Preparation and Characterization of Biodegradable Film Embedding Antimicrobial Emulsion for Food Packaging

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September 2021

Preparation and Characterization of Biodegradable Film Embedding Antimicrobial Emulsion for Food Packaging

A Dissertation Submitted

To

The Graduate School of Integrative and Global Majors (SIGMA)

University of Tsukuba

In Partial Fulfilment of the Requirements

for The Degree of Doctor of Philosophy in Food Innovation

(Doctoral Program in Tsukuba Life Science Innovation)

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Abstract

Foodborne outbreaks are a serious problem that occurred worldwide, either in developed or developing countries. The consumption of food contaminated with foodborne pathogens can be a massive risk to consumers and public health. World Health Organization (WHO, 2015) reported that foodborne illness is responsible for more than 2 million annual mortality globally, among them 600,000 children in Asia, while in Africa 91 million are affected. Every 10 people, 1 healthy life lost because of food poisoning diseases. Furthermore, foodborne outbreaks cause a dramatic economic loss for the food industry and economies. Therefore, producing safe food with high quality becomes an urgent need to protect consumers' health and avoid the enormous economic losses that happened mainly in the food industry and reflected in the national economy.

Chitosan-based film incorporated with natural active antimicrobial agents would be an innovative solution to build smart antimicrobial food packaging. Essential oils are considered the perfect candidate to be incorporated with food film packaging materials. However, they have some limitations that restrict their food industry applications, such as instability, high reactivity, odor, and low solubility. For that reason, encapsulation would be an optimum way to overcome all these challenges.

Thyme essential oil was formulated in nanoemulsions form and checked for droplet size, distribution, and physical stability. Thyme essential oil nanoemulsions TH-NE was incorporated with chitosan-based films, which induced remarkable changes in the film properties. TH-NE effects on the morphological character included yellowish film color with less transparency, a higher light barrier, and more antioxidant property. In addition, TH-NE has a positive impact on reducing water solubility and enhancing the wettability of the film (contact angle). At the same

time, there was a reduction in the water vapor barrier property, which may slightly limit the investigated films to be used in high water content food products. Meanwhile, TH-NE gave the film more flexibility to be easily used in food wrapping applications. Furthermore, the thermal stability was improved by adding TH-NE with a higher degradation temperature.

TH-NE remarkably improved the antimicrobial activity of chitosan-based films against foodborne pathogens (*Bacillus subtilis* and *E.coli* spp.). Thus, chitosan-based film incorporated with thyme oil nanoemulsions would be a promising antimicrobial food packaging material with considerable packaging properties and substantial growth inhibitors of foodborne pathogens.

Understanding the releasing mechanism of thyme essential oil nanoemulsions from the packaging materials to the food is critical to control their concentrations on the food surface and predict the food shelf-life. Thyme essential oil was analyzed to identify the main bioactive components which are thymol. Thymol was chosen to be the target compound that would be tracked during the study. Thymol release was investigated using physical fat food simulant (in vitro release test) under different temperatures, humidity condition, and different chitosan concentrations. Both time-kill and in vitro challenge microbiological tests were performed to evaluate the antimicrobial activity.

It was found that storage food simulant containing the investigated films at a higher temperature of 40 ℃ resulted in a higher concentration of the released thymol. In contrast, higher humidity 88% caused lower released thymol concentration. The results showed a slower, gradual release of thymol, revealing that both temperature and humidity conditions could be used to control the releasing at a certain point. The results suggested that keeping the films at higher temperatures and low humidity would accelerate the release of the thymol from the film matrix to the food surface.

The investigated films showed remarkable antimicrobial activity against *E. coli* and *Bacillus*

subtilis spp. at the first 4-6th hours after contacting the bacterial culture. In addition, they showed the lowest viable bacterial cells count at the 6-8th h. At the same time, the investigated films showed remarkable antimicrobial activity against *Bacillus subtilis* and *E.coli* spp. when it kept under challenging conditions for 14 days.

Chitosan-based film incorporated with thyme essential oils nanoemulsions was evaluated for its the controlled release of thymol from the film matrix to the real food system by using animal butter product. The investigated films showed a gradual control release of thymol from the film material to the food product. Around 99% of the initial thymol concentration was released to the butter surface by the end of the 4th storage day and followed by a 48% reduction of the released thymol. In addition, the investigated films had a considerable low swelling %, approving the film suitability for high-fat content food application.

Meanwhile, chitosan-based films showed remarkable bacteriostatic antimicrobial activity by reducing the total *bacterial* count of the fresh food products (fresh salmon fish, ground, and chicken meat) under the standards limit. Chitosan-based film could extend the shelf life of fresh food products kept at refrigerator temperature. Furthermore, it could minimize the bacterial risk during the first 24 h for food products kept at 25 ℃.

Chitosan-based films incorporated with thyme essential oil nanoemulsions would be a promising control release antimicrobial food packaging materials and highly recommended for food applications.

CHAPTER 1 - GENERAL INTRODUCTION

General Introduction

Foodborne diseases are a serious issue all over the world. It presents a health risk for all the people, either they are in developed or developing countries. Consumption of unsafe food was estimated to cause 600 million food poisoning cases yearly, resulting in more than 400,000 deaths. According to the World health organization reports, each year, 33 million healthy human being lose their life due to eating contaminated food. In general, 1 in every 10 people got food poisoning disease due to eating unsafe food (WHO, 2015).

1.1.The burden of foodborne diseases

Foodborne diseases are not a massive burden to public health only, but it also dramatically affects the economies. According to the world bank records (Jaffee et al., 2018), foodborne diseases caused a loss in total productivity, mainly in low and middle-income countries estimated to 95.2 billion US dollars yearly. While the cost of the medical service treats foodborne illness is evaluated at 15 billion US dollars.

Based on the economic analysis and studying the effect of foodborne diseases on the food industry, it has been found that it is more economical for the food industry to invest in preventive measures than treating the food poisoning consequences (Ribera et al., 2012). Moreover, Foodborne diseases may cause unexpected expenses to the food industry through recalls and disposal, decrease sales and profits (Hussain & Dawson, 2013).

1.2. Relation between food packaging and food safety

Food packaging has a significant relationship with food safety. Good food packaging has many roles that help improving food quality and serve the food industry. The importance of food packaging can be seen in retaining the food desired quality throughout extending the shelf

life (Robertson, 2014). Although the main roles of food packaging are protecting the food products from physical damage and contamination, they have many other essential functions related to food safety and consumer's health. Food Packaging retards the food product deterioration and spoilage, improves the food quality, and protects food products. This protection comes in three different ways; chemically, physically, and biologically (Marsh & Bugusu, 2007).

Chemical protection throughout reducing the chemical changes triggered by environmental factors such as oxygen, moisture, or light. Physical protection against any mechanical damage such as crushing and abrasions damage. While biological protection comes in providing a barrier to the pathogenic and spoilage microorganisms, thereby preventing foodborne disease and food spoilage (Marsh & Bugusu, 2007).

1.3. Natural food packaging

Plastic films are the most common food packaging materials in the market. They are used extensively due to their inherent plastic physical and mechanical properties. However, this kind of packaging material has caused terrific damage to the environment. On the other hand, natural biodegradable food packaging materials give a promising solution to preserve the environment. They are biodegradable, biocompatible, non-toxic, easily disposable, and eco-friendly (Huang et al., 2019).

Natural food packaging has made from natural polymers or biopolymers. The exploitation of natural polymers and their blends for the green synthesis of polymer-based packaging material provides a sustainable and innovative alternative to plastic materials. It is because of the numerous advantages of these polymers, including cost-effective materials, flexible processability, biodegradability, and ecofriendly (Aliotta et al., 2019). Developing natural food packaging using biobased or biodegradable materials and their composites, essential oils, plant

extracts, and nanomaterials brings down the harmful effect of plastic packaging on the environment (J. W. Han et al., 2018).

1.4. Biobased natural polymers

They are natural materials that have been directly extracted from some plants or derived from animal biomass. The common examples for plant origin materials are starch, zein, pectin, gluten, cellulose, alginate, and carrageenan. While the animal origin materials could be chitin, casein, and gelatin. These polymers can be subjected to further modification to produce valuable biobased materials. A typical example is chitosan, which resulted from a chemical or enzymatic reaction of chitin. Both plant and animal origin materials may show some degree of biodegradability. Many studies have been done to improve the characteristics of biobased polymers materials to obtain high packaging performance. These studies included modifying the natural polymers through either chemical or enzymatic processes, such as starch acetate, cellulose nitrate, hydroxymethyl starch, or hydroxymethyl cellulose (Bajpai, 2019). Some examples of biobased natural polymers used for food packaging are mentioned in Table 1.1.

In general, biopolymers could be classified into three categories based on their chemical structure: polysaccharide, protein, or oil-based polymers. This chemical structure plays a vital role in the physical and chemical characters of the food packaging materials (Sothornvit & Krochta, 2000).

1.4.1. Polysaccharide-based films for food packaging

Polysaccharides have been reported as a raw material for film preparation that can be as food packaging material aiming at extending food shelf life (Cazón et al., 2017). They can form food packaging films with excellent physical and structural properties. However, they have hydrophilic nature with a poor water vapor barrier property (Falguera et al., 2011). The water barrier property of the packaging film is critical to avoid moisture absorption through retarding

the dehydration of the fresh food products and prevent losing the crispness of the dry products (Cazón et al., 2017). At the same time, polysaccharide-based films have been known for their effective gas barrier property such as O2 and Co2, allowing to control the fruits ripening and reducing food oxidation process (Vieira et al., 2011).

Despite the hydrophilic nature of polysaccharide-based films, there are some polysaccharides such as carrageenan and alginate that are highly hygroscopic. This property gives them the advantage to be applied as thick films or coating on the food product surface to absorb water and prevent moisture loss (Huber & Embuscado, 2009). Moreover, polysaccharides are primarily neutral and, in exceptional cases having a positive charge, such as chitosan. The hydroxyl group and the hydrogen bonds in chitosan play the most crucial role in the film formation and the physical and chemical properties of the film (J. H. Han, 2013).

The polysaccharides-based packaging materials are characterized by low cost, abundancy, availability, owing to functional properties. Furthermore, they can be incorporated with a wide variety of bioactive compounds, increasing their effectiveness to extend the food products' shelf life and improve the food quality (Cazón et al., 2017).

1.4.2. Protein-based films for food packaging

Proteins are one of the most widely used materials for food packaging. They are extracted from many different sources, including animals and plants by-products such as milk, grains, or oilseeds. Protein can form food packaging films due to its composition complexity and structure. They owe intermolecular binding capacity to interact with other molecules to form more substantial and better films (Calva-Estrada et al., 2019). Their ability to create food packaging films significantly depends on the molecular structure (Koshy et al., 2015).

Table 1. 1 Biobased natural polymers for food packaging (Association, 2015; Crevel, 2016 and Bajpai, 2019).

Polymers	Source	Biodegradability	Current Application	Market Information
Starch	Starch crops, such as potatoes, sweet potatoes, rice, cassava, wheat, corn.	Biodegradable	Films, bags, wrap films, trays, coffee machine capsules	In 2014, it presented 10 % of the global production capacities of bioplastics.
Cellulose	Fiber crops, such as cotton, jute, kenaf, industrial hemp, sun hemp, and flax	Biodegradable	Flexible films, films for different food products	Have not been yet on the commercial scale as food packaging materials
Chitosan	Crustaceans, such as crabs, lobsters, shrimp, and pawns.	Biodegradable	Health care, pharmaceuticals, and water treatment applications	Have not yet on the commercial scale as food packaging materials
Polylactic acid (PLA)	Sugar crops, such as sugar can, sugar beet, bagasse, and cassava	Non-biodegradable	Containers, films, bags, jars, and barriers	In 2014, it presented 12 % of the global production capacities of bioplastics.
Biobased polyethylene	Sugar crops, such as sugar can, sugar beet, bagasse, and cassava	Non-biodegradable	Water bottles, trays, films, containers	In 2014, it presented 35.4 % of the global production capacities of bioplastics.
Polyhydroxybutyrate	Sugar crops and oils and fats from oil crops such as soybean, sunflower, and olive oils	Biodegradable	Biobased additives, films, coatings, and trays	In 2014, it presented 2 % of the global production capacities of bioplastics.

There is a great interest in recycling the agro-industrial by-products through their utilization to form protein-based food packaging films (Oymaci & Altinkaya, 2016). There are many examples of protein-based films that were previously studied. For instance, gelatin-based films; the gelatin were extracted from meat, fish, and poultry by-products of industrial waste. The films showed excellent gas and aroma barrier properties with a moderate mechanical property (Liu et al., 2017). While in the case of casein films, casein was obtained from milk, it produced water-insoluble films with opaque optical property (Khwaldia et al., 2010). However, to prepare protein-based films with acceptable physical, chemical, and mechanical properties, crosslinking with other chemical compounds is highly recommended.

1.4.3. Lipid-based films for food packaging

Lipids are natural substances, hydrophobic with interesting water resistance properties. Therefore, they are a great solution to reduce moisture losses in food products. Furthermore, they have low surface energy, with soft solids shape at room temperature. According to previous studies, it is better to combine lipids and protein or polysaccharides to form food packaging materials with enhanced functions. The efficiency of the lipid-based films highly depends on the lipid's nature, such as chemical structure, hydrophobicity, the physical state, either solid or liquid, and how the lipids interact with other film's components (J. H. Han, 2013)

Waxes and fats-based food packaging films are characterized by their significant moisture barrier property. However, they were found to have a lousy gas barrier property causing undesirable physiological changes because of anaerobic respiration (Lee & Wan, 2005). Therefore, it is better to incorporate lipid with other polysaccharides materials to obtain successful food packaging films. Currently, in the food market, hydrocolloid-lipid coatings were practically applied to many food products such as cereals, dried fruits, fresh-cut fruits, and vegetables, aiming to reduce rancidity, respiration, flavor, and moisture loss (Pérez-Gago

& Krochta, 2005). Furthermore, incorporating lipid as natural bioactive compounds such as essential oil to polysaccharide or protein-based film matrix gives more functional properties to the films, including desirable antimicrobial and antioxidant activities (Pérez-Gago & Krochta, 2005).

1.5. Biobased food packaging film materials preparation techniques

In fact, the technique used for the film preparation depends on the material chemical structure. Giving an example, polysaccharide-based films are prepared by solvent removal. Simply, the hydrocolloid is dispersed into a solvent, such as dispersion of chiton into the acetic acid solution. Following by adding the plasticizer to give some flexibility to the films. Then, the prepared filmogenic mixture is cast on a flat surface and kept in a drying oven to remove the solvent forming a film (Durango et al., 2006). While in the case of protein-based films, it is prepared either through solvent removal, pH modification, protein thermal denaturation, or gelatinization of the starch, followed by casting the filmogenic mixture (Khwaldia et al., 2004). Also, the extrusion technique could be a way for film preparation. It is a high-temperature short-time process used to produce many other food products. However, the effect of the extrusion technique on the biological activities and chemical structure of the film-forming materials has not been studied yet.

Table 1. 2 Examples of polysaccharide and protein-based antimicrobial films.

Kind of the film	Film Polymer	Used Antimicrobial Agent	References
Polysaccharide-based films	Alginate apple puree	Oregano and cinnamon essential oils	(Rojas-Graü et al., 2007)
	Chitosan	Potassium sorbate	(Yoshida et al., 2010) (Sangsuwan et al., 2015)
	Corn starch	Potassium sorbate	(López et al., 2013)
	Fish Gelatin	Cinnamon essential oil	(Wu et al., 2017)
	Carrageenan	Potassium sorbate	(Choi et al., 2005)
	Corn starch	Nisin	(Meira et al., 2017)
	Fish gelatin	Citric acid	(Uranga et al., 2019)
	Carrageenan	Grape seed extract	(Kanmani & Rhim, 2014)
Protein-based films	Corn zein	lysozyme	(Mecitoğlu et al., 2006)
	Corn zein	Nisin	(Teerakarn et al., 2002)
	Whey protein	Potassium Sorbate	(Ozdemir & Floros, 2001)
	Corn zein	Sorbic acid	(Carlin et al., 2001)
	Soy protein isolate	Nisin	(Ko et al., 2001)
	Whey protein	Oregano and garlic essential oils	(Seydim & Sarikus, 2006)

1.6. Active natural food packaging.

Incorporating bioactive compounds into the natural polymeric film matrix enhances the film's properties. It adds more functions such as antioxidant and antimicrobial activities. Antimicrobial food packaging films are prepared by incorporating active antimicrobial agents into the film matrix. The idea behind those kinds of packaging is the ability of these

antimicrobial agents to control release from the film to the food surface. The controlled release highly depends on the nature of the antimicrobial agents and their interactions with both packaging and food matrix (Irkin & Esmer, 2015).

Antimicrobial food packaging enhances food product quality by extending shelf life, inhibiting microbial growth, and improving sensory qualities (Malhotra et al., 2015). The controlled release of antimicrobial agents from the food packaging materials to the food surface has an impact on reducing the food spoilage microorganisms growth, and consequently improve food safety and ensure the hygienic quality (Irkin & Esmer, 2015). Active antimicrobial food packaging is currently attracting both consumers' and scientists' attention due to the great desire for high-quality and safe food products based on the application of natural antimicrobial materials.

1.7. Control release antimicrobial food packaging

Control released packaging is known as CRP. It is a new innovative form of food packaging materials. The main concept of CRP is incorporating the food packaging film matrix with other active compounds such as antimicrobial or antioxidants agents. In this kind of packaging, there is a great possibility of the active control release of these bioactive compounds from the food packaging materials to the food surface. This allows the food packaging to be more efficient in protecting the food products against deterioration. As shown in Fig. 1.1. the bioactive compounds such as antimicrobial agents are immerged into the film matrix. Then, they are released gradually to the food surface at a specific releasing rate to improve both food safety and quality (Lacoste et al., 2005).

The significance of control release active packaging mainly regulates the released concentration of the bioactive compounds from food packaging materials to the food surface. Based on that principle, the released concentration should be found in an adequate amount that

effective to inhibit bacterial growth and prevent food deterioration over a certain amount of time. Hence, it is also known as time-release packaging due to its ability to control release rate at a certain time (Malhotra et al., 2015).

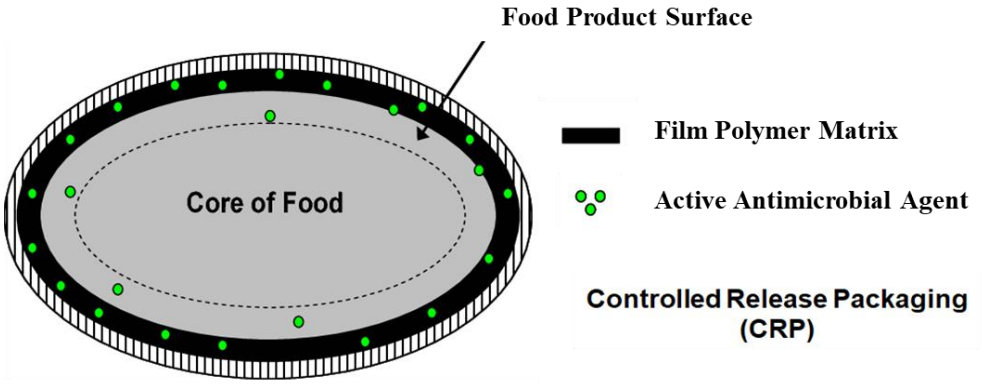


Figure 1. 1 Control release of active food packaging

The concept of release control has already been used in the pharmaceutical market, such as antibiotics, vitamins, and other medical prescription drugs. Although many studies have been done on the controlled release, it is still entirely understandable, especially for the food packaging field. It is believed that determining the accurate parameters affecting the controlled release and building a mathematical model would give a huge impact on understanding the control release mechanism. It would also help predict the released concentration and be accurate about the food product shelf life wrapped with the target food packaging materials (Mastromatteo et al., 2010).

1.8. Research outline

- Chapter 1 is a general introduction and recent review literature.
- Chapter 2 is developing chitosan-based film as a natural polymer incorporated with a natural antimicrobial agent (thyme essential oil nanoemulsions), aiming to enhance the antimicrobial activity. As well as studying the effect of the essential oil nanoemulsions on the film's properties including morphological, physical, mechanical, molecular, and microbiological.
- Chapter 3 is studying the release of the antimicrobial agent (thyme essential oil nanoemulsions) from chitosan-based film using physical food stimulant, as well as exploring the antimicrobial kinetic release.
- Chapter 4 is the food application of chitosan-based films with thyme essential oils nanoemulsions.
- Chapter 5 is a general conclusion with and summarizing the obtained results from the research.

CHAPTER 2 - Chitosan-Based Film Incorporated with Essential Oil Nanoemulsions Foreseeing Enhanced Antimicrobial Effect

2. Chitosan-Based Film Incorporated with Essential Oil Nanoemulsions Foreseeing Enhanced Antimicrobial Effect

2.1. Abstract

Foodborne diseases are a serious problem that occurred worldwide. It is known for causing huge economic losses, as well as representing a threaten for the consumers' lives. Chitosan-based film incorporated with natural active antimicrobial agents is an innovative solution as an active antimicrobial food packaging. Essential oils are considered the perfect candidate to be incorporated with food film packaging materials. However, they have some limitations that restrict their food industry applications, such as instability, high reactivity, odor, and low solubility. For that reason, encapsulation would be an optimum way to overcome all these challenges. Thyme oil is one of the promising powerful antimicrobial essential oil. It was formulated in nanoemulsions form and investigated for their droplet size, and thermal stability. Thyme essential oil nanoemulsions were added into the chitosan-filmogenic mixture through and stirred till complete homogenization. The whole filmogenic mixture was cast in petri dishes and dried in an oven at 50-55 ℃ for 24h. The dried prepared films were investigated for their morphological, physio-chemical, and mechanical properties. As well as evaluating the antimicrobial activity against both gram-negative (*E. coli* spp.) and gram-positive (*Bacillus subtilis* spp.) bacteria. Thyme essential oil nanoemulsions exhibited a small droplet size (89-90 nm) which showed a considerable stability when stored at different temperature conditions. Incorporating thyme oil nanoemulsions with the chitosan-based film has made remarkable changes to the film morphological, physicochemical and mechanical properties. It is also

enhanced the antimicrobial activity of chitosan-based films against food poisoning microorganisms. Chitosan-based film incorporated with thyme oil nanoemulsions is considered a promising innovative antimicrobial food packaging material with valuable packaging properties and remarkable growth inhibitor of foodborne pathogens.

