.8, (b) S-8.7, (c) S-14, and (d) S-16.5 samples

6.4.2.2 Thermogravimetric analysis

TGA was used to study the thermal stability of films containing 6.8, 8.7, 14, and 16.5 wt% LAM powder. The TGA curves for the various produced LLDPE/LAM film samples has been shown in Fig. 6-4. Decomposition of pure LLDPE began near 290 °C, but increasing the LAM powder content to 16.5 wt% (S-16.5) results in a temperature shift from 290 to 230 °C, indicating a reduction in the thermal stability of the film samples. However, the results indicate that the thermal decomposition pattern of the LLDPE/LAM films has not changed with the presence of LAM. Gaikwad et al. previously reported a similar effect of LAE on the thermal stability of LDPE/LAE film. [24].

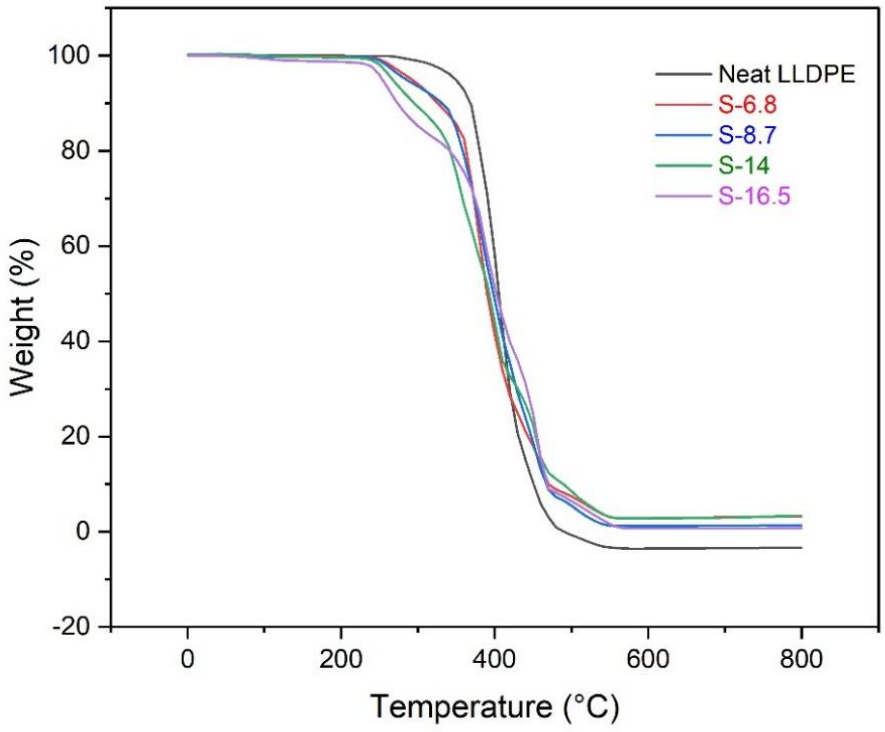

Figure 6-4 TGA of films with different concentrations of LAM

6.4.3 Antimicrobial performance

The antimicrobial performance of LLDPE films containing various concentrations of LAM has been determined towards *E. coli* and it was presented in Figure 6-5. As it can be seen samples S-6.8 and S-8.7 containing 0.68 and 0.87 wt% LAE presented a poor antibacterial activity with less than one log (CFU/mL) reduction against *E. coli*. However, LLDPE film samples S-14, and S-16.5 containing 1.4 and 1.65 wt% LAE, respectively, reduced the *E. coli* cell density by 7.5 log (CFU/mL) compared to the LLDPE reference film, and no live bacteria were recovered from film surfaces of these samples after incubation. Therefore, samples S-14 and S-16.5 were selected as the active films for further experiments. Similarly, Muriel-Galet et al. [35] used a solvent casting technique to create EVOH-based films with varying concentrations of LAE. They claimed that films containing 5 and 10 wt% LAE could completely stop the growth of *L. monocytogenes*, *E. coli*, and *S. enterica*..

