INHIBITORY EFFECTS OF LIQUID- AND VAPOR-PHASE THYMOL VIA DIFFERENT MODES OF DELIVERY ON THE GROWTH OF *ESCHERICHIA COLI* DH5α

By

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Kit L. Yam

And approved by

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New Brunswick, New Jersey

January 2020

ABSTRACT OF THE DISSERTATION

Inhibitory Effects of Liquid- and Vapor-Phase Thymol via Different Modes of Delivery on the Growth of *Escherichia coli* DH5α

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Although thymol is a natural antimicrobial with broad-range activities, it is not suitable to be added directly as an ingredient into the food formulation (a delivery mode known as "instant addition"), because effective microbial inhibition requires high concentrations of thymol, while its strong aroma is undesirable to consumers. Meanwhile, high concentrations mean high cost, which discourages food manufacturers from substituting synthetic preservatives with thymol. In order to expand the applications of thymol as a food antimicrobial, an immediate challenge is to reduce the required concentration to below sensory and economical threshold. This challenge may be addressed by exploring novel delivery modes instead of the traditional instant addition.

Controlled release packaging (CRP) is an innovative technology, which releases active compounds from packaging material at targeted rates in a controlled manner over extended storage, in order to enhance the quality and safety of foods. Previously,

controlled release of the antimicrobial nisin has been reported to be more effective in inhibiting the growth of *Micrococcus luteus* than instant addition. It has also been proposed that there's an optimum range of release profiles, called "target release profile", which exhibits effective antimicrobial activity using as little antimicrobial as possible. This study aims to further understand the relationship between release profiles, the resulting concentration profiles and microbial inhibition, and to adopt the unique characteristics of controlled release to extend the potential applications of thymol as a food antimicrobial.

In the first part of the study, liquid-phase thymol was released via different modes of delivery in order to compare their effectiveness, and the microbial growth kinetics were measured to link back to the release kinetics, in order to further unveil the underlying mechanism for the dependence of inhibitory effects on delivery modes.

Since most studies on the antimicrobial activity of liquid-phase thymol were conducted via instant addition, there is a lack of knowledge about how different modes of delivery affect the inhibitory effect of thymol, especially when a combination of two or more delivery modes is used, such as instant addition plus controlled release. The rationale behind using a combined mode of delivery lies within a practical consideration: in practice, due to technical limitations, a single delivery mode may not be able to suffice the requirement of effective inhibition, while a combined mode provides more flexibility. Therefore, the objective of the first part is to compare the inhibitory effects of liquid-phase thymol via three delivery modes (instant addition, controlled release, and combined mode) on the growth of *Escherichia coli* DH5α, and to find the relationship between the release (and the resulting concentration) profile and the corresponding inhibitory effect,

in order to propose basic requirements for an effective release profile and to narrow down the range of the optimum release profile.

The results showed that via controlled release and combined mode, the minimum inhibitory concentrations of thymol were lowered by 25% and 50% respectively, as compared to instant addition. It could be proposed that in order to effectively inhibit microbial growth, an instant inhibitory effect must be achieved in the beginning, followed by sustained release at later time to maintain the effect. Combined mode of delivery in the study was able to satisfy both requirements, making it the most effective. Specifically, a minimum of 123 mg/L thymol must be released during the inherent lag phase (1.14 hours), in order to provide an instant inhibitory effect as proposed. This minimum dosage can be achieved using a combination of instant addition and controlled release. Based on the amount instantly added at time zero, a subsequent release profile can be designed using Crank's diffusion model. By varying the amount instantly added, various release profiles can be generated, to accommodate the variable conditions in real-life situations and to best guarantee effective inhibition throughout the shelf life of foods.

In the second part of the study, vapor-phase thymol was released via instant addition and controlled release, and the correlation between the actual concentration profile and microbial growth was investigated, followed by linking back to the release profile.

Few previous studies have shown that via instant addition, thymol and thyme oil in vapor phase are effective against bacteria and fungi, utilizing less concentration than those in liquid and solid phase. However, current evidence is still lacking, and more research is needed to substantiate the prominent antimicrobial activity of vapor-phase thymol. Moreover, upon addition, thymol's vapor concentration is subject to constant change.

There is little knowledge of thymol's actual concentration profile upon its addition, and a lack of understanding of how the profile may affect microbial growth over time. It is plausible to hypothesize that concentration profiles of varying shapes will affect the inhibitory effect differently. Therefore, it is important to verify the antimicrobial activity of vapor-phase thymol, as well as to identify the actual concentration profile and correlate it with the antimicrobial behavior over time.

Furthermore, controlled release of a deliberate design has rarely been exploited in vapor-phase antimicrobials, leaving a knowledge gap concerning the effects of vapor-phase antimicrobials via controlled release. Thus, it is of necessity to test the feasibility of controlled release using vapor-phase thymol. It can be hypothesized that by controlling the release of vapor-phase thymol, it may be possible to generate an effective concentration profile with lower amount used, thereby enhancing the possibility of thymol as a food antimicrobial. Therefore, the objective of the second part is to investigate the inhibitory effect of vapor-phase thymol against *Escherichia coli* DH5α via instant addition and controlled release.