This chapter published as:

Samar Elshamy, Kubra Khadizatul, Kunihiko Uemura, Mitsutoshi Nakajima , and Marcos A. Neves. Chitosan-based film incorporated with essential oil nanoemulsion foreseeing enhanced antimicrobial effect. J Food Sci Technol (2021).

2.2. Introduction

Food poisoning disease is a serious issue for all people all over the world. Currently, there is a great awareness about the importance of consuming safe food to avoid all the diseases and malnutrition problems that resulted from contaminated food with bacteria, viruses, parasites, or even chemical residues.

Food spoilage and decomposition happen mainly because of the growth and multiplication of microorganisms. Although reducing the water activity and increasing the protection against moisture may help to prevent the food spoilage, still there is a high chance for the microbial growth and food deterioration because of temperature fluctuations during storage (Torres et al., 1985). Therefore, there is a great need to use antimicrobial food packaging to control the microbial growth, prevent food spoilage, ensure food hygiene and safety.

Many chemical antimicrobial agents have already been used in the food industry and can be loaded into films such as benzoic acid, propionic acid, potassium sorbate, sodium benzoate, and sorbic acid (Cha & Chinnan, 2004). However, there is a huge need for using natural food-grade ingredients because of the microbial resistance against antimicrobials and antibiotics. Microbial resistance happened mainly because the massive and random use of synthetic antibiotics resulted in the development of resistance against them (Kraemer et al., 2019). For example, *E. coli* spp. is one of these microbes that was able in developing resistance against a wide spectrum of antimicrobials, antibiotics, and medicinal drugs (Chouhan et al., 2017). Therefore, it is a must to find alternative natural antimicrobial agents to be applied in the food system aiming to protect the consumer's health and ensure hygienic food quality.

Essential oils are considered strong alternatives to the chemical elements and antibiotics, especially in the food production (Burt, 2004). They are characterized by owning many distinctive properties such as; powerful natural antibacterial, antiviral, antifungal, insecticide,

antiparasitic (Chouhan et al., 2017), and antioxidant through free radical-scavenging properties (de Sousa Barros et al., 2015). These properties make essential oils an excellent candidate to be used in food applications like the hygienic quality of food due to their potentiality as an antimicrobial agent (Safaei-Ghomi & Ahd, 2010). As well as preventing the deterioration of the foods organoleptic by possessing anti-free radical properties (Hale et al., 2008). Moreover, they are capable to be combined with polysaccharide-based films such as rosemary, garlic, and cinnamon essential oils (Rojas-Graü et al., 2007).

Considering all the distinctive characteristics of essential oils, still, many limitations make their applications in the food industry quite challenging. Essential oils are volatile, thermo-labile, and unstable. They are easily subjected to natural changes in their components and compositions (Turek & Stintzing, 2013). Essential oils are reactive substances through interactions with other food components, and their antimicrobial activity may be go through impairing due to changes in either pH or temperature (Rattanachaikunsopon & Phumkhachorn, 2010). Essential oils are characterized by owing aroma, which may affect the organoleptic characters of the food products that affect the consumer's acceptability (Lv et al., 2011).

From this point, we consider the encapsulation of essential oils in nanoemulsions form is a great way to eliminate all these limitations. Especially those related to using a high dose of essential oils, aroma, volatility, stability, and solubility in water.

Therefore, in this research, the authors propose chitosan-based film incorporated with thyme essential oil nanoemulsions TH-NE. The research is aiming to produce an active antimicrobial food packaging material. Chitosan has been chosen to be the film polymer because of its significant characteristics, making it a promising solution to be used as a food packaging material. Chitosan is widely known for being a distinct, abundant polysaccharide. It is edible, biodegradable, non-toxic, non-allergic material, as well as biocompatible (Ban et al.,

2014). Moreover, chitosan is widely recognized for being a distinct food preservative agent because of its antimicrobial effect against food pathogenic microorganisms (Lekjing, 2016). Besides, it possesses valuable film-forming properties (Domard & Domard, 2002) and produces transparent stretchable films (Kanatt et al., 2008). Furthermore, Chitosan owns a positive charge, making it to interact with other negatively charged particles such as fat and lipid molecules (Youn et al., 2009). This makes chitosan the ideal polymer to be incorporated with essential oils.

Through this research, our aim is developing a chitosan-based film loaded with thyme essential oil nanoemulsions as a smart antimicrobial packaging against foodborne microbes. Thyme essential oil, with its significant antimicrobial activity (Rota et al., 2008) was encapsulated in a nanoemulsions form then loaded to the chitosan-based filmogenic mixture. The prepared dried chitosan-based films were examined to investigate the effect of thyme essential oil nanoemulsions TH-NE on the chitosan-based film properties. The film's assessments included morphological, physio-chemical, and mechanical properties. Moreover, the antimicrobial activity against both gram-negative (*E. coli* spp.) and gram-positive (*Bacillus subtilis* spp.) bacteria.

2.3. Materials and methods

Chitosan (medium molecular weight 500 KDa with 75 to 85 % deacetylation) and thyme oil (*Thymus vulgaris* spp.) were purchased from Sigma-Aldrich (USA). Food-grade acetic acid, Polyoxymethylene sorbitan monooleate (Tween 80), and phosphate pH standard equimolar solution (pH 6.68 at 25 ºC) were provided by Wako Pure Chemical Industries Ltd., (Tokyo, Japan). Glycerol (film plasticizer) was bought from MP Biomedical, Inc. (France). Modified lecithin SLP-White H was supplied by Tsuji Oil Co., Ltd, (Japan).

2.3.1. Preparation of thyme essential oil nanoemulsions TH-NE

Thyme essential oil (*Thymus Vulgaris* spp.) was encapsulated into nanoemulsions form through the following steps: continuous phase contained phosphate pH standard equimolar solution at pH 6.68, natural stabilizer modified lecithin SLP (0.1 wt%), and a non-ionic surfactant Tween 80 (2.0 wt%). While dispersed phase contained soybean oil (1.0 wt%) and thyme essential oil (1.0 wt%). Both of two phases were homogenized together using a rotor-stator homogenizer (Polytron PT-3100, Kinematic, Switzerland) at 7000 rpm for 5 minutes, followed by a high-pressure homogenizer (NanoVater NV200, Yoshida Kikai, Japan) for 3 cycles at 100 MPa.

2.3.2. Measurement of thyme essential oil nanoemulsions TH-NE droplet size.

The droplet size and distribution of thyme essential oil nanoemulsions were measured using a laser diffraction particle size analyzer (L13320, Beckman Coulter, Brea, USA). The average droplet size was evaluated by recording the Sauter mean diameter (d3,2).

2.3.3. Thyme essential oil nanoemulsions TH-NE thermal stability.

Nanoemulsions were investigated for their physical stability. TH-NE were divided into three different sealed groups. All the samples' groups stored under different temperature

conditions (5, 25, and 40 °C). The droplet size was evaluated daily for 7 successive using a laser diffraction particle size analyzer (L13320, Beckman Coulter, Brea, USA).

2.3.4. Preparation of chitosan-based film loaded with TH-NE.

Chitosan powder 1.5 wt% chitosan powder was dispersed in a previously heated 1.0 wt% acetic acid solution (pH 5.3 - 5.6) at 50 - 55 ºC, followed by stirring for 30 minutes with rising the temperature till reaching 70 - 75 ºC to ensure the dissolving and form the chitosan filmogenic mixture. 2.0 wt. % Glycerol (film plasticizer) and TH-NE with different concentrations (1, 2, 3, or 4 wt%) were added to filmogenic mixture. The filmogenic mixture was stirred till complete homogenization. After preparing the chitosan-based filmogenic mixtures had loaded with TH-NE, the filmogenic mixtures were cast into Petri dishes (φ 90 x 15 mm). Both water and solvent were removed by drying in the oven at 50 - 55 ºC for 24 h. All the prepared dried chitosan-based films were kept at 25 ± 5 % RH and 25 ºC. All the films were assessed for their morphological, physio-chemical, mechanical, and antimicrobial properties against *Bacillus subtilis* and *Escherichia coli* spp.

2.3.5. Morphological characterization of chitosan-based film incorporated with TH-NE.

2.3.5.1.Transmittance T% and transparency A600 / mm

A UV/VIS spectrophotometer at 600 nm (V-530, JASCO Corporation, Japan) was used to measure both transmittance T% and transparency A600 / mm, according to the procedures mentioned by Hosseini et al. [19]. A piece of the chitosan-based film was cut into a rectangular shape with (3 × 1 cm) and put into the spectrophotometer test cell, and an empty test cell was used as the blank value.

Transmittance T% was assessed by using equation 1.1.

$$T\% = 10\,(2 - A) \tag{1.1}$$

A is the absorbance at 600 nm.

Transparency was assessed through the following formula equation 1.2.

$$\text{Opacity} = \frac{A_{600}}{mm} \quad (1.2)$$

A600 is the absorbance at 600 nm, while mm is the film thickness in the mm unit.

All measurements were calculated and evaluated in triplicate at six different locations in the films.

2.3.5.2. Color profile

The color profile of the films was assessed with a reflecting colorimeter (CR400, Konica Minolta Sensing, Inc.). A standard white and black plate was used to calibrate the calorimeter. A piece of the film with (30 mm × 30 mm) size was placed into the calorimeter to be measured. The total color difference (ΔE) was evaluated with the following equation 1.3.

$$\Delta E = \sqrt{(L^* - L^\circ)^2 + (a^* - a^\circ)^2 + (b^* - b^\circ)^2} \quad (1.3)$$

L* (black 0 to light 100), a* (red 120 to green -120) and b* (yellow 120 to blue -120) values as L*, a*, b* are the color coordinates of the film. While the standards values are $L_\circ$= 90.97, $a_\circ$ = 0.08 and $b_\circ$ = -0.28 (Acevedo-Fani et al., 2015).

2.3.5.3. Microstructure examination.

A piece of the film was sticked to a circular metal cylinder stub (φ 15 × 5 mm) b using double-sided adhesive tape. Then it was dried entirely at 40 ºC overnight. The film specimen was coated with platinum using Ion sputter device (Hitachi E-1045). Later it was examined for its microstructure with a scanning electron microscope SEM with an acceleration voltage 5 kV (JSM6330F Field Emission SEM, JEOL).

2.3.6. Physicochemical and mechanical characterization.

2.3.6.1. Viscosity and pH measurement

Both viscosity and pH of the prepared chitosan-based filmogenic mixtures loaded with TH-NE were measured with a viscometer (Vibrio Viscometer SV-10) and (pH meter). All measurements were calculated in triplicate. Three different measurements for each filmogenic mixture were done, and then calculating the mean values.

2.3.6.2. Film thickness

The thickness of the previously prepared chitosan-based films loaded with TH-NE was assessed with a digital caliper with an accuracy ±1 μm (Gigimatic Caliper, Mitutoyo Corporation., Japan). Five different thickness measurements were taken randomly, and then calculating the mean values.

2.3.6.3. Surface density

A piece of the chitosan-based film with size (3 × 3 cm) was cut and weighed with a balance scale to the nearest 1 mg and divided by the sample's area equal to 9 cm^2. The surface density (ρs) was evaluated through to the following equation 1.4.

$$\rho_s = \frac{m}{(A \times \delta)} \quad (1.4)$$

A is the film surface area (9 cm^2), δ is the film thickness (cm), while m is the dry mass (g). Surface density was written as ρs is the dry matter density (g cm^{-3}) (Kumari et al., 2017).

2.3.6.4. Moisture content

A piece of the chitosan-based film with size (3 × 3 cm) was cut and weighed before and after drying in the oven at 105 °C for 24 h. The moisture content was assessed through to the following equation 1.5.

$$\text{Moisture Content } \% = \frac{M_0 - M_F}{M_F} \times 100 \quad (1.5)$$

Where M_0 is the initial weight of the chitosan-based film (mg), and M_f is the final wight of the film (mg). Moisture content was written as mg of water per mg of dry solids [22].

2.3.6.5. Water solubility

The water solubility was evaluated using the previously mentioned method (Sánchez-González et al., 2010) with a little modification. A piece of the chitosan-based film was weighted into 500 mg and immersed into 50 mL of water. The specimen was placed into a shaking incubator at 25 ℃ for 24 h. Later, the wet film was placed into an oven 105 ℃ for 24 h for drying. The water solubility of chitosan-based film solubility was evaluated through the following equation 1.6.

$$\text{Water Solubility \%} = \frac{\text{Wt. of initial dry film - Wt. of undissolved film}}{\text{Wt. of the initial dry film}} \times 100 \quad (1.6)$$

2.3.6.6. Water vapor permeability

A piece of the chitosan-based film was placed and sealed on an aluminum test cell containing 15 mL of distilled water. The aluminum test cell was put into a desiccator containing previously dehydrated silica gel. (the dehydration of silica gel was done by placing it inside a drying oven at 105 ℃ for 3 - 5 h). The desiccator was kept at the room temperature for 24 h. The test cell was weighed before and after being placed inside the desiccator. The weight loss was calculated through the following equation 1.7.

$$\text{WVTR} = \frac{\Delta W}{(\Delta t \times A)} \quad (1.7)$$

WVTR is the water vapor permeability value. ΔW is the test cell's weight loss, Δt = storage time (24h), and A= the exposed area of the chitosan-based film (Singh et al., 2015).

2.3.6.7. Contact angle.

The contact angle was assessed with a contact angle analyzer (Kyowa Interface Science Co., Ltd, Japan). A piece of the prepared film with size (3 × 5 cm) was put on the silver steel sample stage. A drop of 0.1 µl water was added on the surface of the chitosan-based film sample using a micro-syringe. The contact angle (θ) of the water droplet was assessed at room

temperature with relative humidity at 25 ± 5% (Rhim et al., 2006). All measurements were done in 10 replications.

2.3.6.8. Tensile measurement.

For measuring the film's tensile, a piece from of the prepared film with size (30 mm wide × 50 mm long) was cut, mounted, and clamped with the texture analyzer probe (EZ-SE Texture Analyzer, Shimadzu, Japan). Followed by stretching the film sample at a speed of 0.1 mm / s at room temperature until the break. and The tensile strength and elongation at break EB were calculated using stress-strain and force-distance curves, respectively (Zúñiga et al., 2012). All measurements were done in triplicate.

2.3.6.9. Thermogravimetric thermal analysis

Differential scanning calorimeter DSC 60 Plus (Shimadzu, Japan) was used to perform the thermogravimetric thermal analysis. Chitosan-based film samples were heated under a nitrogen atmosphere from 40 °C to 500 °C at a heating rate of 10 °C / min in unsealed DSC aluminum pans. Also, the film samples were cooled from room temperature to -20 °C at a cooling rate of -3 °C / min. DSC measurements were done in triplicate.

2.3.6.10. Fourier-transform infrared FTIR spectroscopy

FTIR spectra of the chitosan-based films were assessed using FT/IR-300 spectrometer (JASCO Co., Ltd. Hachioji, Japan). The wavelength of FTIR spectra was estimated to be ranged from 400 cm^{-1} to 4000 cm^{-1} under transmission mode with a resolution of 4 cm^{-1} with 32 scans per minute.

2.3.7. Antimicrobial activity of chitosan-based film loaded with TH-NE.

2.3.7.1. Bacterial cultures

The standard strains *Bacillus subtilis* spp. (2015/014) and *E. coli* spp. (JCM.1649) served as test pathogens for the antibacterial activity tests. They were obtained from "Food

Technology Unit, Food Technology Unit, Food Research Institute NARO, Japan." All the test pathogens were kept in a glycerol at –18 ℃. regarding *E. coli* spp., the sub-culturing was performed using a nutrient broth for 24 h before the experiment.

2.3.7.2. Agar well diffusion test

Following the standards procedures by (Clinical and Laboratory Standards Institute, 2015), the pathogen test either *Bacillus subtilis* spp. (2015/014) or *E. coli* spp. (JCM. 1649) were serial diluted to obtain concentrations of 10^4 CFU/mL. 100 µL of the prepared bacterial dilution was spread on a standard plate count agar with a sterile swab. An 8 mm diameter well was punctured into the agar medium using a sterilized tip. The wells were filled with 25 µL of chitosan-based filmogenic mixtures incorporated with TH-NE at different concentrations (0, 1, 2, 3, or 4 wt%). The control well was filled with 25 µL sterilized distilled water. All the petri dishes were incubated at 37 ℃ for 24 hours. The antimicrobial activity was assessed by measuring the inhibition zone diameter, including the diameter of the well 8 mm. A digital caliper with an accuracy ± 1 µm (Gigimatic Caliper, Mitutoyo Corporation., Japan) was used to do all the measurements. All the samples were done in triplicate.

2.3.7.3. Agar dilution test

According to the standards procedures by (Clinical and Laboratory Standards Institute, 2015), 1 mL of *Bacillus subtilis* or *E. coli* spp. with concentrations of 10^4 CFU/mL was inoculated into sterilized petri dishes (φ 90 x15 mm). 1 mL of chitosan-based filmogenic mixtures incorporated with TH-NE with varying concentrations (0, 1, 2, 3 or 4 wt%) was mixed with 25 mL of the standard plate count agar and poured inside the petri dishes. After the solidification of the agar, all the inoculated petri dishes are incubated at 37 ºC for 24 hours. After that all the grown bacterial colonies were counted. All the samples were done in triplicate.

2.3.7.4. Application to fresh raw beef meat

A fresh raw beef meat was bought from a local supermarket (Hana Masa) located in Tsukuba city, Japan, and transported to the laboratory (National Food Research Institute NARO, Tsukuba, Japan). The beef meat was investigated for any contamination with *E. coli* spp. by swabbing it, then inoculated into a selective agar media (Deoxycholate Agar) and incubated at 37 °C for 24 h. After the confirmation of *E. coli* spp. detection negativity in the meat and under sterile conditions, the meat portion was cut and weighted into small pieces 5 g approximately. All the meat samples were swabbed with *E. coli* spp. 1×10^4 (JCM.1649). Followed by dividing the samples into two groups; the first group was the unwrapped samples representing the control group, while the second group was the wrapped samples with chitosan-based film loaded with TH-NE with different concentrations (1, 2, 3, and 4 wt%). All the samples were placed into small polypropylene bags and stored at 4 °C for successive 7 days.

2.3.8. Statistical Analysis.

Statistical analysis was done to determine the significant differences of all chitosan-based film samples at a 95% confidence interval and alpha equal to 0.05 using Statistix 8.1 program.

2.4. Results and Discussion

2.4.1. Thyme essential oil nanoemulsions TH-NE droplets size distribution and stability

Nanoemulsions are identified as an emulsion with a small droplet size ranged between 5-200 nm. They are known by being kinetically stable, transparent, and owning tunable rheology (Gupta et al., 2016).

The results showed in Fig.1.a. illustrated the droplet size distribution of thyme essential oil nanoemulsions TH-NE. Thyme essential oil was successfully formulated in the form of nanoemulsions with 89-92 nm as an average droplet size. The obtained average droplet size of TH-NE was smaller than those results previously reported by (Xue & Zhong, 2014). The mentioned authors had formulated thyme oil in a nanoemulsions form, co-emulsified with sodium caseinate NaCas and lecithin resulting in an average droplet size of more than 100 nm. The formulation of nanoemulsions is owing to two main factors through using Tween 80 as a non-ionic surfactant as well as high energy preparation method.

Non-ionic surfactant (Tween 80) played a vital role in processing nanoemulsions with smaller droplet sizes. Non-ionic surfactant is hydrophilic with a polar head and without electric charge. Moreover, it owns three oligo side groups binding to polysorbate sugar which increases the hydrophilicity of the head group, besides having a hydrophobic tail. This chemical structure gives tween 80 a substantial solubility in polar solvents such as methanol, acetic acid and water (Rapp, 2017). Moreover, tween 80 as a non-ionic surfactant has the ability to reduce aggregation by hydration, thermal changes interactions, and steric hindrances (Aswathanarayan & Vittal, 2019). At the same time, the high-energy method (high-pressure homogenizer) worked on breaking up the oil droplets causing their dispersion into the continuous phase (Odriozola-serrano et al., 2014).

Fig.1.b. illustrated the exquisite physical stability of the formulated TH-NE when it was

stored different temperature conditions (5, 25, and 40 °C) for consecutive 7 days. They were checked for their physical stability by measuring the droplet size daily along the storage period. In spite of the difference in storage temperature, TH-NE showed relative stability. The results can be explained due to using modified lecithin SPL as a natural stabilizer and co-emulsifier. Modified lecithin is one of the phospholipid emulsifiers group containing phosphatidylcholine. Phosphatidylcholine is recognized for its an substantial emulsions stabilization effect (Pathania et al., 2018). The application of both small molecular surface surfactant (tween 80) and phospholipid (modified lecithin) worked on producing nanoemulsions with small droplet size and enhanced the stability. In general, the type of emulsifier plays an essential role in the average droplet size and stability of nanoemulsions, especially when they are stored at a different temperature, pH, and long-term storage. This role was cleared by a reduction of the interfacial tension between the dispersed and continuous phases leading to the formulation of nanoemulsions with smaller droplet sizes (McClements, and Rao, 2011).

2.4.2. Morphological characterization

2.4.2.1.Transmittance and transparency

Transmittance % is an indicator of the film light barrier property. Its importance is showing the film's capability to block lipid oxidation produced by Ultraviolet light UV (Leceta et al., 2013). The lower the transmittance % values, the higher the barrier property. Table 2.1. illustrated that the transmittance % was significantly ($P \leq 0.05$) subsided by rising TH-NE concentration. The transmittance % values were dropped from 48 to 23 %, which are lower and superior than chitosan-Zain-based films loaded with anise, orange, and cinnamon (72, 71 and 69.3%), respectively (Escamilla-García et al., 2017). The obtained results suggessted that combining TH-NE with chitosan-based film boosted both the ultraviolet barrier and antioxidant properties.