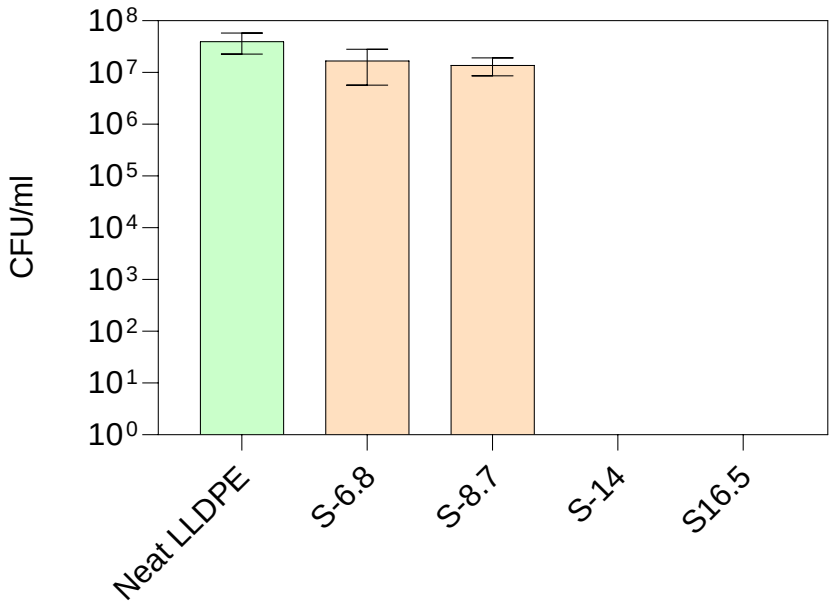

Figure 6-5 Antimicrobial properties of LLDPE/LAM films against *E. coli*.

6.4.4 Antimicrobial activity after washing LLDPE/LAM films

In order to wash out all eluted components on the surface of the films, both samples S-14 and S-16.5 were immersed into a flask containing demineralized water for one and ten days. The films were removed from the water after these periods and dried at room temperature for 30 minutes. Then, as can be seen in Figure 6-6, the antimicrobial performance of the resulted films was tested towards *E. coli*. The obtained results on samples after one-day immersion in water showed that sample S-14 lost its antimicrobial activity by 4 log (CFU/mL) and sample S-16.5 retained its antibacterial activity compared to the active fresh film. Moreover, both films exhibited a dramatic reduction in antimicrobial properties after ten days of immersion in water compared to the previous films that were immersed in water only for one day. The high polarity along with the low oil in water coefficient of LAE results in high solubility in water caused the release of all active compounds from film surfaces [36].

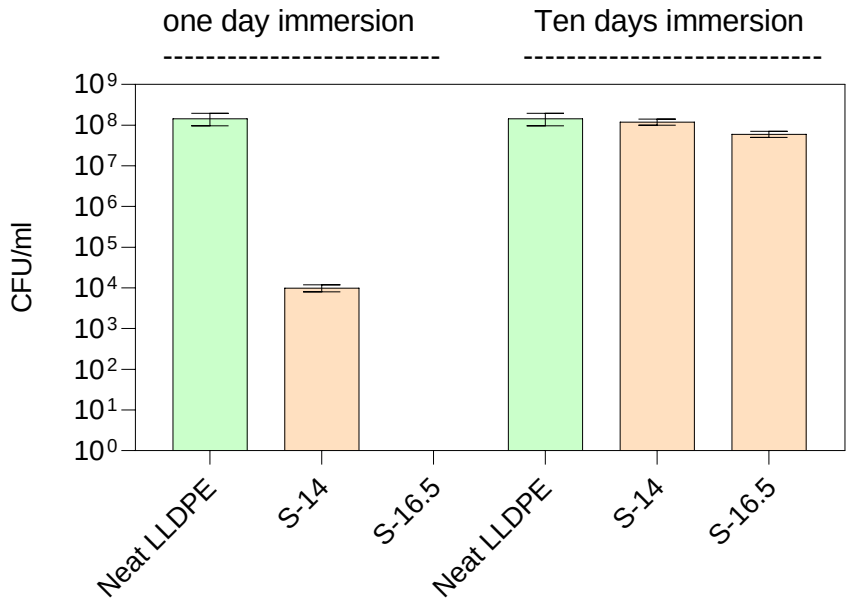

Figure 6-6 Inhibitory effect of LLDPE/LAM films after one and ten days of immersion in the water against *E. coli*.

6.4.5 FTIR spectroscopy

As shown in Figure 6-7, the presence of LAE in the bulk of S-14 film was confirmed after ten days of immersion in water using transmission FTIR spectra. These results also demonstrate that the migrated LAE into the film surface predominantly is responsible for the inhibitory effect against *E. coli*. Characteristic absorption bands for LAE can be assigned at 1740 cm^{-1} (ester) due to the ν(C=O) stretching vibration, and at 1647 cm^{-1} (amide) due to the ν(C=O) to a combination band of the ν(C=N), and δ(N–H_2) [37, 38]. Despite low molecular interactions between the LLDPE and the additive, there were no significant differences in the intensities or positions of the characteristic LLDPE peaks for fresh S-14 film and S-14 film after ten days in water, indicating that these characteristics were unaffected by washing or LAE migration to water. Similarly, Gaikwad et al. [24] showed that the chemical bonding properties of LDPE were not affected after the incorporation of various concentrations of LAE in the polymer matrix. Haghighi et al. [39] incorporated the different concentrations of LAM (69.3% LAE, 30.7% maltodextrin) in chitosan-polyvinyl alcohol blend films (CS-PVA) via the film-forming solution method for food application. It was found no severe changes for the absorption band of CS-PVA after the incorporation of LAE in concentrations up to 2.5% due to low interaction between the polymer matrix and LAE. At higher LAE concentrations, however, there was a greater interaction between the C=O, NH_2, and NH functionalities of this additive and the functional groups of the CS-PVA film matrix. Furthermore, Gamarra-Montes et al. [40] demonstrated that varying LAE concentrations have no effect on the properties of a poly(-glutamic acid) (PGGA) film.

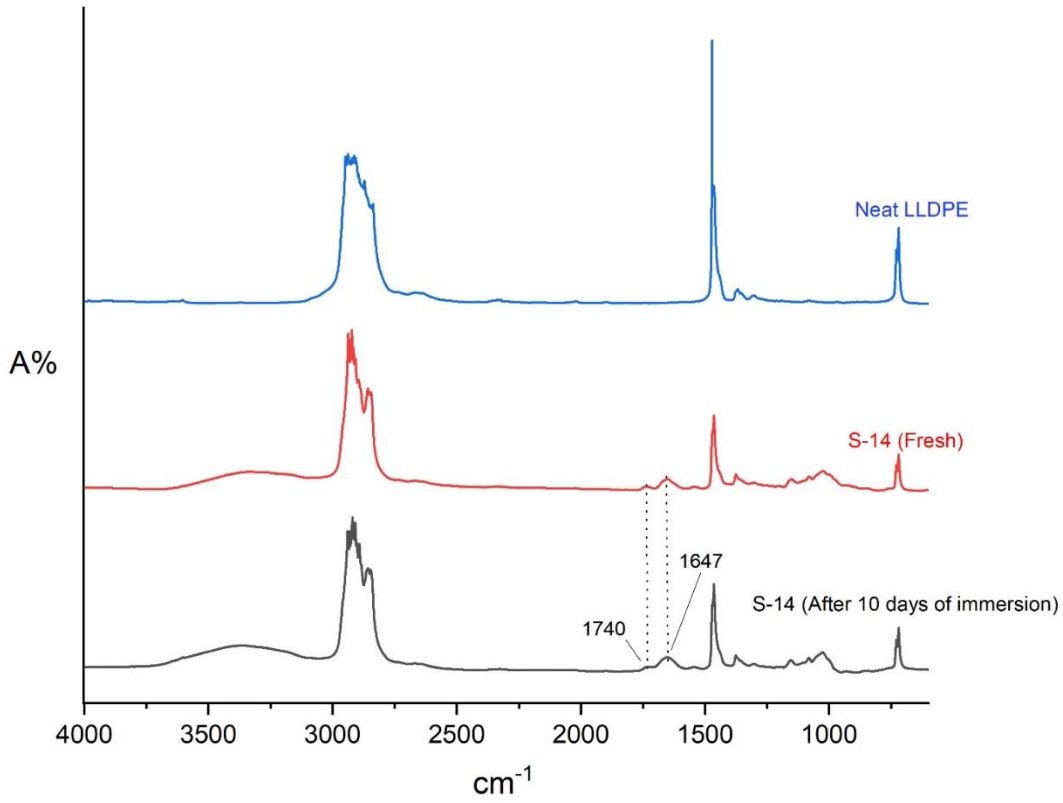

Figure 6-7 Compared FTIR spectra of neat LLDPE, fresh S-14, and S-14 after 10 days immersion in water.