Translucent food packaging is a critical parameter to entice the consumer's recognition. The lower transparency, the higher the film opacity. In Table 2.1., the results revealed a slight increase in the opacity of the chitosan-based by adding TH-NE to chitosan-based film. The obtained data ranged from 1.50 to 2.35 A600 / mm, which were similar to those of chitosan film loaded with tea polyphenols (Wang et al., 2013). The results were possibly because of the TH-NE particles that are implanted in the film matrix. These particles produced light scattering, producing higher absorption values and higher film's opacity (Siripatrawan & Harte, 2010). Furthermore, the effect of the film's thickness on transparency increases the film opacity by increasing the thickness (Pires et al., 2013).

2.4.2.2. Color profile

The color profile was determined to show the impact of TH-NE on the color profile of the chitosan-based films. As shown in Table 2.1., L* value presents the lightness, a* refers to the redness, and b* value points to the yellowness of the film. Merging TH-NE to chitosan-based films caused significant ($P \leq 0.05$) decreasing in coordinate L* value, without noteworthy effect between different TH-NE concentration. At the same time, there was reduction in coordinate a* value, giving negative value revealing the green color occurred due to adding TH-NE to the chitosan-based films. While coordinate b* gave positive results, which increased by adding TH-NE to the chitosan-based film, indicating the yellowish color of the films. Thus, the investigated films had light yellowish color with an acceptable level of transparency. The obtained data of total color difference are like those of chitosan-based films loaded with cinnamon essential oil (Ojagh et al., 2010) and alginate-based film loaded with thyme essential oil (Acevedo-Fani et al., 2015).

The visual properties of food packaging films are highly affected by their incorporating of essential oils. This effect highly depends on the type and concentration of adjoined essential

oils (Du et al., 2009). In general, less transparency of the films may have more vantages to the consumers such as providing enhanced light barrier characteristic, superior antioxidants effect, and heavier in their active compounds.

2.4.2.3. Microstructure examination.

Microstructure investigation was critical to prove the implanting of nanoemulsions droplets into the film matrix. Furthermore, elucidate the consistency of the film structure. However, there are many factors that affect the film microstructure, for example, the filmogenic mixture, drying time and temperature, and the concentration of oils inside the filmogenic mixtures (Lorena Atarés & Chiralt, 2016).

The photographs in Fig.2.3. a, b, c, and d. showed the morphological properties of chitosan-based films combined with different concentration of TH-NE. The films lean to have a marginally yellowish color with lessen transparency. The obtained results were coherent with the data in Table 2.1. The films loaded with TH-NE appeared to have a considerably rougher surface with comparing to other films without TH-NE. These results suit those results previously stated by (Sánchez-González et al., 2011). It can be elucidated because of the migration of nanoemulsions particles from the film matrix to the surface (Acevedo-Fani et al., 2015). The films' cross-sections in Fig.2.3. e, f, g, h, and i. were examined by using SEM. The images revealed that oil droplets were implanted into the film matrix. The cross-section images did not illustrate any cracks, fissures, holes, or pores in the film's matrix, indicating the considerable film compatibility. Incorporating TH-NE with chitosan-based films did not have any negative or undesirable effect on the microstructures of the films.

2.4.3. Physicochemical and mechanical characterization

2.4.3.1. Viscosity and pH

The estimation of the filmogenic mixture's pH is a critical procedure to be considered

during the film's processing and determine the antimicrobic activity of the chitosan-based film. The obtained data in Table 2.2. varied between 5.10 ± 0.08 and 5.47 ± 0.18, revealing that TH-NE had non-significant effect ($P > 0.05$) on the pH value of the filmogenic mixtures.

The pH values lower than 6.3 would have an essential role in the solubility of chitosan and enhance its antimicrobial activity (Aider, 2010). By lowering the pH value than 6.3, the electrostatic interaction strikes between the amino group NH3+ as a positive charge and the bacterial surface as a negative charge, resulting in an alteration in the bacterial cellular membrane permeability, osmotic imbalance, and restrain the bacterial growth (Dutta et al., 2009). Oppositely, by increasing the pH values to the alkaline level, it can interact with the chitosan cations and lead to hindering the reactivity of amino groups NH3+, and consequently reducing the antimicrobial activity (Aider, 2010).

Viscosity is an indirect sign of the uniformity and water activity of the chitosan-based film (Fundo et al., 2011), in addition to its significant influence on the stability of nanoemulsions and filmogenic mixture (Acevedo-Fani et al., 2015). The obtained data results in Table 2.2. illustrated that TH-NE has a little significant ($P \leq 0.05$) influence on the viscosity of filmogenic mixture. It rose the viscosity from 236 ± 9.8 to 287 ± 3.36 cP. The obtained values are less than those reported by (Acevedo-Fani et al., 2015) by adding essential oils to sodium alginate filmogenic mixture caused an increase in the viscosity of the whole mixtures to be 452.0, 616.0, and 473.0 cP, upon adding thyme, lemongrass, and sage oils, respectively. Therefore, regarding the obtained results, incorporating chitosan-based film with TH-NE did not trigger a high rise in the viscosity, preserving the film consistency and water activity.

2.4.3.2. Film thickness and surface density

The obtained data in Table 2.2. illustrated the influence of TH-NE on the film's thickness (μm). They revealed that incorporating TH-NE has a significant effect ($P \leq 0.05$) on the

thickness of the films by increasing from 113 ± 1.27 to 197 ± 1.07 μm. The results can be explained as implanting TH-NE into the film matrix caused large molecular contact, flagging the polymer chain, decreasing the chain aggregation forces, and accordingly untying the matrix and producing higher film thickness (Escamilla-García et al., 2017).

The importance of surface density as a parameter is being a sign of several critical film properties, such as mechanical properties, corrosion resistance, and refractive index (Mattox, 2010). Therefore, high surface density hardness is a favorable property for having high-quality films for food packaging.

The results in Table 2.2. revealed that inserting TH-NE into chitosan-based film reduced the surface density values from 1.24 ± 0.08 to 0.62 ± 0.08 $g.cm^{-3}$. The results indicate that adding TH-NE caused less surface density and the smoother surface of the chitosan-based films. The reason possibly due to the TH-NE ability to downgrade the formation of Z agglomerates, producing a film with a smoother surface (L. Atarés et al., 2010). However, several factors were stated to show remarkable effects on the films' surface density, such as the drying temperature, preparation approach, and viscosity and water amount inside the filmogenic mixture. Increasing or decreasing any of them leads to significant changes in both film density and thickness (Fajardo et al., 2010).

2.4.3.3. Moisture content and water solubility

Moisture content % is a decisive parameter used to indicate the water activity of the chitosan-based films, which have influences on both the mechanical and physical characteristics of the films [21]. The obtained data in Table 2.3. disclosed that incorporating TH-NE produced a 36.7 % increase of moisture content %, with slight significant difference values between different concentrations of TH-NE. Adding TH-NE possesses an enormous influence on the hydrophobicity of chitosan-based films and increases the film’s capacity to

hold water. However, monitoring moisture content is vital to create a high-grade chitosan-based film with enhanced barrier properties as a food packaging material (Aguirre-Loredo et al., 2016).

Water solubility becomes a more critical parameter, especially when the films are applied to food products with high water content. It is used as a sign of the film's hydrophobicity or hydrophilicity, water resistance as well as film's integrity (Singh et al., 2015). The showed results in Table 2.3. cleared that there is a decline in the water solubility % values with rising TH-NE concentrations without any statistical significance ($P > 0.05$). The results are possibly due to the aptitude of chitosan as a polymer to steady the film structure with the presence of TH-NE inserted into the film matrix (Gómez-Estaca et al., 2010).

2.4.3.4. Water vapor permeability

The significance of water vapor permeability as a parameter is for being a guide for the film's resistance to transfer moisture. In addition to indicating the ability of the films to decrease moisture transfer between the food and the environment or even between heterogeneous components of the food products (Fakhreddin Hosseini et al., 2013).

The shoed results in Table 2.3. clarified that there is a rising in the water vapor permeability of the films from 0.0019 to 0.22 $g/m^{-2.d}$. The results indicate that TH-NE caused a reduction in the water vapor barrier properties. These data are in contrast with previously reported by (Pires et al., 2013) and (L. Atarés et al., 2010) by incorporating essential oils with hake protein and sodium caseinate films, respectively. Both authors were working on the incorporation of hydrophobic essential oils with a hydrophilic film matrix. However, a combination of different mixtures with different chemical compounds and structures, like in the case of nanoemulsions, may influence the hydrophobicity of the oils. Consequently, alter their ability to enhance the water vapor permeability (Lorena Atarés & Chiralt, 2016).

Furthermore, adding the essential oils may work on increasing the intermolecular interactions of the film structural matrix and triggering the water vapor passing through the film (Pranoto et al., 2005).

2.4.3.5. Contact angle.

The contact angle is one of the wetting properties of the film surface, which should be measured to determine the hydrophilic or hydrophobic film's properties (Sharp et al., 2018). In Table 2.3., the results illustrated that TH-NE marginally improved the film's contact angle giving a contact angle ranged between 83.80 and 85.90 deg with a non-significant difference between various concentrations of TH-NE. The obtained results are much greater than those previously recorded by (Ojagh et al., 2010) through studying the incorporation of chitosan-based films with cinnamon essential oil producing a contact angle of about 70 deg. The obtained data indicate that the application of thyme essential oil into the form of nanoemulsions has the capability to keep the film surface's hydrophobicity and improve the wetting characteristics. The results can be explained due to the hydrophobic nature of the oil included in the nanoemulsions, furthermore the possibility of reducing the number of hydroxyl groups related to the chitosan chemical structure (Noshirvani et al., 2017).

2.4.3.6. Tensile Measurements.

Tensile strength and elongation at break are the mechanical properties of chitosan-based films. Tensile strength is used to indicate the endurance to tension forces, at the same time, elongation at breaks is used to determine the stretching capacity of the film (Acevedo-Fani et al., 2015).

Table 2.4. cleared that increasing TH-NE decreased the film's tensile strength significantly from 0.66 to 2.13 MPa. Oppositely, it increased the elongation at break from 72 to 119 %. The obtained results could be clarified through the capability of TH-NE to minimize inter and

intramolecular interactions and reduce the polymer cohesion network forces causing lower tensile strength and greater film flexibility (Escamilla-García et al., 2017).

However, tensile strength and elongation at break are greatly reliant on several factors for instance; the film's chemical structure (Dufresne & Vignon, 1998), various interactions between polymer networks, kin of oils, and their chemical constituents (Shen & Kamdem, 2015), chitosan natural source, filmogenic mixture's acidity degree, as well as other experimental elements such as pH, relative humidity, and emulsifiers (Sánchez-González et al., 2010).

2.4.3.7. Thermal analysis

Table 2.4. reported the thermal analysis of the investigated films as T_{onset} is the temperature at the degradation starting point. T_{peak} is the maximum degradation temperature, while T_{endset} is the temperature at the degradation process ending point. Degradation temperature refers to the point that all the components of the films are decomposed, including chitosan, the main component of the film (Siracusa et al., 2018). The results revealed that there was no significant effect at both the T_{onset} and T_{endset} temperature. In contrast, rising TH-NE concentrations incorporated with chitosan-based film produced a significant increase in the T_{peak} from 205 to 266 °C, revealing a rise in the thermal stability of the films. The obtained results consent with results previously reported by (Souza et al., 2019) when the author incorporated chitosan-based films with ginger essential oil. The author explained the improvement of the film's thermal stability due to the well-constructed film matrix with a homogenous structure (Noshirvani et al., 2017) and (Souza et al., 2019).

2.4.3.8. Fourier-transform infrared FTIR spectroscopy

The structural alteration of chitosan-based films incorporated with TH-NE was examined using FTIR spectroscopy. The showed results in Fig.2.4. illustrated that there were a little

alteration in the peaks placed between 3300 and 3309 cm^{-1}. This relates to the extending of the O-H band contained in the hydrogen bond (Chentir et al., 2019). On the other hand, there was invisible alteration at the peaks placed at 2925, 2926, and 2927 cm^{-1}, indicating that there was no major influence on the C–H band. At the same time, there was a little shift at 1560 cm^{-1} with inserting TH-NE with various concentrations into the film. This can be possibly due to the vibrations of both N–H bending and C-N expanding bands (Xu et al., 2020). Also, there was no obvious alteration at the peaks that appeared at 1400 and 1030 cm^{-1} which connected to the C-O band and O-H group of glycerol (the film plasticizer) (Chentir et al., 2019). Overall, FTIR spectra investigation results showed that merging the chitosan-based films with TH-NE did not cause major intermolecular interaction or structural changes in the film matrix.

2.4.4. Microbiological Investigation

2.4.4.1. Agar well diffusion test.

The agar well diffusion test is highly known for being the ideal test to investigate the antimicrobial activity of essential oils (Magaldi et al., 2004). The test mechanism depends on releasing the active antimicrobial agents and their diffusion through the agar to work as a microbial growth inhibitor (Balouiri et al., 2016).

The showed data in Fig.2.5. illustrated the antimicrobial activity of chitosan-based filmogenic mixture combined with TH-NE against *Bacillus subtilis* and *E. coli* spp. The antimicrobial activity was evaluated by measuring the formed inhibition zone diameter around the well. The results illustrate that the control sample did not show any antibacterial effect without inhibition zone formation. At the same time, chitosan-based filmogenic mixture without TH-NE exhibited a mild antibacterial impact against *Bacillus subtilis* and *E. coli* spp. by having almost the same inhibition zone diameter of 10.10 and 10.25 mm, respectively. Conversely, filmogenic mixtures loaded with TH-NE revealed a significant antibacterial

activity ($P \leq 0.05$) through making inhibition zone diameters ranged from (11.75 to 14.7 mm) and (16.75 to 17.70 mm) against *Bacillus subtilis* and *E. coli* spp., respectively.

In contrast to the results previously stated by (Ahmad et al., 2012), by incorporating gelatin-based films with bergamot and lemongrass essential oils. As well as the results found by (Pires et al., 2013) during studying the combination of hake protein-based film with citronella, tarragon, thyme, and coriander essential oils. The author's results did not get any antimicrobial activity against *E. coli* spp. Furthermore, we found that chitosan-based filmogenic mixtures with TH-NE had potent antibacterial activity against *E. coli* spp. The result can be possibly due to the encapsulation of thyme essential oil into nanoemulsions form. Nanoemulsions allow the hydrophobic molecules of thyme essential oil to be in greater contact with the bacterial cell membrane, causing cellular rupture, leakage of its components, and lysis (Guo et al., 2020).

2.4.4.2. Agar dilution test.

The antimicrobial activity of chitosan-based filmogenic mixtures loaded with TH-NE using the agar dilution test is shown in Fig.2.6. There was increasing was found in the bacterial count related to the control group by nearly 5.1 and 4.9 log CFU/mL for both *Bacillus subtilis* and *E. coli* spp., respectively. While chitosan-based filmogenic mixture without TH-NE had 2.3 and 1.4 log CFU/mL decline for both *Bacillus subtilis* and *E. coli* spp., respectively, in comparison with the control group. At the same time, the filmogenic mixtures incorporated with TH-NE with their various concentrations (1, 2, 3, or 4 wt%) illustrated an outstanding antimicrobial activity by reducing both *Bacillus subtilis* and *E. coli* spp. counts. In comparison with the control group, the bacterial count reduction was evaluated to be nearly 3.6 and 4.8 log CFU/mL for *Bacillus subtilis* and *E. coli* spp., respectively, without a significant difference ($P > 0.05$) between different concentrations of TH-NE. The obtained results are coherent with our

results stated in the previous section.

2.4.4.3. Application to fresh raw meat

The obtained data in Fig.2.7. showed the transformations in *E. coli* spp. count of fresh raw beef meat samples wrapped with chitosan-based films loaded with TH-NE, kept at 4 ºC for 6 successive days. The control group exhibited the greatest *E. coli* spp. count exceeding 8 log CFU/mL by the 6^{th} day of storage period. The control group was accompanied by a foul smell and alteration in the organoleptic properties, starting from the 4^{th} day of the storage period. Compared to the control group, all the samples wrapped with the investigated films began to exhibit a significant bacterial reduction ($P \leq 0.05$) in the *E. coli* spp. count since the 1^{st} day of storage period. Although there was increasing in *E. coli* count in both control and wrapped groups, there was a significant ($P \leq 0.05$) difference in the count between them. The greatest reduction was noticed at the 5^{th} and 6^{th} which was nearby 2.25 and 2.78 log CFU/mL, respectively, with non-significant difference ($P > 0.05$) between various TH-NE concentrations. All the wrapped samples were in sound organoleptic conditions and without any foul smell or color alteration.

The obtained data confirm the capability of chitosan-based films merged with TH-NE to downgrade the inoculated (*E. coli* spp.) bacterial count over the storage period. Contrary, the unwrapped control samples attained the threshold infective dose reported by the Advisory Committee on the Microbiological Safety of Food (ACMF, 1995). Chitosan-based films incorporated with TH-NE has the ability to release their bioactive compounds to the fresh raw meat surface to inhibit the bacterial multiplication. The results suggest that the chitosan-based films incorporated with TH-NE are valid to expand the expected shelf-life of the fresh raw meat kept at refrigerator conditions (4 ºC) to 6 days instead of 3-5 days (Food & Drug Administration, 2018), through inhibition the growth and multiplication of foodborne

pathogens.

2.5. Conclusion

This study shows that the encapsulation of thyme essential oil into nanoemulsions and its incorporation with chitosan-based films provoke noteworthy modifications in the film characteristics. TH-NE influences on the morphologic character involved yellowish film color with less translucence, greater light barriers, and further antioxidant property. TH-NE possesses a positive influence on declining the water solubility and improving the film's wettability. Contrary, TH-NE has a little negative influence on the mechanical characteristics by reducing the tensile strength and rising the film stretchability. The thermal stability was enhanced by inserting TH-NE with a higher degradation temperature. Incorporating TH-NE with chitosan-based films enhanced the film's antimicrobial activity against *E. coli* and *Bacillus subtilis* spp. The film expanded the shelf-life of fresh raw beef meat inoculated with *E. coli* spp. to reach 6 days. In summary, TH-NE has improved the functional properties of chitosan-based films and is suggested for potential applications in the food industry.

Table 2. 1 Effect of TH-NE concentration on the morphological properties of the chitosan-based films; color profile, Transmittance T%, and Opacity (A600/mm).

Nanoemulsions concentration wt%	L*	a*	b*	ΔE	Transmittance (%)	Opacity (A_{600} / mm)
0.0	37.39 ± 1.18^{a}	-0.19 ± 0.05^{a}	2.55 ± 0.36^{a}	56.85 ± 1.19^{a}	48 ± 0.01^{d}	1.5 ± 0.10^{a}
1.0	30.77 ± 1.43^{b}	-0.21 ± 0.19^{b}	3.64 ± 0.65^{b}	60.30 ± 1.45^{b}	46 ± 0.16^{c}	1.55 ± 0.09^{b}
2.0	30.30 ± 0.89^{b}	-0.28 ± 0.06^{b}	3.89 ± 0.39^{b}	60.73 ± 0.91^{b}	46 ± 1.19^{b}	1.48 ± 0.05^{c}
3.0	30.50 ± 1.55^{b}	-0.29 ± 0.05^{b}	3.23 ± 0.18^{b}	60.57 ± 1.55^{b}	33 ± 0.75^{ab}	1.85 ± 0.08^{c}
4.0	30.87 ± 0.81^{b}	-0.27 ± 0.08^{b}	3.18 ± 0.64^{b}	62.12 ± 0.84^{b}	23 ± 0.27^{a}	2.35 ± 0.06^{c}

Values are given as mean ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

Table 2. 2 Effect of TH-NE concentration on the physical properties of the chitosan-based films.

Nanoemulsions concentration wt%	pH Value	Viscosity (cP)	Thickness (μm)	Surface Density (g/cm^{-3})
0.0	5.10 ± 0.08^{a}	236 ± 9.8^{c}	113 ± 1.27^{a}	1.24 ± 0.08^{a}
1.0	5.42 ± 0.27^{a}	287 ± 3.36^{a}	137 ± 2.04^{b}	0.85 ± 0.12^{b}
2.0	5.47 ± 0.18^{a}	283 ± 7.50^{b}	148 ± 0.13^{b}	0.79 ± 0.06^{b}
3.0	5.44 ± 0.22^{a}	278 ± 4.57^{b}	188 ± 2.18^{c}	0.69 ± 0.04^{b}
4.0	5.41 ± 0.21^{a}	273 ± 5.03^{b}	197 ± 1.07^{c}	0.62 ± 0.08^{b}

Values are given as mean ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

Table 2. 3 Effect of TH-NE concentration on the moisture content, water solubility, water vapor permeability, and contact angle of the chitosan-based films.