6.4.6 Effect of storage time

After confirming the LAE removal from the film surface of samples S-14 and S-16.5 through conducting the previous experiments, a new study was performed to survey the LAE migration capability from bulk to the film surface. The samples obtained after 10 days of immersion in water were stored at room temperature for 5 months in order to ensure that LAE has enough time for migration to the film surface. Then, the antimicrobial properties of films were determined again and the results were presented in Figure 6-8. It was found that samples S-14 and S-16.5 showed antibacterial activity against *E. coli* with a reduction of 2 log (CFU/mL). The results indicate the migration of LAE from the bulk toward the film surface.

The plasticizer effect of LAE and the low interaction with LLDPE polymer chains are the main reasons to describe the migration behavior of LAE to the film surface [24, 41, 42]. These results

are in agreement with previous finding of the FTIR data, which demonstrated low interactions between the polymer and the LAE. In contrast, a potential contribution of maltodextrin to interact with LAE could have occurred. However, interactions between charged LAE surfactant and uncharged maltodextrin polysaccharide were found to be very weak [16]. As a result, LAE's homogenous dispersion in the film, combined with its high solubility, may be an effective method for achieving a sufficient release rate of this additive from the film surface. Aznar et al. [21] investigated the migration of LAE from polyethylene terephthalate (PET)-based antimicrobial film containing LAE. It was reported that the LAE compound migrated into two types of food simulants and packaged chicken meat. Vidal et al. [43] studied the release rate of LAE from core/shell electrospun fibers, whose inner and outer structures were composed by PVOH/LAE and PLA, respectively. They confirmed that migrated LAE concentrations into aqueous and fatty food simulants were effective enough to inhibit *L. innocua*.

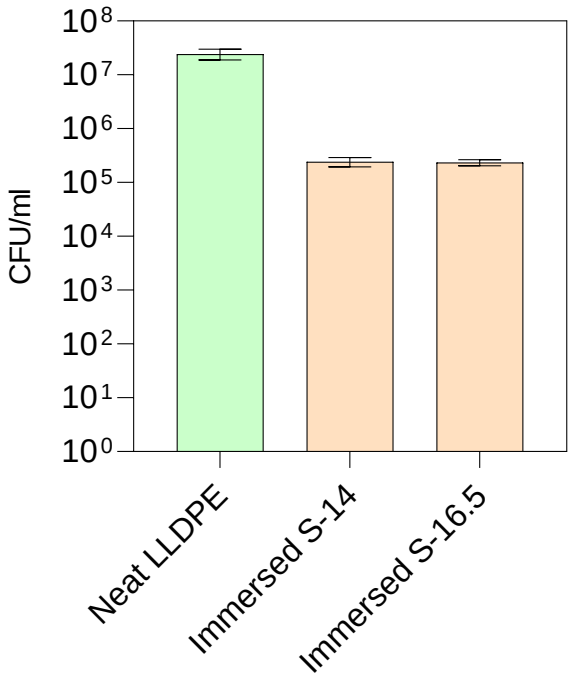

Figure 6-8 Antimicrobial properties of immersed LLDPE/LAM films in water after 5 months storage at room temperature against *E. coli*

6.4.7 Virucidal properties

The type of virus, the amount of antiviral additive, and the contact time are the main effective variables that affect the antiviral performance of active material [44]. Active film S-14 with a lower concentration of LAE (1.4 wt%) than S-16.5 was chosen for the antiviral activity test, and it was shown an effective antiviral performance in reducing viral titers of *Human Coronavirus*, Strain 229E (*HCoV-229E*) depending on contact time. As it can be seen in Table 6-2, the evaluated test substance sample S-14 demonstrated an average 0.70 log $TCID_{50}$/mL reduction in viral titer (80.05 %) at 1 hour and an average 1.18 log $TCID_{50}$/mL reduction in viral titer (93.39%) at 2 hr. The antiviral performance of sample S-14 might be attributed to the amount of LAE released from the film surface that is close to the minimum inhibitory concentration for *HCoV-229E* inhibition [45, 46]. Castro-Mayorga et al. [29] developed active silver nanoparticles-based (AgNPs) systems performing antiviral activity against *FCV*, *Human noroviruses (HuNoV) surrogates*, and *Murine norovirus (MNV)*. They reported that incorporated AgNPs into poly (3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV could remove all FCV in 24 hour contact time, at 100% RH, and 37 °C, while this system was capable to reduce MNV viral titers by only 0.86 log $TCID_{50}$/mL in the same condition. In a similar study, the solvent casting method was employed for the fabrication of PLA-based antiviral films containing different concentrations of silver ions for active packaging applications [28]. The antiviral assay carried out with *FCV* using the Japanese industrial standard (JIS Z 2801) for 24 hr at 25 °C showed 2 log $TCID_{50}$/ mL reductions for films at a concentration of 0.1 wt% of silver, while in films containing 1 wt% of silver, *FCV* infectivity was totally removed.