Nanoemulsions concentration wt%	Moisture Content %	Water Solubility %	Water Vapor permeability ($g/m^{-2}.d$)	Contact Angle (deg)
0.0	39 ± 0.15^{c}	66.19 ± 0.81^{a}	0.0019 ± 0.0002^{c}	83.95 ± 1.36^{a}
1.0	62 ± 1.04^{b}	62.46 ± 0.25^{a}	0.02 ± 0.005^{b}	85.40 ± 2.50^{a}
2.0	61 ± 0.21^{ab}	62.18 ± 0.71^{a}	0.03 ± 0.013^{b}	85.90 ± 2.20^{a}
3.0	60 ± 0.78^{ab}	60.09 ± 0.46^{a}	0.18 ± 0.004^{a}	85.40 ± 1.20^{a}
4.0	59 ± 1.08^{a}	58.84 ± 0.52^{a}	0.22 ± 0.013^{a}	83.80 ± 2.0^{a}

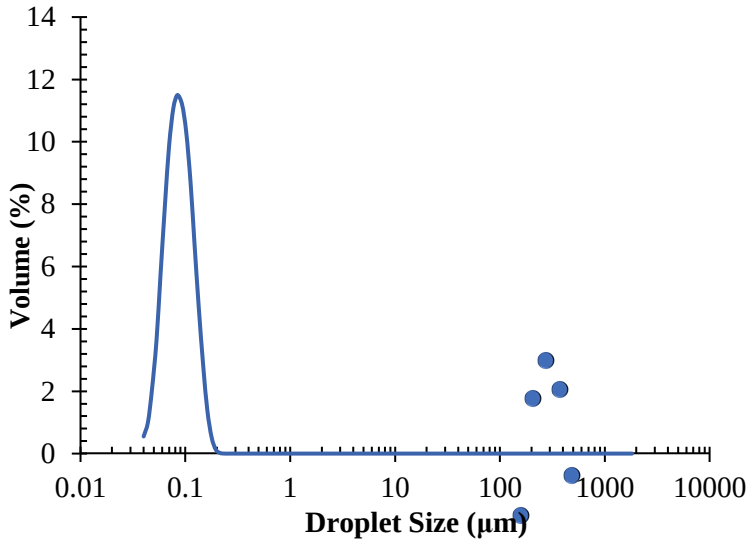
Values are given as mean ± standard deviation. Different letters in the same column indicate significant differences ($p \leq 0.05$).

Table 2. 4 Tensile measurement and thermal analysis of chitosan-based films incorporated with TH-NE.

Nanoemulsions concentration wt%	Tensile Strength (MPa)	Elongation at Break (%)	T_{onset} (°C)	T_{peak} (°C)	T_{endset} (°C)
0	6.66 ± 0.17^{c}	72 ± 3.15^{b}	204.6 ± 2.30^{a}	205.4 ± 1.05^{ab}	220.8 ± 1.45^{a}
1	3.05 ± 0.27^{b}	118 ± 1.46^{a}	216.8 ± 2.21^{a}	228.9 ± 2.43^{ab}	234.4 ± 1.97^{a}
2	2.24 ± 0.15^{a}	118 ± 0.07^{a}	233.8 ± 0.70^{a}	258.2 ± 1.27^{ab}	258.8 ± 1.58^{a}
3	2.15 ± 0.08^{a}	117 ± 3.85^{a}	229.7 ± 1.64^{a}	263.3 ± 1.85^{ab}	257.4 ± 0.46^{a}
4	2.13 ± 0.22^{a}	119 ± 2.22^{a}	232.3 ± 0.91^{a}	266.7 ± 0.66^{a}	257.9 ± 1.67^{a}

Values are given as mean ± standard deviation. Different letters in the same column indicate significant differences ($p \leq 0.05$).

(a)



(b)

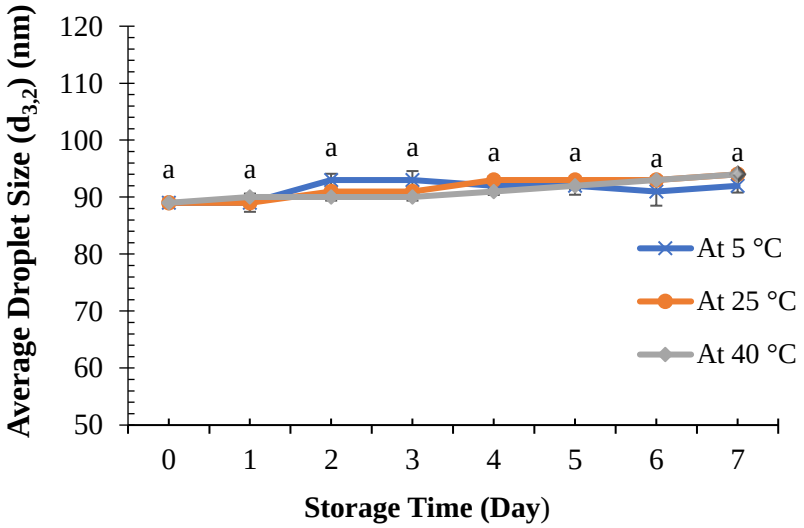
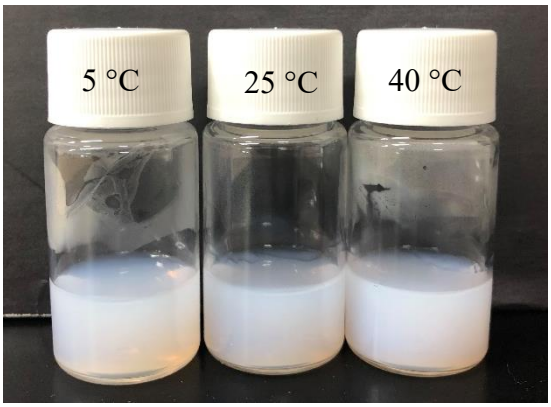


Figure 2. 1 The droplet size distribution and thermal stability of TH-NE through storage for 7 days at different temperatures (5, 25, or 40 ˚C).

(a)



(b)

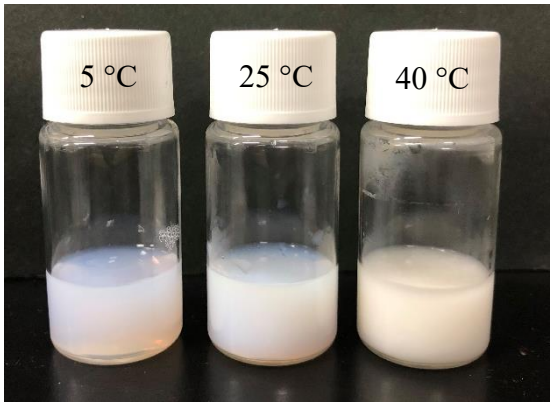


Figure 2. 2 The visual appearance of TH-NE; (a) fresh prepared TH-NE (b) TH-NE after storage for 7 days at different temperature conditions (5, 25, or 40 ˚C).

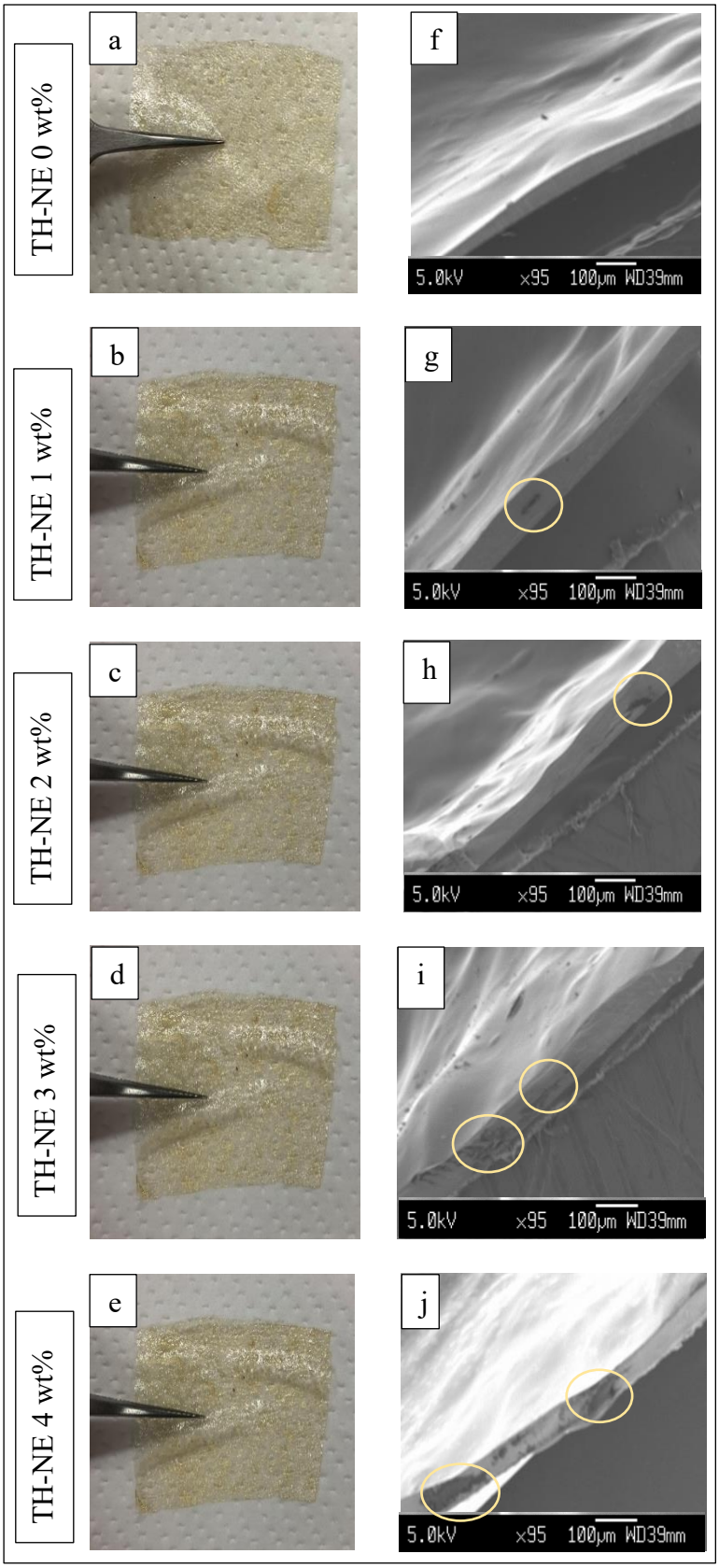


Figure 2. 3 Photographs and cross-sections observed by scanning electron microscope of chitosan films incorporated with different concentrations of TH-NE (0, 1, 2, 3, or 4 wt%).

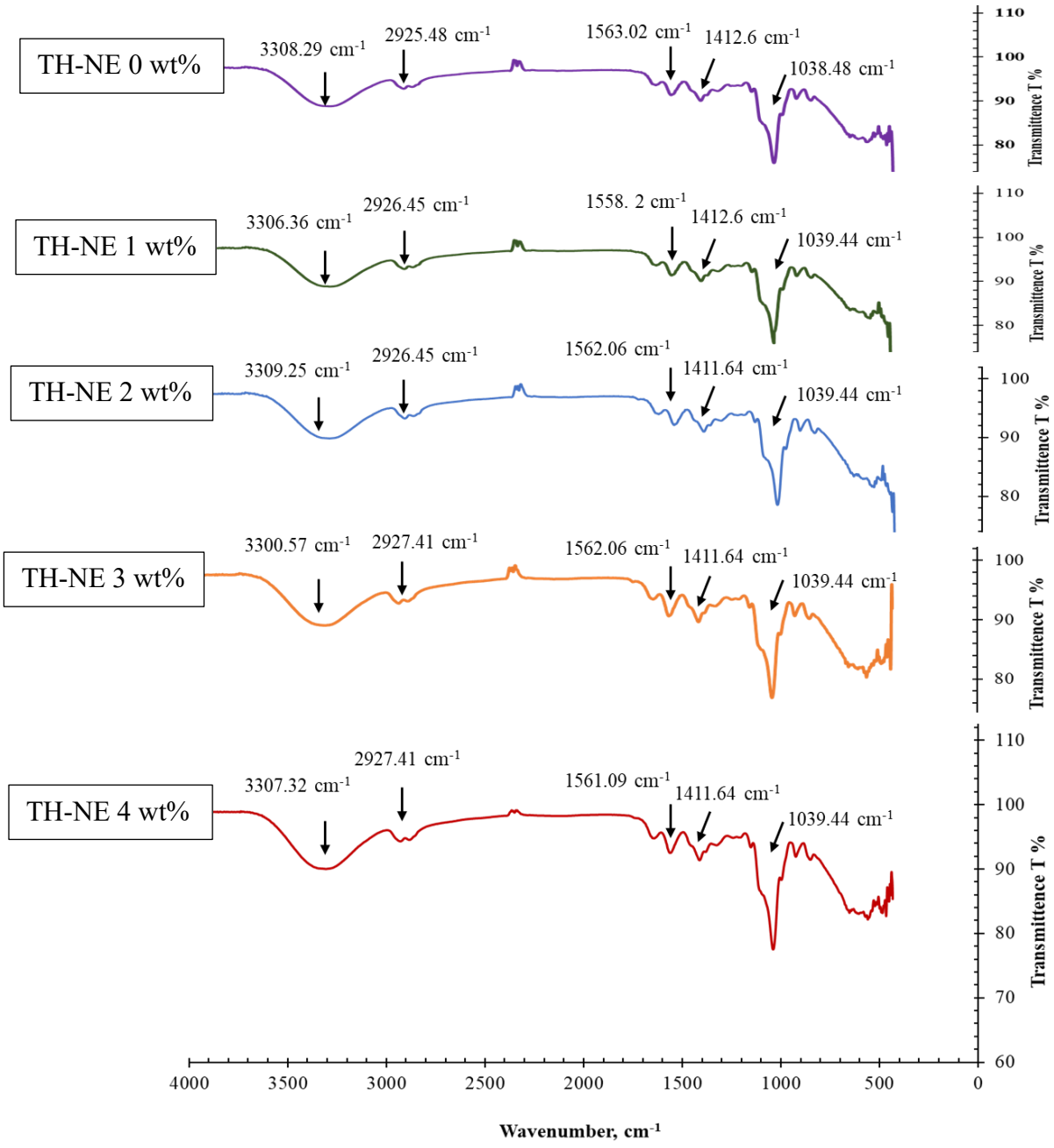


Figure 2. 4 FTIR spectra of chitosan-based films incorporated with different concentrations of TH-NE (0, 1, 2, 3, or 4 wt%).

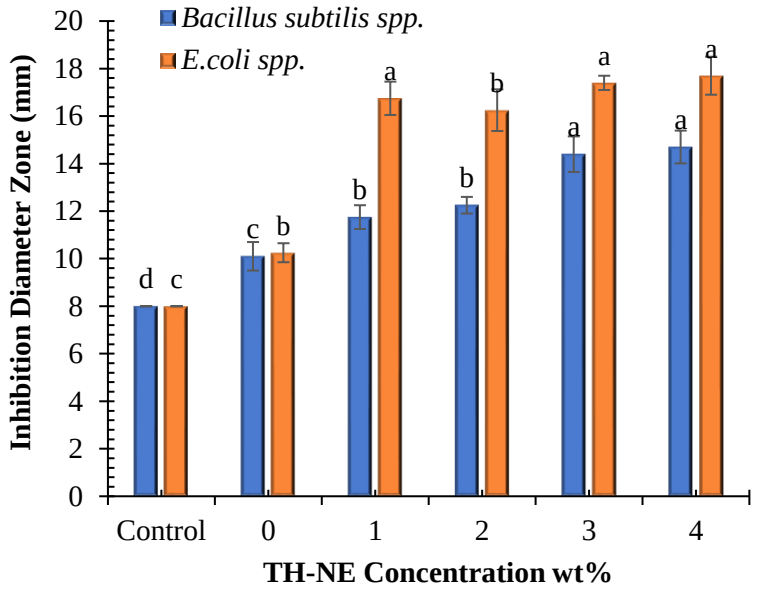


Figure 2. 5 Antimicrobial effect of chitosan-based filmogenic mixture loaded with TH-NE against *Bacillus subtilis* and *E. coli* spp. Agar well diffusion test with well diameter = 8 mm and sterilized distilled water as a control. All the plates were inoculated with 10^4 CFU/mL and incubated at 37 ºC for 24 h.

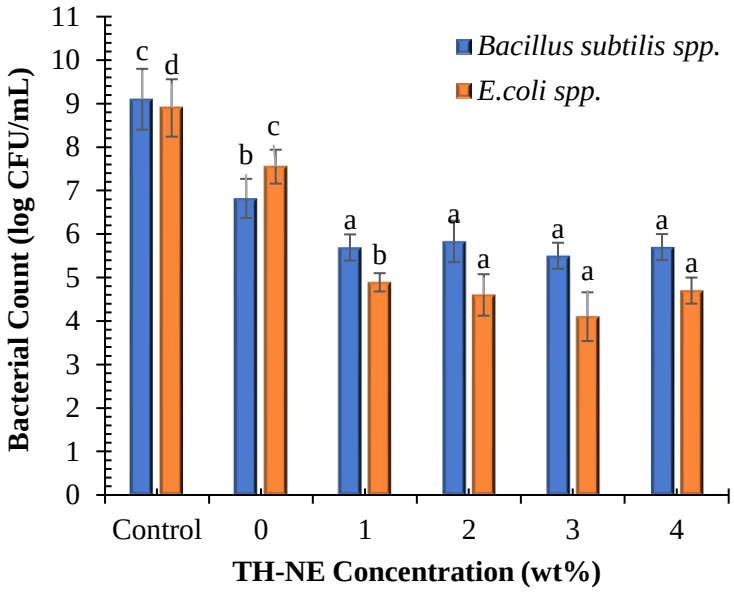


Figure 2. 6 Antimicrobial effect of chitosan-based filmogenic mixture loaded with TH-NE against *Bacillus subtilis* and *E. coli* spp. Agar dilution test, sterilized distilled water as a control. All the plates were inoculated with 10^4 CFU/mL and incubated at 37 ºC for 24 h.

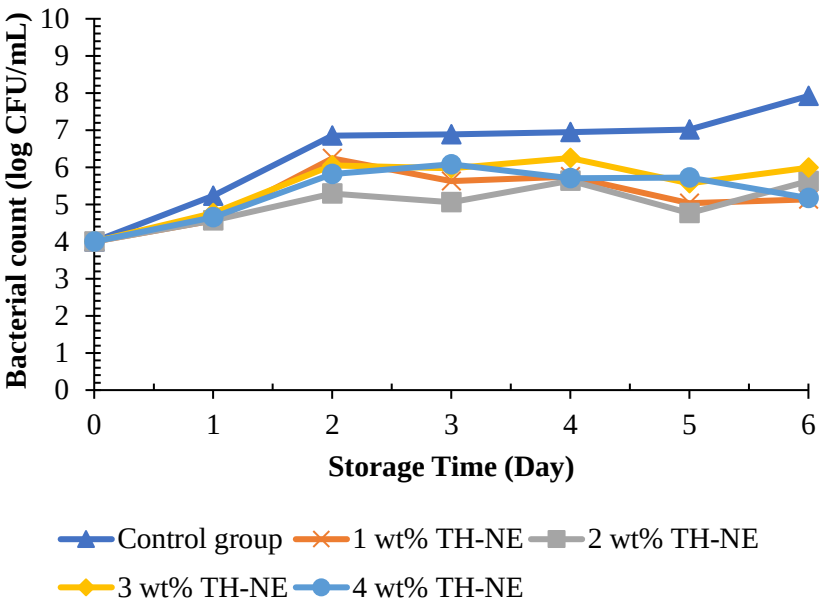


Figure 2. 7 Antimicrobial activity of chitosan-based films incorporated with TH-NE against *E. coli* spp. in fresh raw meat samples. All the meat samples were swabbed with 10^4 CFU/mL and incubated at 4 ºC for successive 6 days.

CHAPTER 3 - Evaluation of Thyme Essential Oil Nanoemulsions Release from Chitosan-based Films and Their Antimicrobial Efficacy

3. Evaluation of Thyme Essential Oil Nanoemulsions Release From Chitosan-based Films and Their Antimicrobial Efficacy

3.1. Abstract

Understanding the releasing mechanism of antimicrobial agents from the packaging materials to the food surface is critical to control their concentrations on the food surface and predict the food shelf-life. Thyme essential oil was identified for its bioactive components using Gas chromatogram-Mass spectrometer. Thyme essential oil bioactive component (thymol) release was investigated using physical food simulant under different storage conditions. Both time-kill and *in-vitro* challenge microbiological tests were performed. The results revealed storage food simulant containing the investigated films at higher temperature 40 ℃, accelerate thymol releasing, while higher humidity 88%, slower the releasing. The investigated films showed remarkable antimicrobial activity against foodborne pathogens (*E. coli* and *Bacillus subtilis* spp.) at the first 8 hours with 2 Log reduction by the end of the experiment. Chitosan-based films integrated with thyme essential oil nanoemulsions could be a potential antimicrobial control released food packaging materials.

This chapter will be submitted as:

Samar Elshamy, Emna Abdennour, Teetach Changwatchai, Kunihiko Uemura, Mitsutoshi Nakajima, Marcos A. Neves. Evaluation of Thyme Essential Oil Nanoemulsions Release from Chitosan-based Films and Their Antimicrobial Efficacy.

3.2. Introduction

Foodborne outbreaks have become a serious concern for all people worldwide. They may dramatically affect consumers' health and safety and affect the food industry with substantial economic losses. They are caused mainly because of food spoilage microorganisms, including bacteria, viruses, and fungi. Active food packaging is considered an innovative way and a key to minimize the risk of pathogenic microorganisms' growth and multiplication. It can enhance food safety, extend shelf-life, and reduce food waste (Malhotra et al., 2015). Moreover, it provides several functions, such as antioxidants, oxygen scavenging, moisture, light, and gas barriers (Quintavalla & Vicini, 2002).

Antimicrobial food packaging material can be either films or coating to be applied directly to the food products. Antimicrobial films are considered the most active and influential food packaging material. It carries antimicrobial agents that interact with the food surface and prevent microbial growth. It can also control these antimicrobial agents' releasing rate and ensure an adequate amount of them onto the food surface (Amidani et al., 2016). They are prepared by incorporating either natural or synthetic antimicrobial agents into the film matrix. Their efficacy highly depends on the concentration of antimicrobial agents incorporated into the film matrix and the amount released over a particular time (Zhong et al., 2020).

Natural antimicrobial films are a promising innovative way to protect consumer health and preserve the environment. They are biodegradable, biocompatible, non-toxic, easily disposable, and eco-friendly (Huang et al., 2019). Several studies discussed natural antimicrobial films and their powerful antimicrobial activities against different kinds of foodborne pathogens. Such as corn starch film loaded with nisin against *Listeria monocytogenes* and *Clostridium perfringens* spp. (Meira et al., 2017), chitosan films loaded with potassium sorbate against mold (Sangsuwan et al., 2015), fish gelatin loaded with citric

acid against *Escherichia coli* (Uranga et al., 2019), carrageenan loaded with grapefruit seed extract against *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus*, and *Listeria monocytogenes* (Kanmani & Rhim, 2014).

Chitosan is a potent polymeric antimicrobial food packaging material. It is natural, biodegradable, biocompatible, and environmentally friendly (Sung et al., 2013). It is an abundant material produced through chitin's deacetylation process (Reesha et al., 2015). It can be combined with other natural antimicrobial agents to give more broad properties. In previous studies, chitosan films were incorporated with rosemary and cinnamon essential oils. Both oils enhanced the antimicrobial activity, showing better results than those of pure chitosan films. Moreover, cinnamon essential oil improved the film's performance by reducing water affinity (Ojagh et al., 2010 and Abdollahi et al., 2012).

Essential oils are well known for their antimicrobial activities. They are considered an excellent alternative to chemical preservatives. They are rich in phenolic and terpenoid compounds that have potential antimicrobial activity against a broad spectrum of foodborne pathogens. These active components inhibit pathogenic microorganisms' growth by disrupting the cytoplasmic membrane causing its rupture and inhibiting protein synthesis. There are many examples of essential oils used in food packaging, such as thyme, carvacrol, clove, limonene, cinnamon, basil, and garlic essential oils (Sung et al., 2013). However, they may have some limitations to challenge them to be used in food applications. They are highly volatile, owning a strong aroma, low soluble in water, unstable, and high reactive with other components (Fernández-López & Viuda-Martos, 2018). For this reason, we are considering the encapsulation of essential oil into the form of nanoemulsions will be an innovative solution to overcome all these challenges.

Although many studies were done about incorporating natural films with essential oils,

still, there is a need to study the release kinetic of essential oils active components from the polymeric matrix to the food surface. It can estimate the relative concentration and predict the time in which those active components remain in an adequate concentration on the food surface to inhibit the growth of foodborne pathogens. Studying the controlled release is necessary to give the possibility to produce durable and sustainable antimicrobial packaging materials. This work aims to understand the releasing mechanism of thyme essential oil active components from chitosan-based films using a physical release model. The main goal is to extend the application of chitosan-based films incorporated with thyme essential oil nanoemulsions, where we can control the releasing and make decisions.

3.3. Materials and methods

Chitosan (high molecular weight 800 KDa) and thyme essential oil (*Thymus vulgaris* L. spp.) were supplied by Sigma-Aldrich (USA). Food-grade acetic acid, Polyoxymethylene sorbitol monooleate (Tween 80), unroasted sesame oil, phosphate standard equimolar solution (pH 6.68 at 25 ºC), and thymol standard (2-isopropyl-5-methyl-phenol) were brought from Wako Pure Chemical Industries Ltd., (Tokyo, Japan). Glycerol was provided by MP Biomedical, Inc. (France). Modified lecithin SLP-White was bought from Tsuji Oil Co., Ltd, (Japan). Deoxycholate agar, standard method agar, and nutrient broth were ordered from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan).

3.3.1. Preparation of thyme essential oil nanoemulsions TH-NE

Thyme essential oil (*Thymus Vulgaris* spp.) was encapsulated into nanoemulsions form through the following steps: continuous phase contained phosphate pH standard equimolar solution at pH 6.68, natural stabilizer modified lecithin SLP (0.1 wt%), and a non-ionic surfactant Tween 80 (2.0 wt%). While dispersed phase contained soybean oil (1.0 wt%) and thyme essential oil (1.0 wt%). Both of two phases were homogenized together using a rotor-stator homogenizer (Polytron PT-3100, Kinematic, Switzerland) at 7000 rpm for 5 minutes, followed by a high-pressure homogenizer (NanoVater NV200, Yoshida Kikai, Japan) for 3 cycles at 100 MPa.

3.3.2. Preparation of chitosan-based film incorporated with TH-NE

Chitosan 1.5 wt% was dissolved into 2.0 wt% acetic acid solution (pH 5.3 - 5.6), with continuous stirring for 30 min at 70 - 75 ºC. Then, glycerol 2.0 wt% was added as a film plasticizer, followed by 3 wt% of the previously prepared TH-NE. The whole filmogenic mixture was stirred for 10 min at room temperature for complete homogenization. The

filmogenic mixture was cast in plastic Petri dishes (φ 90 x 15 mm) and dried in an oven at 50-55 ºC for 24 h.

3.3.3. Determination of thyme oil composition.

Gas chromatograph-mass spectrometer (GCMS-QP2010 Plus) was used to determine thyme oil constituents through the following condition: the initial temperature was 60 ºC for 2 min and increased with a heating rate 30 ºC/min up to 240 ºC. The sample injection temperature was 270 ºC, at a 1:10 split ratio and 1 µl amount. Helium (99.9 mass %) was used as a carrier gas at 101.5 kPa constant pressure. The GC column was a crossband silica capillary column (5% diphenyl 95% dimethyl polysiloxane) with 30 m in length and a film thickness of 0.25 µm. Mass spectrometer MS ion source and interface temperatures were 200 and 250 ºC, respectively. MS was operated in the electron impact (EI) mode with electron energy = 70 eV, a total ion current mode ranged from 35 to 500 m/z, and GC-MS solution software. Thyme essential oil components were identified based on the retention time and their molecular mass compared to those of the literature and MS library (Satyal et al., 2016). A calibration curve was performed to evaluate thymol concentration.

3.3.4. *In-vitro* Releasing test

Chitosan-based film incorporated with 3 wt% TH-NE was cut into small pieces with 2 ×2 cm. The film piece was immersed in a sealed beaker containing 20 ml unroasted sesame oil as a fat food simulant. For evaluating the effect of temperature and humidity on the releasing, the specimens were kept at different temperatures (5, 25, and 40 ºC) and humidity (85, 33, and 8 % RH) conditions. While evaluating the effect of polymer concentration on the releasing, other specimens containing chitosan-based films prepared from different chitosan concentrations (1.0, 1.5, and 2.0 wt%) incorporated with 3 wt% TH-NE and kept at 25 ºC.

3.3.5. Determination of the released thymol concentration

Sucking of 3 ml of sesame oil with 1 ml ethanol 99.7% to form a 3:1 ratio. The sample was filtered using a syringe filter (0.45 μm). Thymol concentration was checked by using Gas Chromatography (GC-QP2010 Plus) daily for consecutive 7 days using the previously mentioned method in paragraph 2.3.

3.3.6. Microbiological investigation

3.3.6.1. Time-killing test

The time-kill test is used to show the time-dependent antimicrobial effect. Bacterial suspensions of *E. coli* spp. and *Bacillus subtilis* spp. were prepared into three tubes for each of them. A piece of the film with size 2 × 2 cm was placed into the first and second tubes, while the third tube was used as growth control. All the tubes were incubated at 37 ℃ for varied time intervals (0, 4, 6, 8, 10, 12, and 24 h). The number of living cells (CFU/mL) was determined using the agar plate count method (CLSI, 1999).

3.3.6.2. *In-vitro* microbial challenge test

Bacterial suspensions of *E. coli* spp. and *Bacillus subtilis* spp. were prepared into four tubes for each of them. A piece of the film with size 2 × 2 cm was placed into the first and second tubes, while the third and fourth tubes were used as growth control. All the tubes were incubated at 10 ℃ for 14 days. The number of living cells (CFU/mL) was determined using the agar plate count method daily (Komitopoulou, 2011).

3.3.7. Statistical analysis

All the experiments were repeated at least three times. Statistix 8.1 program was used to determine the significant differences of all samples at a 95% confidence interval and alpha equal to 0.05.

3.4. Results and discussion

3.4.1. Determination of thyme oil composition

Thyme essential oil obtained from *Thymus vulgaris* L. spp. is highly known for owning potential antimicrobial activity against different food pathogens. The main components of thyme essential oil are the natural terpenoid thymol (2-isopropyl-5-methylphenol) and its conformational isomer, carvacrol (5-isopropyl-2-methylphenol). Both are the bioactive constituents responsible for thyme essential oil's antimicrobial properties (Fani & Kohanteb, 2017).

It was critical to identify thyme essential oil bioactive components to determine the main element tracked and evaluated for the controlled release during our study. Therefore, GC-MS was used for the thyme essential oil components' identification. The chromatogram in Fig.3.1.a. showed thyme oil constitutes four basic components: p-cymene, γ-terpinene, thymol, and carvacrol. Thymol was found to have the biggest peak area over the other compounds. From that point, thymol was chosen to be our target active component. Thymol is highly known for its ability to inhibit a wide variety of food spoilage pathogens, protecting the food products during the storage period (Cai et al., 2019). It was classified as 'Generally Recognized As Safe' by the FDA for use as a food additive due to its safety and negligible toxicity (Food & Drug Administration, 2018).

To evaluate thymol concentration in thyme essential oil, a thymol standard was used to prepare a calibration curve. Thymol concentration inside thyme essential oil was found to be is 2.06×10^{-4} mol/ml with 30.9 % relative concentration. The obtained value was slightly less than 33.0 and 35.4 % found by (Šegvić Klarić et al., 2007) and (Dehghani et al., 2019). However, there was a decrement of 8.6 % compared with the result obtained by (Tohidi et al.,

2017). Nonetheless, thymol concentration highly varies depending on the climate conditions, geographical area, plant's age, methods of drying and extraction, etc. (Lorán et al., 2011).

Thymol essential oil concentration into thyme nanoemulsions was estimated to be 1.14 × 10^{-4} mol/ml, with a 44.45 % reduction. This reduction could be related to using the high shear and high-pressure homogenizer HPH as an energy-intensive oil encapsulation technique. Although HPH can produce high stable nanoemulsions physically and biologically, it may lead to the decomposition of essential oils' active components (Ali et al., 2020 and Aouf et al., 2020). Therefore, further studies on the effect of the energy-intensive technique on thyme essential oil's chemical stability are highly recommended.

3.4.2. *In-vitro* release test.

The released test was performed to understand thymol's duration to be fully released from the chitosan-based films to the food products with high-fat content. For that reason, unroasted sesame oil was chosen as a fat food simulant (D2 simulant), based on European Commission regulation (Simoneau, 2015). It has a relatively low smoking point (177 ˚C) compared with other vegetable oils, which would be more appropriate to GC column maximum temperature. The in vitro release profile of thymol from chitosan-based films was studied under different temperature and humidity conditions. Besides, releasing from chitosan-based films with varying concentrations of chitosan and TH-NE.

The initial thymol concentration in the film sample was estimated to be 1.1629 mol/m^3. When the film specimen was kept at different temperature conditions (5, 25, and 40 ˚C), it showed a gradual release of the thymol from the films to the fat food simulant, as shown in Fig.3.2.a. The releasing rate of thymol at the first three days was slower than those reported by (Wu et al., 2017). The mentioned author registered an initial burst releasing of cinnamon oil from fish gelatin films to the fat food simulant followed by a slower releasing rate. The release

rate difference can be explained because of the encapsulation of thyme essential oil into nanoemulsions form. The enforcement of nanostructures such as nanoemulsions or nanofibers to the food packaging materials may positively help the gradual release and maintain physical and mechanical properties (Huang et al., 2019). However, this releasing rate can be a desirable property for fresh food applications such as meat products (dos Santos et al., 2020).

By the end of experiment time, the released thymol concentrations from the chitosan-based films kept at various temperature conditions (5, 25, and 40 ˚C) were evaluated (0.864, 0.777, and 0.942 mol/m^3), respectively. It was found film samples kept at a higher temperature, 40 ˚C, had the highest released thymol concentration representing 81.0 % of the initial thymol concentration in the film sample. Higher temperature impacts the polymer segment's motion (chitosan), increasing the molecules' kinetic energy, causing bigger gaps in the polymer matrix and a higher releasing rate (Mrkić et al., 2007). The kinetic energy is highly relying on the molecule's size and the interaction between polymeric chains.

While the released thymol concentrations of film sample samples kept at (5 and 25 ˚C) were found to represent (74.3 and 66.8%), respectively. Although temperature affects the release of thymol molecules, it also depends on the polymer's crystallinity degree. High molecular weight chitosan-based films known to have a crystallinity degree of about 23.9% (Feng et al., 2012), which is relatively smaller compared to starch amylose-based films 32.6%, (Rindlav-westling et al., 2002), cellulose films 48.5% (Sun et al., 2015), High-Density Polyethylene HDPE 95% and Low-Density Polyethylene LDPE 65% (Riley, 2012). The lower degree of polymer crystallinity, the higher diffusion coefficient, and consequently higher released concentrations with less significance to the temperature effect (Taylor et al., 2007).

Fig.3.2.b. showed the released thymol concentration at different humidity conditions (8, 33, and 80% RH), evaluated to be (1.032, 0.800, and 0.767 mol/m^3). These values were

representing (88.74, 68.8, and 66.0%) of the initial thymol concentration in the film sample. It was noticed decreasing the relative humidity RH% increased the thymol releasing. The hydrophobicity is highly related to hydration and plays a significant role in the binding process. Thymol – sesame oil (fat food simulant) interactions are mainly hydrophobic - hydrophobic spontaneous interactions. Both hydrophobs are nonpolar molecules that do not interact with water molecules and tend to group (Garrett & Grisham, 2013), explaining the higher concentration of released thymol under low humidity conditions. In general, humidity affects the thymol releasing. Moreover, the oil application as a food simulant facilitates the releasing process.

Fig.3.3.a. showed the effect of using different polymer (chitosan) concentrations (1.0, 1.5, and 2.0 wt%) on thymol releasing. The released concentrations were estimated to be (0.663, 0.777, and 0.836 mol/m^3), representing (57.0, 66.8, and 71.9%) of the initial thymol concentration in the film sample. Based on the obtained results, the higher concentration of chitosan caused a higher concentration of released thymol by the end of the experiment. Chitosan makes direct hydrophobic interaction with nonpolar molecules such as oil food simulant (Ken et al., 2013). Therefore, increasing chitosan concentration would increase the hydrophobicity and facilitate the thymol releasing. Moreover, the deacetylation degree may affect the hydrophobicity. As DD deacetylation degree more than 80% increases the hydrophobic interaction (Ken et al., 2013). However, releasing thymol from the chitosan-based film is a complicated phenomenon that includes many factors such as electrostatic interactions, ionic osmosis, and structural changes in the polymer induced by incorporating thyme essential oil nanoemulsions (Sobral, 2013).

3.4.3. Microbiological investigation.

3.4.3.1.Time-killing test

The time-kill kinetic test was used to study the activity of chitosan-based film incorporated with thyme essential oil nanoemulsions against *Bacillus subtilis* and *E. coli* spp. The test was done by counting the viable bacterial cells in the broth following placing the film to determine the bactericidal or bacteriostatic activity over time.

The time kinetic profile of the tested films against *Bacillus subtilis* and *E. coli* spp. showed viable cells count reduction over the 24th h, as shown in Fig.3.4.a. and b. The viable bacterial log reduction started by the 4th, 6th, and 8th h followed by a gradual rise to the 24th h. In the case of *Bacillus subtilis* spp., it hit the lowest viable bacterial cells count at the 6th h by having 2.9 log (CFU/ml) reduction and ended by 2.0 log (CFU/ml) reduction at the 24th in comparison to the control group. While *E. coli* spp. showed the lowest viable cells count at the 8th h and ended by 1.5 log (CFU/ml) reduction at the 24th. Thus, chitosan-based films incorporated with 3 wt% thyme essential oil nanoemulsions could release thymol at the first 4-6th h from contacting with the bacterial culture, showing bacteriostatic antimicrobial activity against both *Bacillus subtilis* and *E. coli* spp.

3.4.3.2. *In-vitro* microbial challenge test

In-vitro microbial challenge test was used to predict the likelihood of bacterial survival under temperature danger zone (10 ℃) up to 14 days. It could be a helpful tool in identifying the prevalence of *Bacillus subtilis* and *E. coli* spp. and determine the ability of the tested films to inhibit bacterial growth (Feroz, 2013).

Fig.3.5.a. and b. showed the changes in both *Bacillus subtilis* and *E. coli* spp. viable cells count in broth following placing the films kept at 10 ℃ for 14 consecutive days. In the case of *Bacillus subtilis* spp., the bacterial count increased noticeably on the 1st day with no difference compared to the control group. However, by the 2nd day, it started to show remarkable bacterial count reduction. The highest reduction of 3.6 log (CFU/ml) was noticed on the 5th day. After

that, the bacterial count showed slight fluctuation, as, by the 7th day, it started to show a gradual slight increase. However, by the 14th day, *Bacillus subtilis* spp. viable cells count showed a remarkable reduction of 3.5 log (CFU/ml) compared to the control group.

While *E. coli* spp. bacterial count showed an increase on the 1st and 2nd days with no difference compared to the control group. The 3rd day showed noticeable bacterial count reduction with a gradual and steady decrease until the 14th day to reach the maximum reduction value by a 3.3 log (CFU/ml).

Therefore, Chitosan-based film incorporated with thyme essential nanoemulsions could inhibit the growth of *Bacillus subtilis* spp. and *E. coli* spp. showing antimicrobial effectiveness under challenging conditions.

3.5. Conclusion

Chitosan-based film incorporated with thyme essential nanoemulsions was evaluated for their control release properties. Chitosan-based films could release thymol as antimicrobial agents from the film materials to the fat food simulant. It was noticed that both higher temperature 40 ℃ and lower humidity 8% increased the released amount of thymol by the end of the storage period. However, it is believed that many different factors may affect the releasing mechanism, such as the polymer chemical structure, molecular weight, and crystallinity. Besides, food simulant is less complex than the actual food system, making it unable to accurately predict the released thymol concentration. For that reason, we highly recommend testing the controlled release properties using the actual food system. On the other hand, chitosan-based films showed remarkable bacteriostatic antimicrobial properties from the first early hours with contacting the bacterial culture and keeping under challenging conditions. Therefore, chitosan-based films incorporated with thyme essential oils nanoemulsions are highly recommended for food applications.

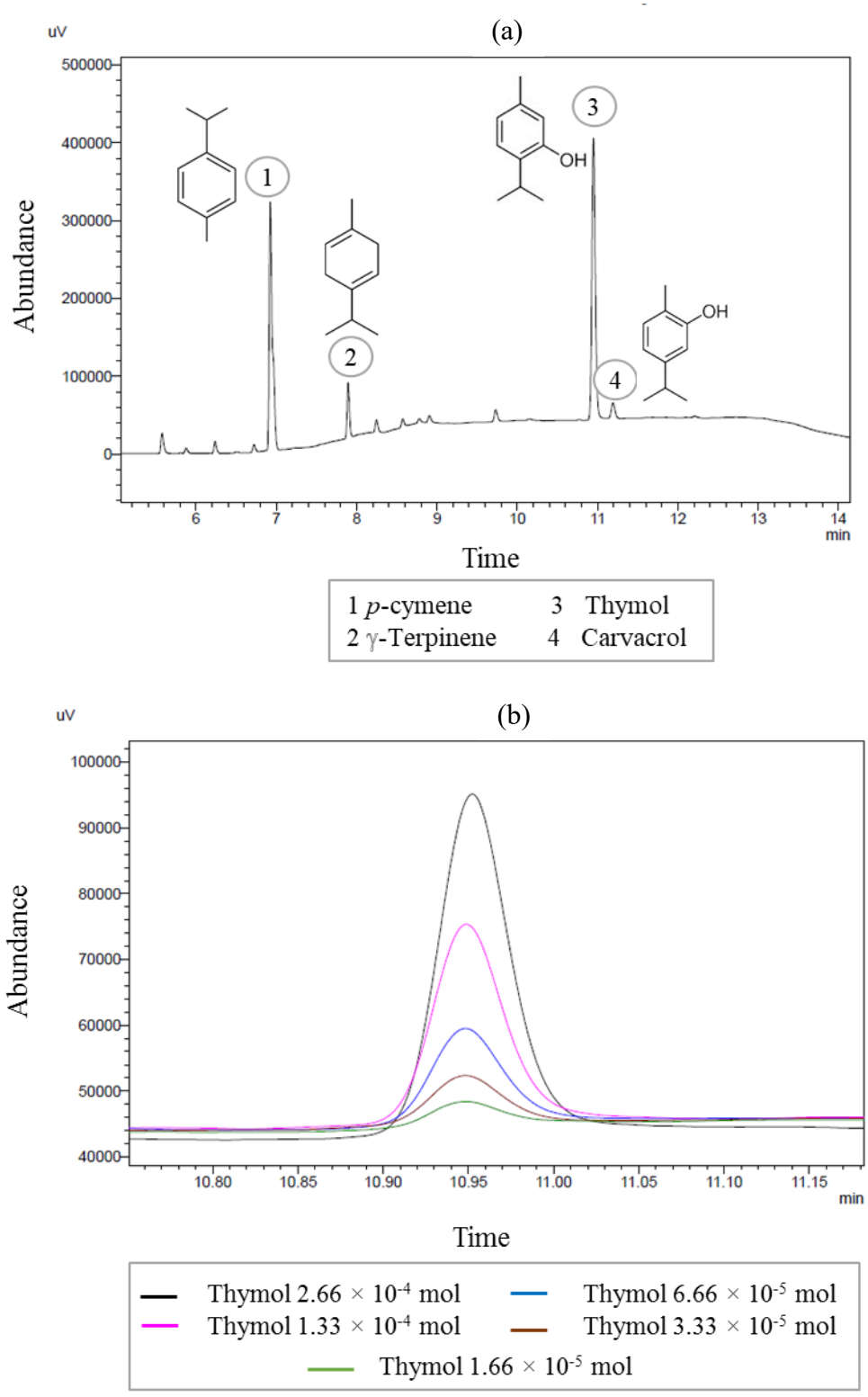


Figure 3. 1 The GC-MS chromatogram of thyme essential oil; (a) Analysis of Thymus vulgaris essential oil; and (b) Thymol standard chromatogram with different concentrations.