Table 6-2 Antiviral effect of LLDPE/LAM film on *Human Coronavirus*, Strain 229E (*HCoV-229E*) after 1 and 2 hr contact time

Virus	Type of films	Contact time			
		1hr		2hr	
		Recover titer (Log $TCID_{50}$/mL)	Reduction	Recover titer (Log $TCID_{50}$/mL)	Reduction
HCoV-229E	Control	5.5		5.84	
	S-14	4.8	0.7	4.66	1.18

6.5 Conclusion

In this work, different concentrations of LAM (10% LAE, 90% maltodextrin) were incorporated in the LLDPE matrix by an extrusion method for active packaging applications. Thermal analysis revealed that the incorporation of the antimicrobial additive changed the initial decomposition temperature and decreased the thermal stability of the films. The addition of LAM to LLDPE dramatically enhanced its antimicrobial performance against *E. coli*. Films from 14 wt% LAM reduced satisfactorily the *E. coli*. The resulted films were flexible and showed interesting release capability of antimicrobial additives. When films containing 14 and 16.5 wt% LAM were immersed in water, LAE release continues within 10 days until lost their activity. However, the characteristic absorption bands in the transmission FTIR spectra of the films showed the presence of LAE in the bulk of films after ten days of immersion in water. The antimicrobial activity of the aforementioned films was recovered after 5 months of storage at room condition with a reduction rate of 2 log (CFU/mL) due to LAE migration from bulk to the film surface. Furthermore, S-14, containing 1.4 wt% LAE, showed antiviral performance against *Human Coronavirus*, Strain 229E (*HCoV-229E*) after 1 and 2 hr contact time. As a result, the suggested LLDPE/LAM film may be deemed ideal candidates for use in food packaging due to its antibacterial and antiviral capabilities.

6.6 Acknowledgements

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6.7 References

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CHAPTER 7 GENERAL DISCUSSION

Incorporating antimicrobial additives into the packaging systems is one of the promising approaches that emerged as a viable technology for the upcoming improvement of food quality and preservation during the processing and storage of various food materials. However, it should be noted that the technology used for the fabrication of antimicrobial films plays an important role. Melt compounding, coating processes, multilayer film production, surface engineering, immobilization, and encapsulation methods all perform differently in terms of migration, inactivation, degradation, and evaporation, and thus the antimicrobial performance of developed active films can vary greatly, even when the same active additives and polymeric material are used. [22].

In this thesis, first, a detailed study on the several different methods for incorporation of active substances into the polymer systems was carried out. Among the main methods used for the incorporation of active additives into the wide range of polymer matrices, melt compounding is the most commonly used technique in the plastic packaging industry. However, the application of this technique in this project may result in major challenges such as inactivation, evaporation, and fast release of active compounds. In this dissertation, we explored the thermal fabrication of the antimicrobial packaging structures, which could provide numerous advantages to improve safety and quality of food products. Thus, it was necessary to create strategies to incorporate active additives into the PE film at the needed levels for effective antimicrobial performance.

Zinc oxide nanoparticles (ZnONPs) have high thermal stability and do not lose their antimicrobial activity during the melt processing of polymer films such as LLDPE. However, the effectivity of antimicrobial action of embedded ZnONPs varies considerably with the process conditions used for the localization of nanoparticles inside the film. The Antimicrobial performance of ZnONPs vary depending on the ZnONPs be inside the film, directly under the film surface, at the interface, or even on top of the film surface.