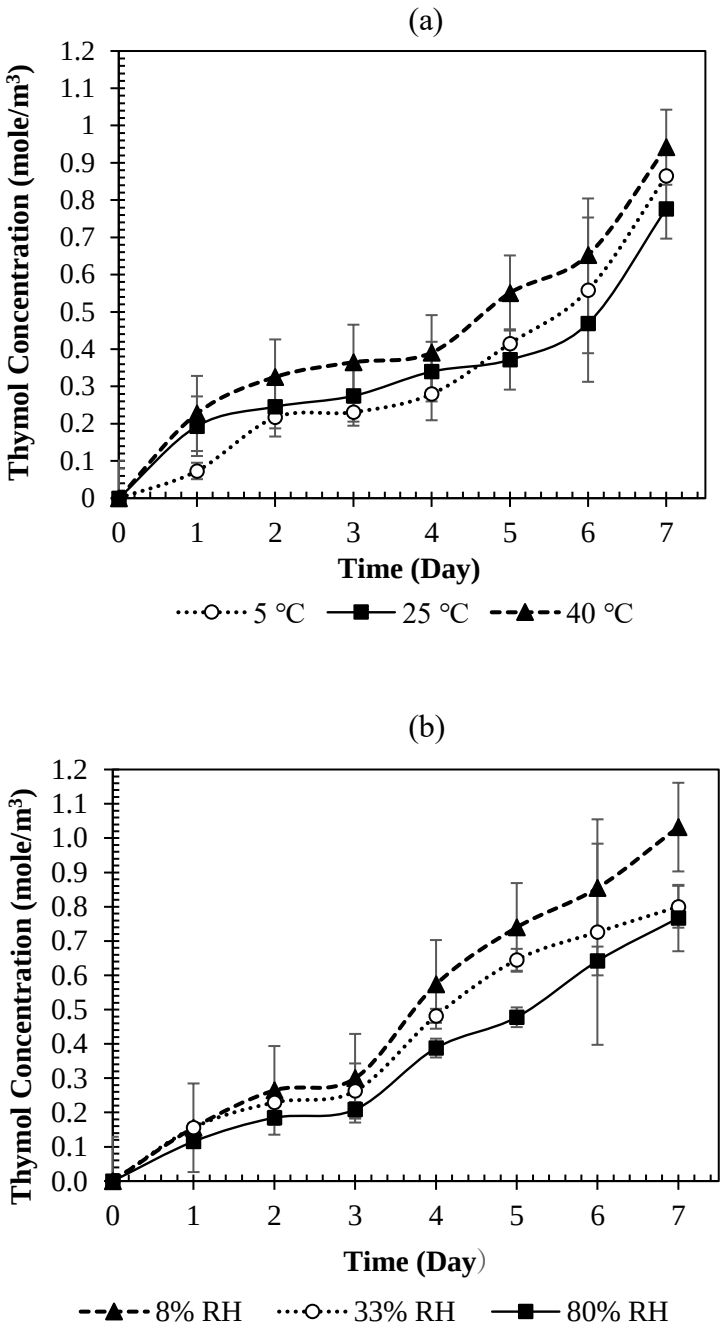


Figure 3. 2 The release profile of thymol from chitosan-based film to the food simulant under different conditions; (a) temperature (5, 25, and 40 °C), and (b) relative humidity RH% (8, 33, and 80%).

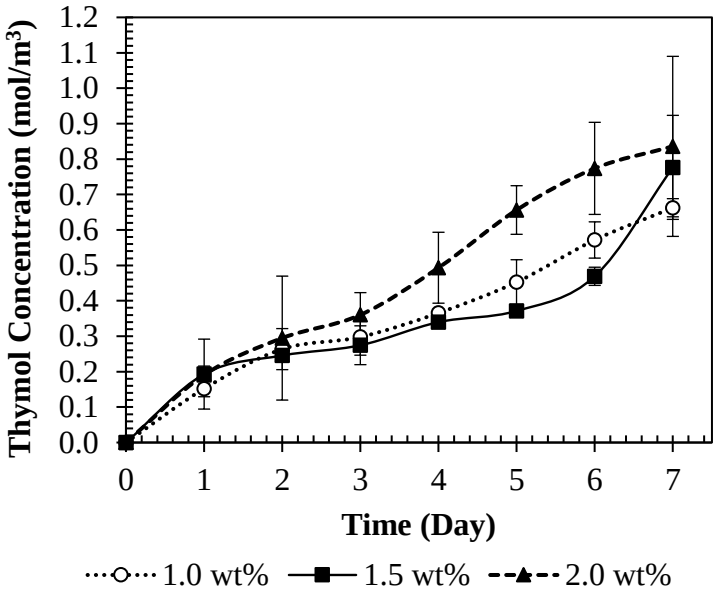


Figure 3. 3 The release profile of thymol from chitosan-based film to the food simulant with different polymer (chitosan) concentrations (1.0, 1.5, and 2.0 wt%).

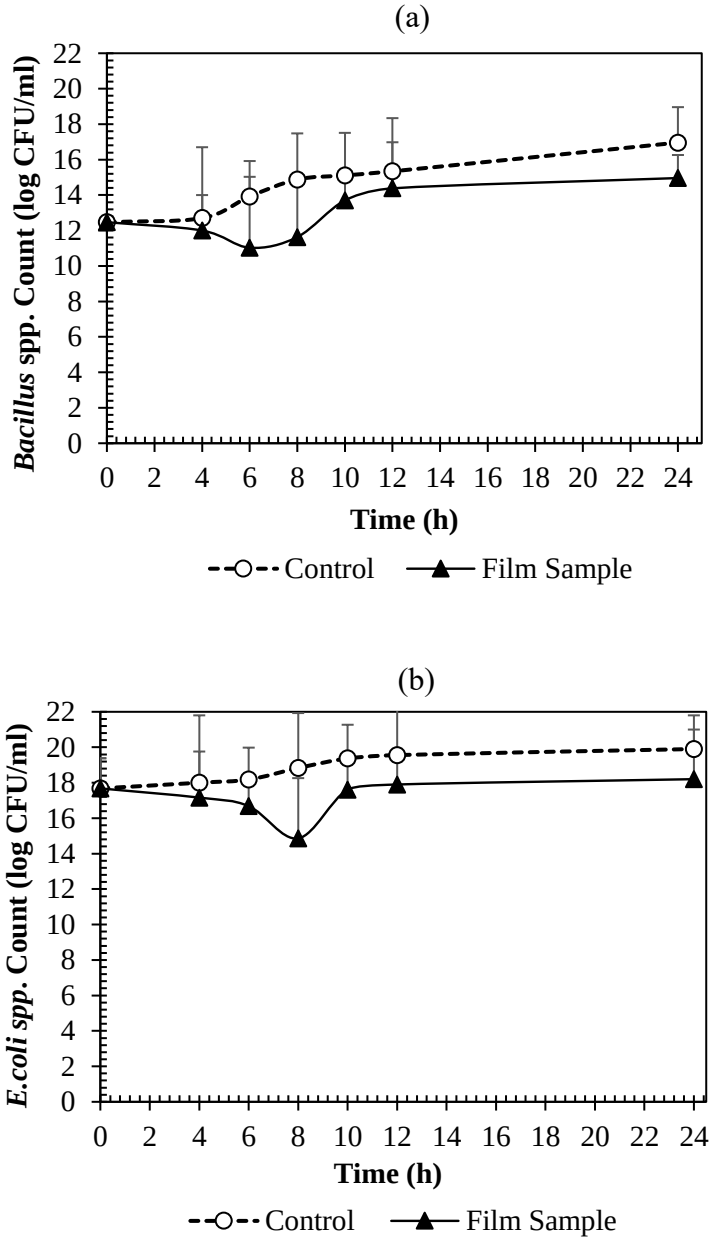


Figure 3. 4 Time-kill kinetics of chitosan-based film incorporated with thyme essential oil nanoemulsions against; (a) *Bacillus subtilis* app., and (b) *E.coli* spp.

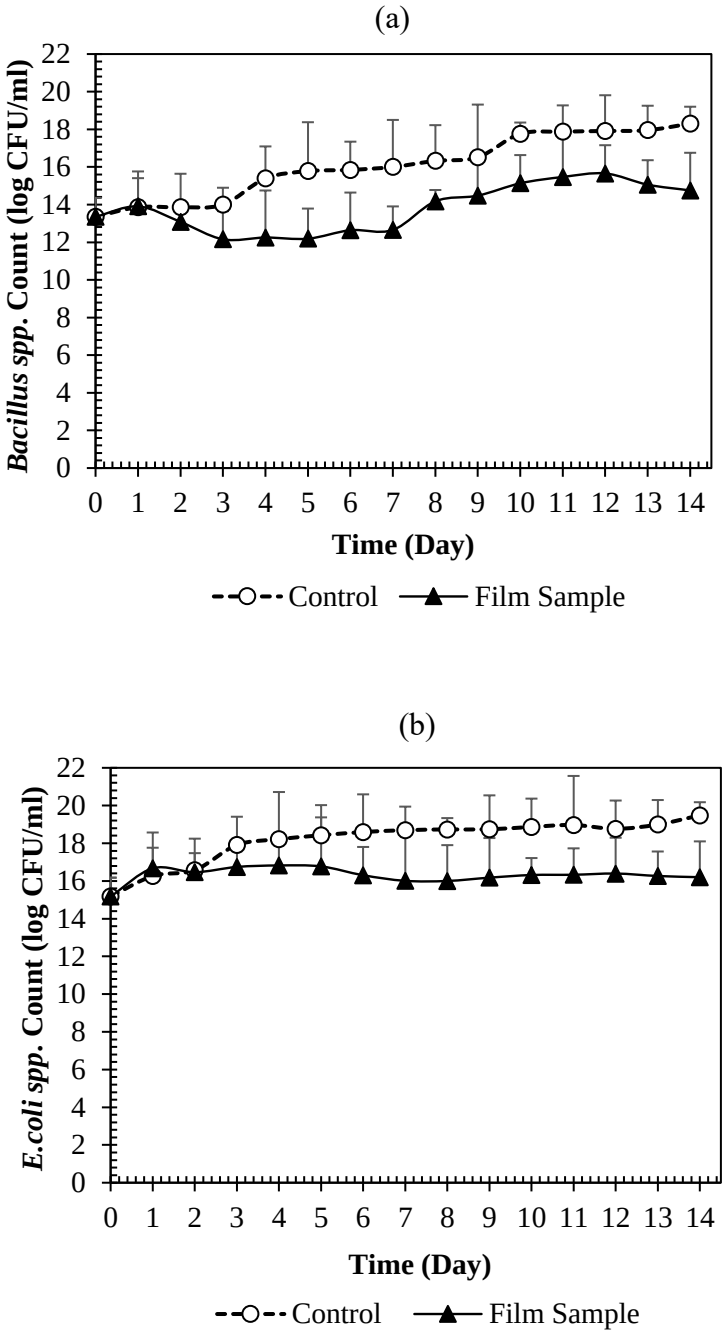


Figure 3. 5 Time challenge test of chitosan-based film incorporated with thyme essential oil nanoemulsions against; (a) *Bacillus subtilis* app., and (b) *E.coli* spp.

CHAPTER 4 – Food Application

4. Food Application

4.1. Abstract

The controlled release antimicrobial packaging is a relatively new food packaging that mainly concerns the antimicrobial agents releasing. Many studies had been done to evaluate the antimicrobial agents releasing using food simulant. However, it is expected that the releasing behavior will have a different pattern when applied to the real food system due to its complexity. In this research, chitosan-based films incorporated with thyme essential oils were applied to animal butter. Thymol release from chitosan-based materials to the butter product surface was evaluated daily using Gas chromatogram GC. The antimicrobial effectiveness of chitosan-based films was assessed by applying the chitosan-based films to fresh food products (fresh salmon fish, ground meat, and chicken meat). Chitosan-based films showed control gradual release of thymol from the film materials to the butter product, confirming the suitability of the film as active food packaging and its application to high-fat content food products. Moreover, the chitosan-based film showed effective antimicrobial activity by reducing the microbial count when it was applied to fresh food products as well as extending their shelf life.

This chapter will be submitted as:

Samar Elshamy, Emna Abdennour, Teetach Changwatchai, Kunihiko Uemura, Mitsutoshi Nakajima, Marcos A. Neves. Evaluation of Thyme Essential Oil Nanoemulsions Release from Chitosan-based Films and Their Antimicrobial Efficacy.

4.2. Introduction

Food spoilage is representing a great concern in the food industry. It causes the loss of a massive amount of food due to color and texture changes—furthermore, the loss of the nutritional value and faster growth of pathogenic food microorganisms.

Food spoilage mainly occurs due to contamination of food products with different spoilage microorganisms. The contamination usually takes place during food processing, packaging, handling, and storage. Traditional food preservation techniques such as freezing, heating, drying, smoking, salting, and fermentation may extend the food products' shelf life. However, subsequent contamination may happen, causing food products' deterioration (Malhotra et al., 2015).

Biodegradable antimicrobial food packaging materials incorporated with natural antimicrobial agents are considered a novel sustainable, and innovative solution. They aim to inhibit the growth of pathogenic food microorganisms and consequently increase the food products' shelf life and improve the hygienic quality (Sung et al., 2013). The antimicrobial packaging prevents the microbial multiplication on the food products' surface through the direct contact of the packaging materials to the food surface. For that reason, antimicrobial food packaging materials should be assured to contact the food surface to facilitate the release of antimicrobial agents throughout the food surface (Malhotra et al., 2015).

The controlled release antimicrobial packaging is considered a relatively new technology; it mainly focuses on the system of antimicrobial agent releasing. It is believed to be an active system where the packaging materials act as a delivery vehicle to effectively deliver the antimicrobial agents under a controlled releasing rate over a specific period (Lacoste et al., 2005). The control released antimicrobial packaging can effectively ensure adequate antimicrobial agent concentration to prevent food deterioration and make it safe for human

consumption. However, after releasing antimicrobial agents to the food, it is expected that the antimicrobial activity could be decreased or negatively affected by their interaction with food components or dilution to be lower than the required levels (Appendini & Hotchkiss, 2002).

Several previous studies had tested the control release packaging materials using food simulants. It was found that food simulants are less complex than the actual food systems. The food system usually has either higher or lower water activity, nutrients, fat, proteins, salts, or sugar contents. All these components are found to interact with the antimicrobial agents (Mauriello et al., 2005). In addition to both the storage and transportation conditions of those food products may affect the antimicrobial agents' characteristics, such as temperature and humidity percentage.

Determination of the releasing rate could be a useful tool to predict the concentration of antimicrobial agents migrating from the food packaging materials to the food surface and the amount of time it takes to reach the adequate, effective concentration over a specific area (Malhotra et al., 2015). Diffusion phenomena could be a primary factor that governs the releasing of antimicrobial agents but not solely; it is believed that many aspects such as swelling, and water uptake could have a significant effect. Although there are many microbiological and chemical analyses to explore the kinetic mechanism of antimicrobial agents released from the packaging materials to the food surface, obtaining the same accurate results in the actual food is challenging (Szente & Szejtli, 2004). It was previously recorded that control release antimicrobial packaging is less effective when tested using the real food system compared to the obtained results using the food simulants (Duan et al., 2007). A previous study had investigated the antimicrobial activity of essential oils using milk and cheese products. It concluded that essential oils should be used in much higher concentrations in order to achieve the desired antimicrobial activity. The authors explained the reason could

be due to the rich content of milk and cheese with organic acid, trace elements, and other nutrients that are of great benefit for microbial cellular repair. In addition, these nutrients may interact with the essential oils and inactivate their actions (Burt, 2004).

For that purpose, this research aims to evaluate the releasing rate and antimicrobial activity of chitosan-based films incorporated with thyme essential oils nanoemulsions using an actual food system. Animal butter product was used to assess the released concentration of thymol from the chitosan-based films. At the same time, fresh salmon fish, chicken, and ground meat were used to evaluate the antimicrobial activity of chitosan-based films at different temperature conditions.

4.3.Material and methods

4.3.1. Evaluate thymol releasing from chitosan-based film to food products.

Chitosan-based film incorporated with thyme essential oil nanoemulsions was applied to animal butter products purchased from the local market, Tsukuba city, Ibaraki, Japan. The butter samples were prepared by cutting into cubes and weighting them into 10 g for each sample. All samples were wrapped with chitosan-based films incorporated with 1 wt% thyme essential oil nanoemulsions and stored at 4℃. The samples were simulated as a food package where all the cube faces were brought into direct contact with the film. All the samples were placed into petri-dish and stored at refrigerator temperature 4 ºC as shown in Fig 4.1.

Solvent extraction of thymol from butter samples was performed according to the (Folch et al., 1956) method with modifications. First, 0.5 g of the outer and inner layer of the butter sample was taken and mixed with 196 mL chloroform and stirred till complete dissolving. Then, samples were filtered using a syringe filter of 0.45 µm. Thymol concentration was checked daily for successive 7 days by using Gas Chromatography GC was through the following condition: the initial temperature was 60 ºC for 2 min and increased with a heating rate 30 ºC/min up to 240 ºC. The sample injection temperature was 270 ºC, at a 1:10 split ratio and 1 µl amount. Helium (99.9 mass %) was used as a carrier gas at 101.5 kPa constant pressure. The GC column was a crossband silica capillary column (5% diphenyl 95% dimethyl polysiloxane) with 30 m in length and a film thickness of 0.25 µm. GC chromatogram was processed by GC-MS solution software. Thymol was identified based on the retention time and their molecular mass compared to those of the literature and MS library (Satyal et al., 2016).

4.3.2. Evaluation film swelling percentage.

Film swelling percentage was evaluated by measuring the film weight before and after applying to the butter samples after completing the storage period (7 days at 4 ºC), using equation 4.1.

$$\text{Swelling } \% = \frac{W2-W1}{W1} \quad (4.1)$$

Where W1 is the film's initial weight, and W2 is the weight of film after swelling.

4.3.3. *In-vivo* assays: antimicrobial effectiveness.

4.3.3.1. Food sample preparation.

Chitosan-based film incorporated with thyme essential oil nanoemulsions were applied to raw fresh Salmon fish, ground beef meat, and chicken meat. All the fresh food samples were purchased from local stores at Tsukuba city, Ibaraki Prefecture, Japan. Prior to the film application, all food samples were aseptically prepared by dividing and weighting them into 10 g for each sample.

4.3.3.2.Total *Aerobic* bacterial count

Samples were divided into four groups, the first and second groups were wrapped with chitosan-based films and stored at 4℃ and 25℃, respectively. The samples were simulated as a food package where all the food faces were brought into direct contact with the film. While third and fourth groups were kept unwrapped as control samples and stored at 4℃ and 25℃, respectively. All samples were investigated for their total bacterial count daily till samples' deterioration.

Each sample (10 g) was placed into a sterile stomacher bag supplied with a filter containing 90 ml buffer solution and mashed using a stomacher. The liquid part was sucked to start the microbiological experiments. Spread swab technique using standard method agar was

performed. All petri-dishes were incubated at 37 ℃ for 24 h. The number of living cells (CFU/g) was determined using the agar plate count method (CLSI, 1999). Bacterial counts were done in triplicates.

4.3.3.3. Evaluation *E. coli* spp. count

Food Samples were inoculated with *E. coli* spp. 10^6. Samples were divided into four groups, the first and second groups were wrapped with chitosan-based films and stored at 4℃ and 25℃, respectively. The samples were simulated as a food package where all the food faces were brought into direct contact with the film. While third and fourth groups were kept unwrapped as control samples and stored at 4℃ and 25℃, respectively. All samples were investigated for their *E. coli* spp. count daily till samples' deterioration.

As mentioned in paragraph 4.3.5., each sample (10 g) was placed into a sterile stomacher bag supplied with a filter containing 90 ml buffer solution and mashed using a stomacher. The liquid part was sucked to start the microbiological experiments. Spread swab technique using Deoxycholate selective agar was performed. All petri-dishes were incubated at 37 ℃ for 24 h. Bacterial counts were done in triplicates.

4.3.3.4. Statistical analysis

All the experiments were repeated at least three times. Statistix 8.1 program was used to determine the significant differences of all samples at a 95% confidence interval and alpha equal to 0.05.

4.4. Results and discussion

4.4.1. Evaluate thymol releasing from chitosan-based film to food products.

Thymol released test was performed using animal butter in order to evaluate the thymol releasing kinetics from the chitosan-based films to the actual food products with high-fat content. For that reason, chitosan-based film incorporated 1 wt% thyme essential oil nanoemulsions was applied to animal butter product. All the wrapped samples were stored at refrigerator temperature 4 ℃ for 7 days and checked daily for their thymol concentration using Gas Chromatogram GC.

The initial thymol concentration in the film sample was estimated to be (0.0565 mol/g). When the wrapped butter was stored at 4 ℃, it showed a gradual release of thymol from the film to the butter surface at the first 4 days, as shown in Fig.4.2. The released thymol concentration was estimated to be around 99% of the initial thymol concentration. The results revealed a gradual full release of thymol by the 4^{th} storage day. At the same time, the existing released thymol concentration started to decrease gradually to reach 48% by the end of the storage period causing a thymol reduction rate of around 51%. The gradual thymol releasing is considered a desirable property for the food application, as it may ensure the existence of thymol as an antimicrobial agent for enough period to prevent the growth of pathogenic microorganisms (dos Santos et al., 2020). However, the decreasing of thymol concentration at the end of the storage period could be explained due to the thymol interaction with the butter nutrients (Burt, 2004).

On the other hand, there might be a possibility that solvent extraction of thymol from the butter is not the best efficient method to obtain accurate results. This because of the volatile property of thymol and the complexity of milk products such as cheese and butter. Milk products are highly known for undergoing several biochemical and rheological changes during

the storage period (Panseri et al., 2014). Therefore, we highly recommend using solvent-free extraction methods such as solid-phase microextraction technique SPME. This is because its sensitivity and providing linear data for a wide range of different concentrations make it a better technique for quantitative and semi-quantitative analysis (Chouhan et al., 2017).