As a result, the overall research hypothesis for this section is that particles put on top of the film will cause the film to have higher antimicrobial capabilities than particles totally embedded inside the film. A detailed analysis of the effect of thickness of LLDPE skin layer film embedded with ZnONPs made by coextrusion and dip-coating processes on antibacterial activity was conducted to substantiate this idea.

According to the findings, increasing the amount of the ZnONPs close to the surface would increase the number of exposed ZnONPs, thereby increasing their antimicrobial efficacy due to the higher interaction with the microbial cell wall, creation of reactive oxygen species (ROS), and release of Zn^{2+} ions. ZnONPs localization closed to the surface can improve antimicrobial properties. However, this system has some drawbacks on film properties such as limited mode of action, a decrease in both heat sealability and stability of embedded nanoparticles.

Incorporated ZnONPs may inhibit the growth of pathogenic and spoilage microorganisms by a surface action or by diffusion and dissolution onto the food surface result in migration. Hence, this system works only against the microbial organisms on the food surface contacting it not food headspace. Increasing ZnONPs content on the film surface possibly leading to the loss of heat sealability. Moreover, migrated ZnONPs into the food may be alter the organoleptic properties of food products or be ingested. Hence, a comprehensive study on the cytotoxicity and safety of ZnONPs are necessary in future works.

Furthermore, sudden leaching out of ZnONPs into the food surface is related to the stability and position of particles inside the film that should be taken into account. Therefore, ZnONPs must be fully embedded inside the film to avoid rapid migration.

Briefly, despite the good performance of ZnONPs, the major constraint in the use of this antimicrobial agent is the strike balance between antimicrobial activity, film quality, and safety aspects due to nanoparticle migration to the food. Therefore, to fulfill the regulatory requirements, this antimicrobial film with a suitable and controllable antimicrobial effectivity can hit the road toward commercialization.

Further modes of action can be used in designing antimicrobial packaging systems depending on spoilage characteristics, needed shelf life, regulatory aspects, and consumer demand. Unlike antimicrobial packaging containing ZnONPs, limonene essential oil (LEO) as a natural antimicrobial component was used for the production of a noncontact antimicrobial packaging system. In this system, the LEO can be evaporated from the active film to the headspace where it is absorbed onto the food surface. As a result, this noncontact system was employed to safeguard the packaged food as well as the headspace surrounding the food product. The major challenge in the second stage of this work was maintaining the effective concentration of the LEO in the films because it was directly related to the antimicrobial properties of the films. Although LEO is a

volatile natural active compound with potential practical applications in food packaging, it was processed by melting compounding. For this reason, to minimize the loss of volatile and heat-sensitive limonene during thermal processing, several mineral carriers were applied to protect LEO from degradation and to reduce the plasticizing effect of the free LEO in the LDPE matrix. The mineral carriers were capable to retain LEO at different levels depending on physical and chemical adsorption or synergistic interaction with them. All carrier-limonene hybrids were added into LDPE films using melt compounding and thermal, mechanical, optical, barrier, and antibacterial properties were reported.

The antimicrobial capabilities of a released-based active packaging are determined by the transfer of volatile molecules via phase equilibrium thermodynamics and diffusion kinetics. Thus, the diffusivity of limonene from the developed films was measured. The results indicated that both films containing HNTs and MSNs carriers retained significantly higher limonene content than other films that were ascribed to the reduction of limonene desorption rate from these carrier's surfaces.

All fresh active films exhibited strong antimicrobial properties towards *E. coli*. Furthermore, the antimicrobial tests were performed within 8 weeks to evaluate the effect of storage time on the antimicrobial performance of the developed films. The findings confirmed that both HNTs and MSNs have an antimicrobial synergistic interaction with limonene, which was in line with the findings of the short-term release study of the films tested. The active films containing HNTs and MSNs carriers succeeded to preserve their antimicrobial efficacy due to the sustained release profile of limonene. However, industrial application of this system might be restricted due to three main reasons: 1) high production cost due to high evaporation rate of limonene during thermal processing; 2) keeping the film activity for a longer storage time; and 3) formation of off-flavors.

Various elements must be considered in the design of antimicrobial film packaging based on the LEO in order for industrial applications to be feasible. One of these is the method of film production in which LEO incorporation should be processed by thermal compounding and the to avoid activity loss or depletion during film storage, the antimicrobial activity should be frozen until the product is packaged.

In addition, essential oils may alter the organoleptic property of food items due to interaction with certain food components. This process not only depends on the properties of active additives like

functional groups, volatility, and polarity but also relies on the chemical and physical properties of the components in the food matrix. Hence, the investigation for high-barrier packaging matrices is essential to preserve the organoleptic property of food items.

In addition, limonene can be dissolved, adsorbed, bound, and entrapped by certain food components. The relative importance of each of these mechanisms varies depending on the properties of the essential oils such as functional groups, volatility, and polarity, and the chemical and physical properties of the food matrix. It can alter the packaged food product by flavor absorption and can also affect the food by releasing off-flavors. [90]. As a result, finding high-barrier packing materials is critical for preserving food's organoleptic properties.

Briefly, although limonene might have a potential application in release systems, high volatility, safety concerns like low flash point, and odor are the main obstacles for its industrial applications. Thermal processing may cause the evaporation of this additive, leaving a profound impact on the developed antimicrobial films. Hence, it is still needed to establish a more robust antimicrobial packaging with few regulatory and operational obstacles for industrial application.

The selection of an appropriate active substance and its mode of action is essential when developing antimicrobial packaging for industrial applications. LAE offers several advantages over ZnONPs and LEO for incorporation in packaging films because of the low effects on human tissues, and high impact on microorganisms. In humans, LAE can easily be metabolized to natural components like lauric acid and arginine [236]. Moreover, the LAE has the potential of being added to the foodstuff or being incorporated into the polymer matrices for developing a migratory antimicrobial packaging. For this reason, the main advantage of using LAE lies in the production of antimicrobial films with few regulatory obstacles that are of special interest to the industry. Hence, in the third stage of this dissertation, evaluating the antimicrobial effectiveness of LLDPE films incorporated with LAE in its commercial form as LAM was investigated. In this case, LLDPE films were supplemented with different concentrations of LAM via melt extrusion. The films with higher concentrations of active compounds (14 and 16.5%) showed interesting antibacterial properties against *E. coli*.

Considering the compatibility of LAE with polymer matrix will control the amount and rate of LAE released from the film surface, the ability of LAE migration to the film surface was investigated as a function of storage time. The obtained results indicate the LAE migration toward

the surface of the films. Furthermore, the LAE capacity to make plastics virucidal was addressed. The film with the 14% LAM has proven to exert antiviral activity against *Human Coronavirus (HCoV-229E)* after 1 and 2 hours contact time.

Despite the advantages of using LAE for developing antimicrobial films, other aspects must be taken into account. First, the incorporation of LAE into polymer matrices leads to antimicrobial films with food contact surfaces characteristics. For this reason, LAE could be used in combination with other volatile additives to protect both food surface and food headspace at the same time. Finally, the food matrix in particular foods with a high level of fat can reduce the performance of films incorporated with LAE due to the poor solubility of LAE in the oil. As a result, tests on real food must be carried out to see how temperature, pH, water content, the presence of dietary components, and other competitor microorganisms affect the antimicrobial effectiveness of the produced films.

In the end, it is worth mentioning that a variety of laboratory methods can be used to evaluate the in vitro antimicrobial performance of packaging films. The most known and basic methods that test for surface antimicrobial activity and efficacy are the disk-diffusion and ISO 22196 methods, but the "desirable" or "acceptable" required level of antimicrobial performance has not been reported in these methods. However, based on the Environmental Protection Agency (EPA), a minimum 3 log reduction of test microbes within 1-2 hours contact time is the required level of performance [237].

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

This thesis focused on the production of different antimicrobial PE-based packaging systems with potential practical applications in food packaging using three classes of antimicrobial substances, including ZnONPs, Limonene, and LAE via melt compounding.