4.4.2. Film swelling percentage.

By evaluating the swelling percentage by measuring the weight of chitosan-based films before and after applying the film to the butter samples, it was found swelling percentage was estimated to 2.7%. This swelling value could be the lowest and the best value obtained till the current time. According to previous literature, no further studies evaluated the swelling percentage after food application. However, chitosan-based films, when placed into a water medium, the swelling percentage was estimated to be 208% (Ghosh & Ali, 2012). Therefore, chitosan-based films are showing outstanding suitability to be used in high-fat content food applications. This result could be explained due to the hydrophobic polysaccharide backbone of chitosan, resulting in low interaction with the butter nutrients, besides the low water content of butter (De Conto et al., 2020).

4.4.3. *In-vivo* assays: antimicrobial effectiveness.

4.4.3.1. Total *aerobic* bacterial count

Application of chitosan-based films incorporated with thyme essential oils nanoemulsions for fresh food products was performed in order to evaluate the antimicrobial activity of the films using real fresh food. Chitosan-based films were applied to three different fresh food products (Salmon fish, ground meat, and chicken meat) purchased from the local market. The total *bacterial* count of all wrapped and control samples was done daily till samples' deterioration.

4.4.3.1.1. Application to fresh salmon fish

Salmon fish wrapped samples stored at 4 °C showed a reduction in the total *bacterial* count, which was noticeable at the 2nd storage day by achieving 1.35 (log CFU/g) reduction compared to the control group, Fig.4.3.a.

While the wrapped samples stored at 25 °C showed a remarkable reduction starting from the first storage day achieving 2.43 (log CFU/g) lower than the control group, Fig.4.3.b. Furthermore, the control group showed complete deterioration by the 2nd storage day. Starting from the 1st storage day, the control group showed a noticeable change in the organoleptic characteristics through color-changing and undesirable odor. In contrast, the wrapped samples were kept in a reasonable organoleptic characteristic.

Chitosan-based films incorporated with thyme essential oils nanoemulsions were able to keep the total *bacterial* count of salmon fish under the standard bacterial limit < 10^6 (FAO; WHO, 2003), during the whole storage period at 4 °C, with extending its shelf life from 2 to 3 days. At the same time, chitosan-based films kept the total *bacterial* count under the standard bacterial limit < 10^6 during the first 24 h for the samples kept 25 °C, revealing the bacteriostatic activity of chitosan-based films.

4.4.3.1.2. Application to ground meat.

Ground meat wrapped samples stored at 4 °C showed a reduction in the total *bacterial* count estimated by 1.97 (log CFU/g) compared to the control group by the end of the storage period, as shown in Fig.4.4.a.

While the wrapped samples stored at 25 °C revealed a total *bacterial* count reduction at 1st day estimated at 1.3 (log CFU/g), lower than the control group, Fig.4.4.b. On the other hand, the control group deteriorated by the end of 1st storage day. Nevertheless, all the wrapped samples were able to keep reasonable organoleptic characteristics.

Chitosan-based films incorporated with thyme essential oils nanoemulsions kept the total *bacterial* count of ground meat samples under the standard bacterial limit < 10^6 (FAO; WHO, 2003) during the whole storage period at 4 ℃, with extending its shelf life from 1 to 3 days. Furthermore, the total *bacterial* count was under the bacterial standard limit < 10^6 during the first 24 h for the samples kept 25 ℃, indicating the remarkable bacteriostatic activity of chitosan-based films.

4.4.3.1.3. Application to chicken meat.

Fresh chicken meat wrapped samples stored at 4 ℃ showed a reduction in the total bacterial count, which was noticeable at the 2nd storage day by 1.13 (log CFU/g), compared to the control group as in Fig.4.5.a.

While the wrapped samples stored at 25 ℃ showed a remarkable reduction starting from the 1st storage day achieving 3.62 (log CFU/g) lower than the control group, Fig.4.5.b. Moreover, the control group showed complete deterioration by the 3rd storage day. The control group was observed to have changed in the organoleptic characteristics. In contrast, the wrapped samples were kept in a reasonable organoleptic characteristic.

Chitosan-based films incorporated with thyme essential oils nanoemulsions were able to keep the total *bacterial* count of fresh chicken meat under the total *bacterial* standard limit < 10^6 (FAO; WHO, 2003), during the whole storage period at 4 ℃, with extending its shelf life from 2 to 4 days. At the same time, chitosan-based films kept the total *bacterial* count under the standard bacterial limit < 10^6 during the first 48 h for the samples kept 25 ℃, revealing the bacteriostatic activity of chitosan-based films.

4.4.3.2. Evaluation of *E. coli* spp. count

According to food safety guidelines and standards, food products should be free from *E. coli* spp. as detection of *E. coli* refers to the bad hygiene and fecal contamination of the food

products (Fasnz, 2001). However, chitosan-based films incorporated with thyme essential oils nanoemulsions for fresh food products inoculated with *E. coli* spp., in order to investigate the antimicrobial behavior of the tested films using actual fresh food.

4.4.3.2.1. Application to fresh salmon fish

Fresh salmon fish inoculated with *E. coli* spp. and wrapped with chitosan-based films showed similar *E. coli* spp. count compared to the control group when kept at 4 ℃ as shown in Fig 4.6.a. However, by the 4th storage day, *E. coli* spp. count was lower in wrapped samples by 1.11 (log CFU/g) than in the control group.

On the other hand, wrapped samples kept at 25 ℃ showed remarkable *E. coli* spp. count reduction at 1st and 2nd storage day by 2.48 and 2.87 (log CFU/g), respectively, as shown in Fig.4.6.b. Moreover, control samples were deteriorated by the 2nd storage day. In the meanwhile, all the control samples produced undesirable odor and color changes.

4.4.3.2.2. Application to ground meat.

Ground meat samples inoculated with *E. coli* spp. and wrapped with chitosan-based films had *E. coli* spp. count close to the control group when kept at 4 ℃ as shown in Fig. 4.7.a. They showed slightly lower *E. coli* spp. count at the 2nd and 3rd day by 0.49 and 0.38 (log CFU/g).

At the same time, wrapped samples kept at 25 ℃ were able to maintain a stable *E. coli* spp. during the 1st and 2nd storage day, as shown in Fig.4.7.b. In contrast, the control group showed a higher increase in *E. coli* spp. count to cause whole deterioration of the samples by the end of 1st storage day. On the other hand, it was noticed that both wrapped and control groups showed undesirable organoleptic characteristics when stored at 25 ℃.

4.4.3.2.3. Application to chicken meat.

Fresh chicken meat samples inoculated with *E. coli* spp. and wrapped with chitosan-based films showed a remarkable reduction in *E. coli* spp. count when stored at 4 ℃, as shown in

Fig.4.8.a. The samples had the lowest count starting from 1st storage day and kept the same count till the end of the storage period. The highest reduction value was estimated at the 5th storage day by 2.37 (log CFU/g) lower than the control group.

At the same time, *E. coli* spp. count in case of wrapped samples kept at 25 °C was stable starting from 2nd day till the end of storage period. On the other hand, control samples showed an increase in *E. coli* spp. count till samples' deterioration by the end of the 2nd storage day. Furthermore, the Control group kept at 25 °C showed changes in the organoleptic properties by the 1st storage day.

Chitosan-based films incorporated with thyme essential oils nanoemulsions showed bacteriostatic antimicrobial activity against *E. coli* spp. The highest bacterial reduction was noticed in all food-wrapped samples stored at 25 °C. While samples kept at 4 °C showed less bacterial reduction compared to the control groups. This could be related to keeping the sample at 4 °C prevent the growth of *E. coli* spp. (Fasnz, 2001), which did not give the clear opportunity to show the actual antimicrobial effectiveness of chitosan-based films under this temperature.

4.5. Conclusion

This work demonstrates that chitosan-based films incorporated with thyme essential oils nanoemulsions showed gradual control release of thymol from the film material to the food product. The film gradually released thymol to animal butter product during the 4 days of the storage period. Chitosan-based films showed a considerable low swelling %, approving the film suitability for high-fat content food application. At the same time, chitosan-based films showed remarkable bacteriostatic antimicrobial activity by reducing the total *bacterial* count of fresh food products under the standards limit. Chitosan-based films could expand the shelf life of fresh food products kept at refrigerator temperature while minimizing the bacterial risk during the first 24h for food products kept at 25 °C. Chitosan-based films showed antimicrobial

effectiveness with application to fresh food products and are highly suggested for potential applications in the food industry.

(a)

(b)

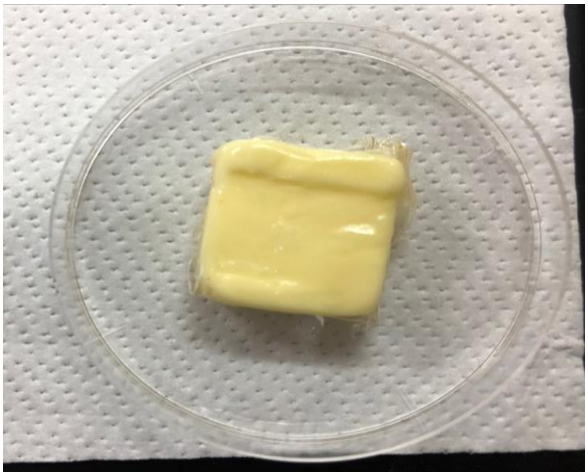


Figure 4. 1 Application of chitosan-based film incorporated with 1 wt% thyme essential oil nanoemulsions; (a) commercial butter product and (b) butter sample wrapped with the film.

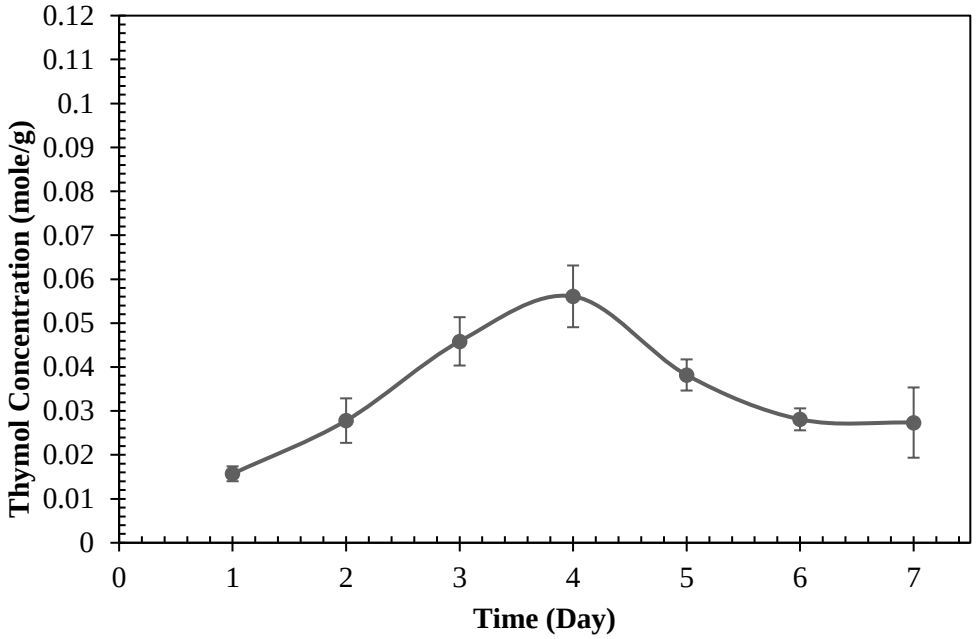


Figure 4. 2 The release profile of thymol from chitosan-based film to the animal butter product stored at 4 ℃ for 7 days.

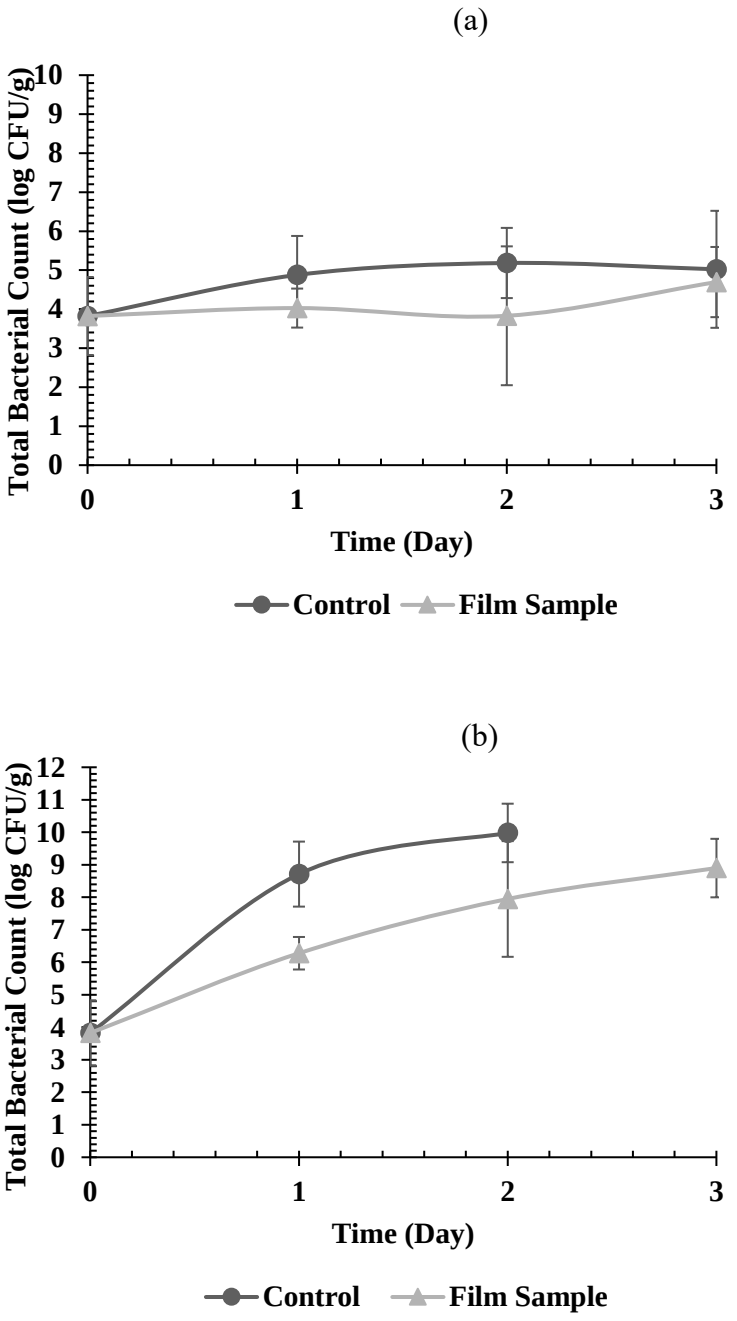
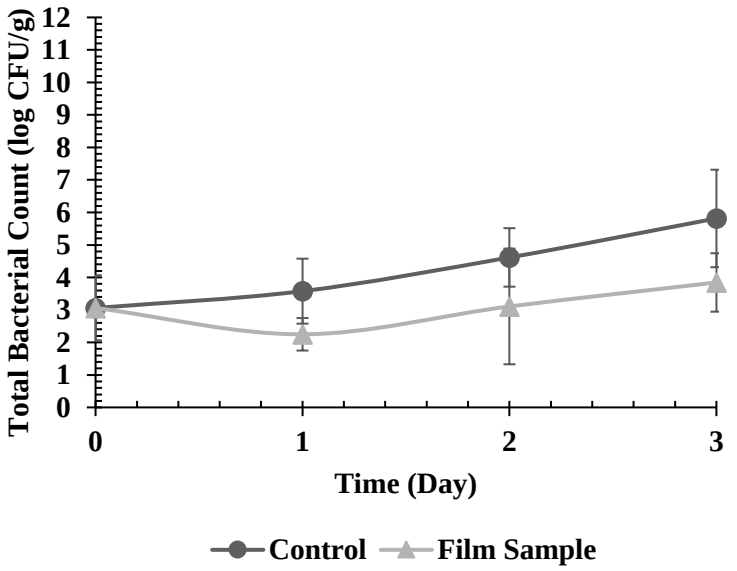


Figure 4. 3 Total *bacterial* count of Salmon fish sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

(a)



(b)

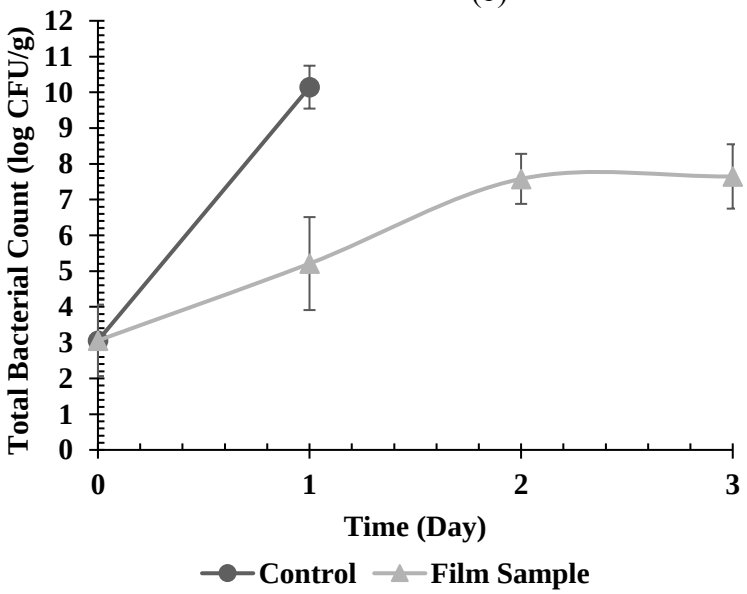


Figure 4. 4 Total *bacterial* count of ground meat sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

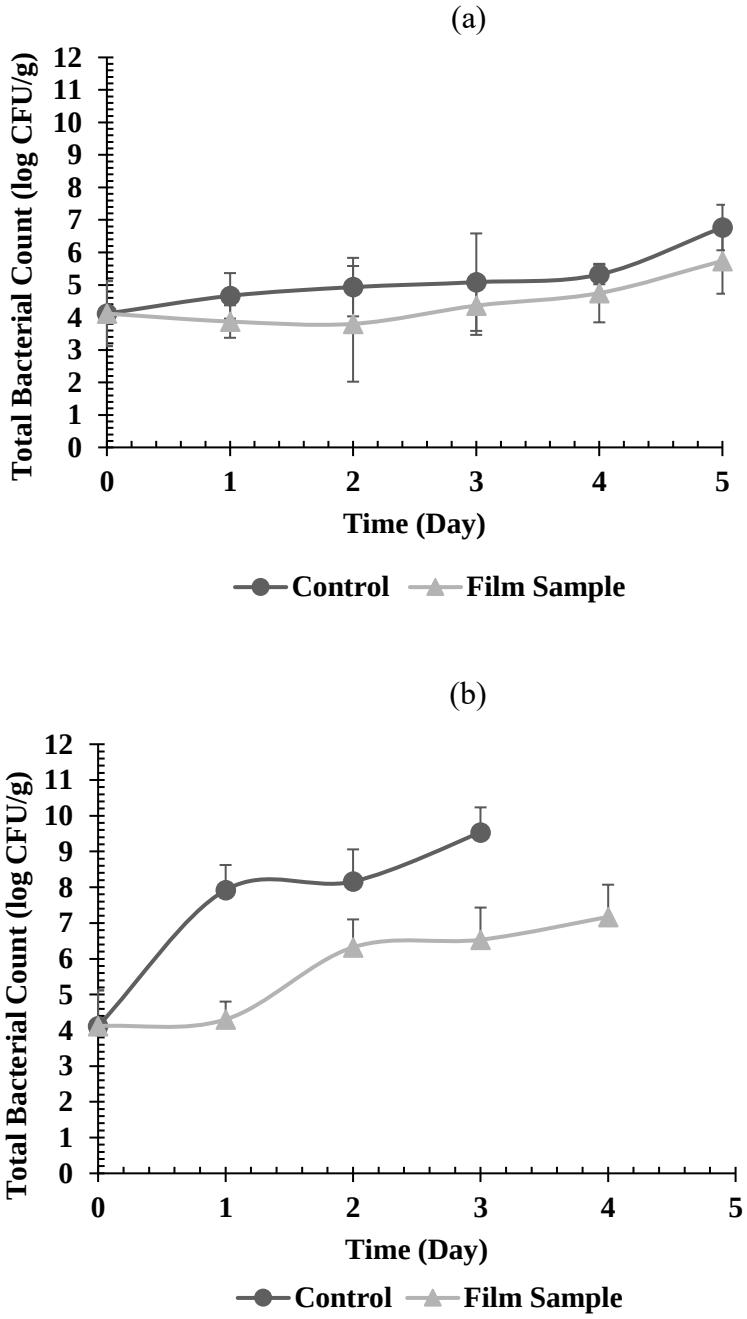
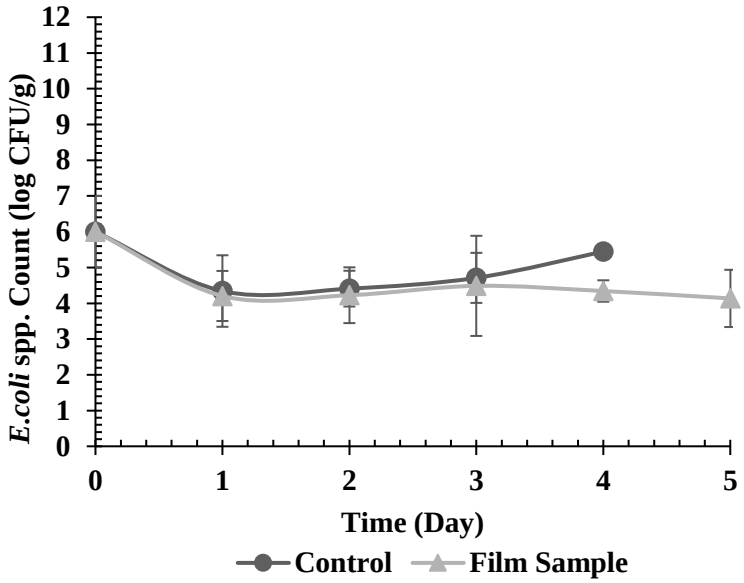