In the first part of this thesis, in order to study the effect of LLDPE thickness on the antimicrobial activity of embedded ZnONPs, several films with varying LLDPE/ZnONPs skin layer thicknesses (1.5-12 μm) were produced using the coextrusion technique. To better understand the effect of required LLDPE thickness to achieve a desired antimicrobial activity in the films, dip-coated ZnONPs in different LLDPE solution concentrations and thicknesses closed to 2 nm were also prepared. All developed films showed antimicrobial activity against both *S. aureus* and *E. coli*. SEM was used to study the dispersion degree of ZnONPs in the cross-section and on the surface of multilayer films as well as to measure the skin layer thicknesses. The formation of a homogeneous thin layer on the surface of the ZnONPs produced by the dip-coating technique was also confirmed by TEM. The antimicrobial activity of embedded ZnONPs was slightly improved by reducing the thickness of LLDPE in both production methods, according to the study results. Sandblasting treatment was done to the surface of multilayer films using fine sandpaper and the antimicrobial performances of treated films were studied regarding the new position of ZnONPs in the active films. According to the results, due to an increased number of exposed ZnONPs, the Sandblasting treatment significantly increased the antimicrobial activity of films against both *S. aureus* and *E. coli* bacteria. Finally, the optical properties of the coextruded multilayer films indicated that the haze value increased due to the presence of ZnONPs.

In the second part of this thesis, in order to minimize the loss of volatile limonene during thermal processing and storage, different mineral carriers were employed and the potential synergistic effects of them with limonene in LDPE films were investigated. First, vacuum pulling treatment was used to maximize the incorporation of limonene into the mineral carriers. All carrier-limonene hybrids were incorporated into LDPE films using melt compounding and thermal, mechanical, optical, barrier, and antimicrobial properties were investigated. Thermal analysis showed that the films containing HNTs and MSNs exhibited higher limonene retention during compounding. All developed films exhibited inferior mechanical and oxygen barrier properties in comparison to the neat LDPE film. However, these films showed superior mechanical properties, compared to the

film containing limonene without a carrier. These behaviors were related to the presence of free limonene in the LDPE matrix acting as a plasticizer. The short-term release study was used to calculate the diffusivity of limonene in the LDPE matrices using isothermal gravimetric analysis. The results exhibited a lower limonene desorption rate from the surface of HNTs and MSNs carriers in the LDPE films. All of the produced films had a strong antimicrobial activity against *E. coli*. The antimicrobial activity of films was evaluated as a function of storage time in order to determine more sustained release systems. These results indicated that HNTs and MSNs carriers due to their strong synergistic interactions with limonene permitted modulating the release rate of active substance and the its potential use for antimicrobial active packaging.

The final part of this thesis was addressed the development of antibacterial and antiviral LLDPE-based films containing different concentrations of LAE in its commercial form as LAM (10% LAE, 90% maltodextrin) using melt extrusion. The morphology of powder and developed films was investigated by SEM. From SEM micrographs, the irregular shape particles of LAM powder were observed embedded in the matrix in the dispersed state. All films were tested for their thermal stability. The results presented that the thermal stability of the active films has diminished with increasing concentrations of the active agent. The presence of LAM, on the other hand, has no significant effect on the thermal degradation patterns of active LLDPE films. The films with higher concentrations of LAM (14 and 16.5%) revealed significantly reduced *E. coli* bacteria counts 7.5 log (CFU/mL) lower compared to reference samples. However, after 10 days in water, these films lost their antimicrobial performance. This means that all active compounds were washed out from the film surface, while the FTIR-transmission technique confirmed the presence of LAM in the bulk of washed films. Finally, the antibacterial activity of films was recovered after 5 months of storage at room temperature as a result of active additive migrating to the film's surface. Furthermore, the film with the LAM has proven to exert antiviral activity against *Human Coronavirus (HCoV-229E)* after 1 and 2 hours contact time.

The following recommendations are proposed based on the obtained results, for future works:

1. Investigate the influence of ZnONPs concentration on the LLDPE film's mechanical, thermal, barrier, and seal properties.
2. Examine the impact of LAM concentration on the LDPE film's mechanical, thermal, barrier, and seal properties.

3. Evaluate the migration of antimicrobial substances into the real food such as meat, chicken or fish and food simulants, and its effect on the food composition.
4. Investigate the cytotoxicity and biocompatibility of active substances in human cells in the event that they are consumed.
5. Study the effect of mineral carriers for LAE for production of antimicrobial film packaging using melt extrusion
6. Study the degradation, reaction products and any identified reaction by-products of active additives in the food or food simulants
7. Test the antimicrobial performance of developed films towards other pathogenic microorganisms including *Thyphimurium*, or *L. monocytogenes*
8. Study the effect of odor/flavor transfer by essential oils to food
9. Evaluation the application of metal–organic frameworks (MOFs) as second-generation antimicrobial agents.

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