Figure 4. 5 Total *bacterial* count of chicken meat sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

(a)



(b)

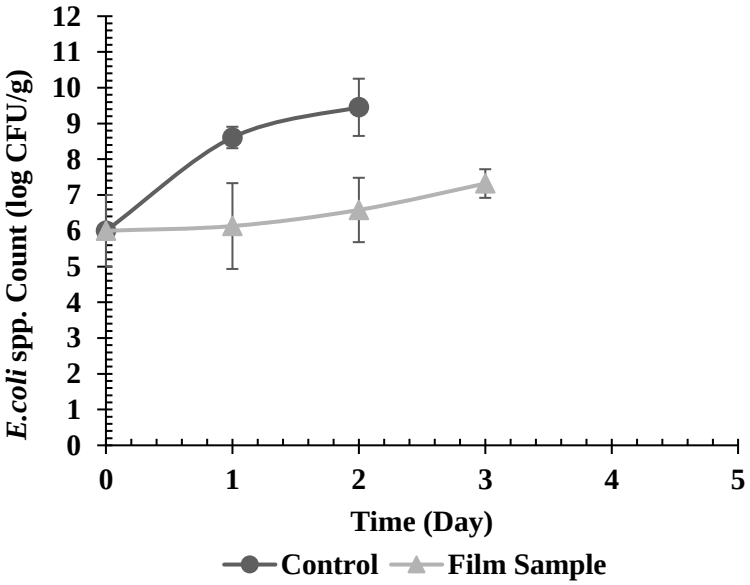
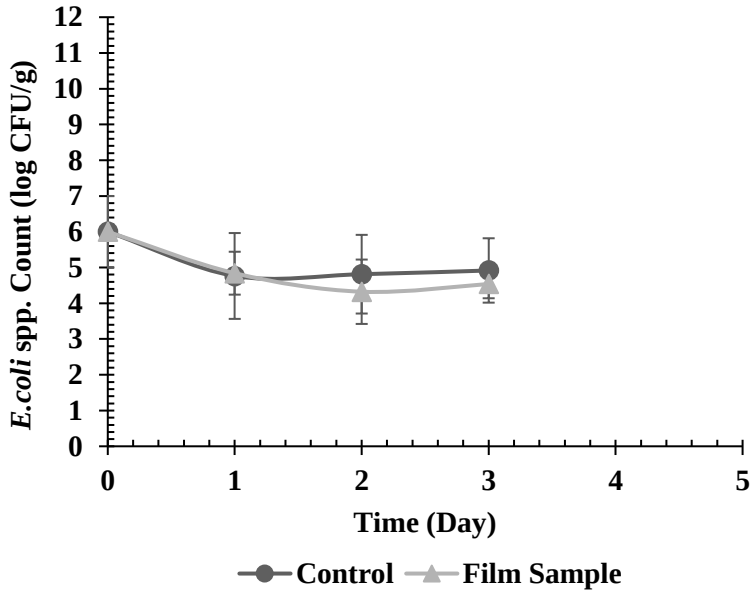


Figure 4. 6 *E. coli* spp. count of Salmon fish sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

(a)



(b)

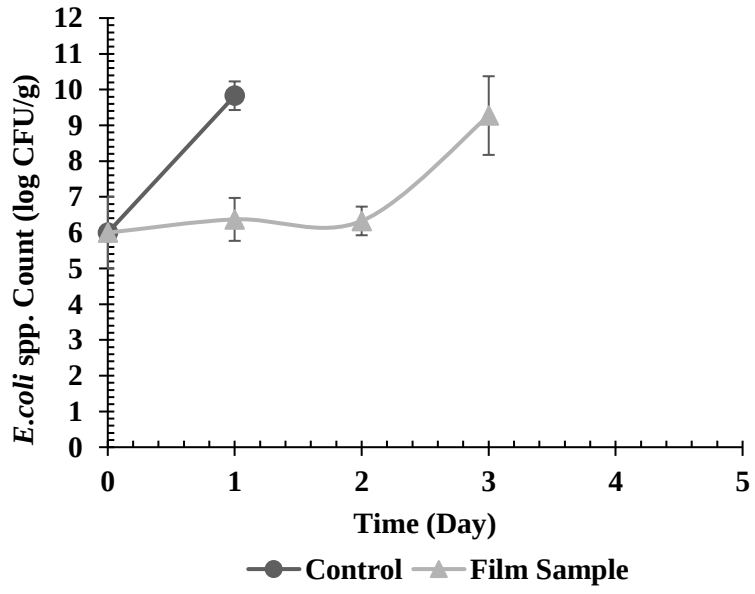
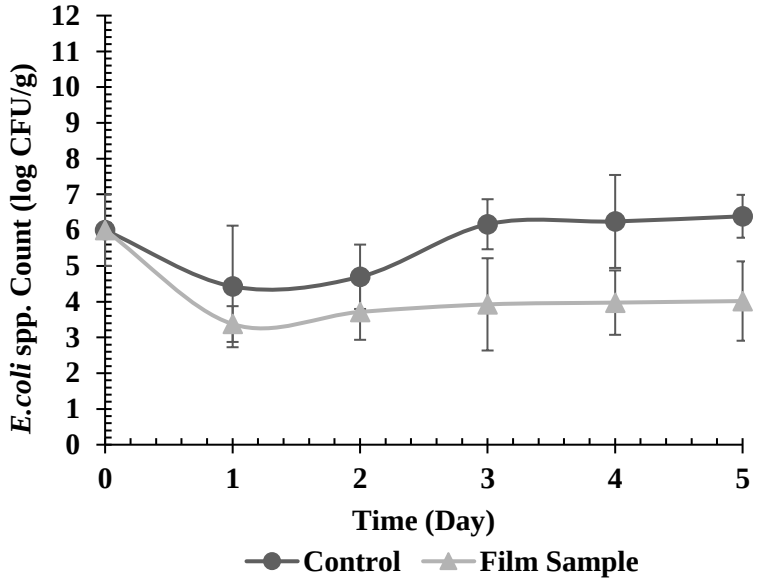


Figure 4. 7 *E. coli* spp. count of ground meat sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

(a)



(b)

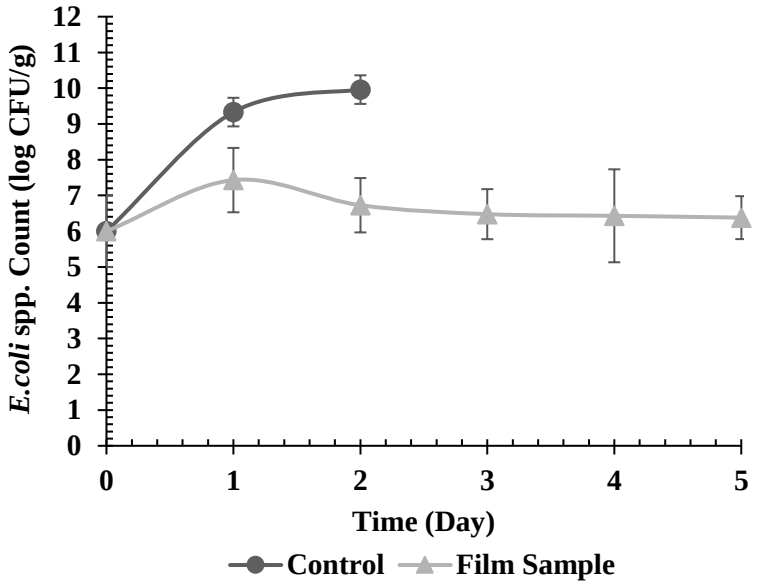


Figure 4. 8 *E. coli* spp. count of chicken meat sample wrapped with chitosan-based film incorporated with thyme essential oil nanoemulsions and stored at 4℃ (a) and 25℃ (b).

CHAPTER 5 – Conclusion

5. Concluding Remarks and Future Perspectives

5.1. Introduction

Foodborne disease is one of the most common problems in the food industry. It also represents a high risk for the consumer’s health. Foodborne illnesses arise from eating contaminated food products with pathogenic microorganisms such as bacteria, viruses, molds, and yeasts. The economic consequences of any foodborne outbreak incidence can be dramatic to both the food industry and companies' reputations. Therefore, there is an urgent need to find a practical solution to food poisoning problems and ensure the consumer’s safety.

5.2. Antimicrobial active food packaging

Antimicrobial active food packaging is an excellent innovative solution for foodborne illness. It is considered a reliable solution as an efficient active packaging material. It plays a vital role in inhibiting the growth of foodborne microorganisms, preventing food spoilage, extending food product shelf life, and enhancing food safety and quality. Antimicrobial active packaging materials are prepared throughout the incorporation of the antimicrobial agents with polymer matrix. Choosing the kind of polymer and the antimicrobial agent is critical to obtain the maximum benefits for the film properties and antimicrobial activity. The powerful effect of active packaging is its property to control the release of antimicrobial agents from the food packaging materials to the food surface. The main concept behind control release is the ability of the packaging materials to release antimicrobial agents at a gradual releasing rate that allows the presence of an adequate amount on the food surface to inhibit microbial growth. Many previous studies have been done to understand controlled release, especially in the pharmaceutical fields. However, it is still unclear regarding the food matrix and packaging materials. It is believed that testing the food packaging materials with physical food simulant

would be a valuable way to understand the controlled release from the food packaging materials to the food surface. Also, it would enable prediction of the released antimicrobial agent amount to the food surface, either in an adequate amount to inhibit the microbial growth or not, and expecting the food product shelf life. Antimicrobial active food packaging is believed to have significant advantages for fresh food products such as meat, fruits, and vegetables by extending their shelf life, maintaining their freshness, sustaining the nutrients content, and consequently reducing food wastes. Furthermore, protecting the consumers' health against foodborne pathogens and eliminating the use of chemical preservatives.

5.3. Chitosan-based film incorporated with thyme essential oil nanoemulsions.

Chitosan-based films have been incorporated with thyme essential oil nanoemulsions. Chitosan was chosen as a natural polysaccharide polymer due to its excellent film and antimicrobial properties. As well as its capability to be combined with other bioactive compounds. Thyme essential oil was selected as natural antimicrobial agents and was encapsulated into the form of nanoemulsion.

The study proves that the encapsulation of thyme oil into nanoemulsion form, and its integration with chitosan-based films persuade noteworthy changes in the film characteristics. TH-NE influences on the morphological property involved yellowish film color with less transparency, a greater light barrier, and further antioxidant property. In addition, TH-NE possesses a positive infuence on decreasing water solubility and improving the film's wettability (contact angle). Simultaneously, there was a declining in the water vapor barrier property, which may slightly restrain the investigated films to be applied to high water content food products.

On Contrary, TH-NE has a little negative impact on the mechanical characteristics by reducing the tensile strength and rising the elongation at break, which gives the film more

stretching capacity to be easily used in food wrapping applications. Furthermore, The thermal stability was enhanced by adding TH-NE with a greater degradation temperature.

Incorporating TH-NE with chitosan-based films bettered the antimicrobial activity of the films against foodborne pathogens (*E. coli* and *Bacillus subtilis* spp.). In addition, the film could expand the shelf-life of fresh raw beef meat inoculated with *E. coli* spp. to reach 6 days. We may suggest chitosan-based films incorporated with 2 wt% TH-NH for the prospective applicantions. TH-NE concentration was chosen based on the antimicrobial activity experimental results. Although increasing TH-NE concentration, it was found that there was non significant difference with a higher concentration in both agar well diffusion and agar diluted tests. In contrary, chitosan-based films incorporated with 2 wt% TH-NH had the ability to maintian the minimum bacterial count through the application to fresh raw beef meat and made an improvment in the antimicrobial activity.

Although we did not observe any smell arising from the chitosan-based film due to its integration with TH-NE, we may suggest a sensory evaluation test to confirm or negate any interferes of thyme oil with the natural flavor and odor of food. In summary, TH-NE has improved the functional properties of chitosan-based films and is recommended for potential applications in the food industry.

5.4. Evaluation of control releasee of thyme essential oil nanoemulsions from the chitosan-based film.

Understanding the releasing mechanism of thyme essential oil nanoemulsions (natural antimicrobial agent) from the packaging materials (chitosan-based film) to the food is critical to control their concentrations on the food surface and predict the food shelf-life. Therefore, thyme essential oil was analyzed to identify the main bioactive components which are thymol. As a result, it was found that thymol was the main bioactive component of thyme essential oil

with 30.9% relative concentration. For that reason, thymol was tracked during the study to evaluate the controlled release of chitosan-based films using physical fat food simulant.

Placing the chitosan-based film samples at a higher temperature of 40 ℃ resulted in releasing a higher concentration of thymol. At meanwhile, higher humidity 88% conditions resulted in releasing a lower concentration of thymol. The results showed a slower, gradual release of thymol, revealing that both temperature and humidity conditions could be used to control the releasing at a certain point. The results suggested that keeping the films at higher temperatures and low humidity would accelerate the release of the thymol from the film matrix to the food surface.

Both time-kill and *in-vitro* challenge microbiological tests were performed to evaluate the antimicrobial activity through time. The investigated films showed remarkable antimicrobial activity against *E. coli* and *Bacillus subtilis* spp. at the first 4-6th hours after contacting the bacterial culture. In addition, they showed the lowest viable bacterial cells count at the 6-8th h. At the same time, the investigated films showed remarkable antimicrobial activity against *Bacillus subtilis* and *E.coli* spp. when it kept under challenging conditions for 14 days. Chitosan-based films incorporated with thyme essential oils nanoemulsions showed an efficient bacteriostatic antimicrobial activity.

Although the obtained results showed an excellent control release property of chitosan-based film incorporated with thyme essential oil nanoemulsions, investigating the controlled release using the actual food system is highly recommended. This could be related to the complexity of the food matrix, which may affect in a different way on the film's control release. In summary, the chitosan-based film incorporated with thyme essential oil nanoemulsions showed a considerable control release and is recommended for potential application in the food industry.

5.5. Food Application

Controlled release antimicrobial packaging is an active system where the packaging materials act as a delivery vehicle. It can effectively ensure adequate antimicrobial agent amount on the food surface to prevent microbial growth. Therefore, there is an urgent need to evaluate the antimicrobial release of packaging materials in the real food system.

Chitosan-based film incorporated with thyme essential oils nanoemulsions was evaluated for its the controlled release of thymol from the film matrix to the animal butter product. The investigated films showed a gradual control release of thymol from the film material to the food product. They released around 99% of the initial thymol concentration to the butter surface by the end of the 4^{th} storage day, followed by a 48% reduction of the released thymol. In addition, the investigated films had a considerable low swelling %, suggesting the film suitability for high-fat content food application.

At the same time, chitosan-based films showed remarkable bacteriostatic antimicrobial activity by reducing the total *bacterial* count of the fresh food products (fresh salmon fish, ground, and chicken meat) under the standards limit. Chitosan-based film could extend the shelf life of fresh food products kept at refrigerator temperature. Meanwhile, it could minimize the bacterial risk during the first 24h for food products kept at 25 ℃. Chitosan-based films showed antimicrobial effectiveness with application to fresh food products and are highly recommended for potential applications in the food industry.

5.6. Comparison with previous literature.

Compared with other chitosan-based biodegradable films incorporated with essential oils, morphologically, in our study, chitosan-based films have a yellowish color with a total color difference similar to chitosan-based film combined with cinnamon oil in its natural form (Ojagh et al., 2010). However, in our study, chitosan-based films showed much better UV barrier and

antioxidants properties by having low transmittance values ranged between 48-23%. In previous studies, chitosan-based films incorporated with anise, orange, and cinnamon essential oils in their natural forms have transmittance values around 72, 70, and 69.3%, respectively (Escamilla-García et al., 2017). It worth mentioning that the lower the transmittance value, the higher the UV barrier and antioxidant properties.

Physically, the chitosan-based film incorporated with thyme essential oil nanoemulsions in our study showed thickness values ranged between 137-197 μm. The thickness is less than reported by incorporating chitosan-based films with rosemary essential oil in its natural form (Abdollahi et al., 2012). The thickness value in our study, making the film more appropriate for food wrapping application.

At the same time, chitosan-based films in our study gave surface density values ranged between 0.85-0.62 $g.cm^{-3}$. In comparison with previous studies, the surface of the film is smoother than incorporating chitosan-based films with citronella (1.10 $g.cm^{-3}$), cedarwood (1.12 $g.cm^{-3}$) (Shen & Kamdem, 2015), polyphenols (1.13 $g.cm^{-3}$) (Wang et al., 2013), anise (1.72 $g.cm^{-3}$), cinnamon (1.54 $g.cm^{-3}$), and orange (1.42 $g.cm^{-3}$) essential oils (Escamilla-García et al., 2017). However, smoother surface density referring to better wetting properties of films.

On the other hand, incorporating chitosan-based films with thyme essential oil nanoemulsions in our study increased the film moisture content to reach 62-59%. While decreasing water solubility to reach 62-58%, which is a higher value than those recorded by (Wang et al., 2013) when incorporating chitosan-based films with polyphenols. The films in our study showed less water resistance property comparing to previous studies. Meanwhile, in our research, chitosan-based films showed higher contact ranged 83.0-85.90 deg than those

reported by (Escamilla-García et al., 2017) around 70 deg. This result is revealing the better hydrophobic properties of chitosan-based films in this research.

Mechanically, incorporating thyme essential oil into the chitosan-based decreased the tensile strength significantly to 3.05-2.13 MPa. Thus, the results are found to be less than those reported by (Ojagh et al., 2010), who found tensile strength of chitosan-based film combined with cinnamon essential oil is around 13.35 MPa. At the same time, tensile strength in our study was increased to reach 117-119%, which much higher than those reported by (Wang et al., 2013), who found elongation at break of chitosan-based film combined with citronella and cedarwood essential oils are 36.54 and 33.0%. Thus, our results showed that chitosan-based films have more flexibility and it more appropriate to be used as wrapping film materials due to their stretchability.

Microbiologically, compared to the previous studies, chitosan-based film incorporated with oregano essential oil in its natural form showed more efficient antimicrobial activity against anise, basil, and coriander essential oils investigated in the same study (Zivanovic, S., Chi, S., Draughon, 2005). However, the chitosan-based film incorporated with oregano essential showed less antimicrobial activity compared to our current study. In the present research work, thyme essential oil was encapsulated in nanoemulsions, which protect thyme essential oils from partial loss of their volatile compounds, especially during the film preparation. Moreover, it positively affects the gradual release and prolonged antimicrobial activity (Huang et al., 2019).

In a previous study, the chitosan-based film incorporated with tea polyphenol was applied to pork meat patties containing 2 wt% sodium chloride stored at 4℃ (Qin et al., 2013). Chitosan-based films were able to inhibit bacterial growth during the 12 days storage period. As a result, the investigated films achieved a shelf-life extension of 6 days and maintained the

acceptable sensory qualities of pork meat patties. However, in our study, chitosan-based films incorporated with thyme essential oil nanoemulsions inhibited the microbial growth of fresh salmon fish, ground, and chicken meat and extended the shelf life for at least one day. In the mentioned study, the author added sodium chloride to the pork meat patties, which played an important role as an antimicrobial agent (SOFOS, 1984), extending the shelf life to 6 days.

In conclusion, chitosan-based film incorporated with thyme oil nanoemulsions would be a promising antimicrobial food packaging material with considerable packaging properties. Furthermore, it showed antimicrobial effectiveness with application to fresh food products and is highly recommended for potential applications in the food industry.

6. Acknowledgement

I am extremely grateful to my supervisor, Associate Professor Marcos Antonio Das Neves, Faculty Member of Graduate School of Life and Environmental Sciences, University of Tsukuba, for the continuous support, and patience during my Ph.D. study. Under whose keen supervision, interest, and criticism this study was carried out. I heartily thank him very much for his constant help to complete this thesis.

I would like to express my sincere gratitude to my esteemed Professor Mitsutoshi Nakajima, Formal Director of Alliance for Research on North Africa (ARENA), and Faculty Member of Graduate School of Life and Environmental Sciences, for the kindness, encouragement, advice, and tutelage during my Ph.D. degree.

My gratitude acknowledgment and heartily thanks to Professor Kunihiko Uemura, Head of Food Technology Unit, Food Research Institute, NARO, for his support, supervision, and all the facilities he provided.

I would like to thank the Electron Microscope Lab., Faculty of Life and Environmental Science, and Chemical Analysis Division, Research Facility Center for Science and Technology, University of Tsukuba, for allowing me to use Scanning Electron Microscope JEOL (JSM6330F), Gas Chromotgram-Mass Spectrometer Shimadzu (GCMS-QP2010 Plus), and Fourier-transform infrared FTIR spectroscopy measurement JASCO (FT/IR -6800) on chitosan-based films incorporated with thyme essential oils nanoemulsions, especially Professor Miyamura Shinichi, Mr. Gonda Hirose, and Mr. Shinohara Yosihiro for their great technical assistance and helpful suggestions.

My appreciation to Mr. CHANGWATCHAI Teetach, my colleague and lab mate, for his continuous assistance, and cooperation. Also, my sincere gratitude to Ms. Cheiko Takahashi, for her kind hospitality and cooperation.

My sincere appreciation and gratitude to Associate Professor Myra Villareal, Faculty Member of Graduate School of Life and Environmental Sciences, for her treasured support which was really influential to continue my Ph.D. especially during COVID-19 time. Also, I would like to express my hearty thanks to Ms. Sano Hiromi, a Member of the Tsukuba Life Science Innovation Academic office, for all her support, understanding, and cooperation. I believe without the great help from Ass. Prof. Myra and Ms. Sano, I would not be able to continue my Ph.D. program.

Last but not least, my gratitude to my beloved husband “Hussein,”. Pandemic has made my Ph.D. life very difficult, and I believe without the love, support, kindness, and motivation that Hussein gave me, I would not be able to survive in a such difficult time.

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