The results showed that via instant addition, vapor-phase thymol was more effective against *Escherichia coli* DH5α than liquid- or solid-phase thymol, with a MID of 8 mg/L headspace, as compared to a MIC of 500 mg/L or 320 mg/L agar, respectively. The results also showed that the headspace concentration, although at a much lower level in comparison to the added dose, was the main contributor to thymol's antimicrobial activity, and a growth phase of 4 hours in the profile when the headspace concentration keeps increasing is necessary for effective inhibition. Controlled release of vapor-phase thymol was able to achieve the same inhibitory effect with only 41% of the amount used

compared to instant addition. The underlying reason for its higher effectiveness is that by continuously supplementing the lost portion, controlled release created a concentration profile that satisfied the 4-hour growth phase requirement and thus effectively inhibited microbial growth with lower amount added.

To conclude, this work identified the important role that delivery modes play in affecting the inhibitory effect of an antimicrobial. It further enhanced our understanding of the relationship between release profile, concentration profile, and microbial inhibition. It provided implications for the design of CRP, using the natural antimicrobial thymol as an alternative to synthetic preservatives.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my advisor, Professor Kit L. Yam, for his help, guidance and support throughout these years. When I first started in Dr. Yam's lab, I was a lost person. Being fresh out of college, I had no idea how to initiate independent scientific research. While my peers all got off to a good start, I was still struggling with a research topic. Dr. Yam had been nothing but understanding and encouraging, which gave me the courage to persist my study. Dr. Yam had taught me nothing but to think independently and critically, which helped me come this far to today. More importantly, Dr. Yam was the most patient coach in communication skills, which, I strongly believe, would benefit me not only academically but also professionally and personally. Last but not least, I'm very thankful for Dr. Yam's open-mindedness, which allowed me to embrace all kinds of opportunities, instead of being confined to the laboratory. I owe what I am today to Dr. Yam, who will be my lifetime role model.

I would like to thank Dr. Paul Takhistov, Dr. Nazir Mir and Dr. Michael Chikindas. They are not only incredible teachers, from whose courses I benefited a lot, but also my mentors, who helped steer my research in the right direction. I would especially like to show my deepest appreciation to Dr. Chikindas, who kindly agreed to step in and serve on my committee upon my presumptuous request when I faced the challenge of losing one committee member due to an unexpected incident. Although it eventually worked out, the generosity of Dr. Chikindas will always be remembered. I would like to thank Dr. Yan Xu for kindly agreeing to be the outside member, for the research and life

suggestions she gave me, and for being very accommodating with regards to my final defense.

I would not have accomplished my work without the kind help of my lab mates. I would like to express my heartfelt gratefulness to Dr. Jianming Sun, a visiting scholar from Henan University of Science and Technology, who selflessly assisted me in running GC programs during unwieldly hours. I would like to thank Xi Chen, Yan Zhang and Mo Chen for their insightful advices and technical assistance in times of need. I would also like to thank all previous lab members, who made the Yam lab a warm family for all.

I would like to thank William (Bill) Sumal and David (Dave) Petrenka for helping me build apparatus for my experiments. Due to the unpredictable and changeable nature of experiments, many adjustments and do-overs had to be done. Bill and Dave gave me great patience and managed to meet my requirements very timely.

Finally, I would like to express my love and appreciation to my family and friends, for their unconditional support. I would like to especially thank my husband, Dr. Heng Pan, who is always there for me, for better or for worse. I will not be me without the people around me, and I cannot be more grateful for their presence in my life.

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1. INTRODUCTION

Foodborne diseases are a growing public health concern worldwide. In the United States alone, Centers for Disease Control and Prevention (CDC) estimated that 31 major pathogens cause 9.4 million cases of foodborne illnesses each year [1, 2]. Microbial contamination is one of the main sources of foodborne illnesses. To control foodborne pathogenic microorganisms, many preservation techniques are used in the manufacture and storage of food products [3]. Currently, food preservatives such as benzoates, lactates, nitrates, and sulfites are primarily used to control foodborne pathogens and to extend the shelf life of foods [4]. Nevertheless, as consumers have expressed the desire to reduce the use of synthetic additives in foods, there is a need to develop novel preservation methods using natural antimicrobials [5].

Thymol, also known as 2-isopropyl-5-methylphenol, is a natural monoterpenoid phenol and a major constituent of many essential oils (EOs), such as thyme oil [3]. Over the past decades, the antimicrobial activity of thymol has been extensively studied against a wide range of microorganisms, including Gram-positive and -negative bacteria, yeasts and molds [6-12]. Thymol has been shown to demonstrate broad range of antimicrobial activities [13]. Although the mode of action is not fully known, thymol is believed to involve in the disruption of inner and outer membranes of microorganisms, and in the interaction with membrane proteins and intracellular targets [3, 14-16]. Thymol is classified as generally regarded as safe (GRAS) by the United States Food and Drug Administration (FDA), and it is registered by the European Commission for the use as

flavoring in foods [3, 13]. Therefore, it is an attractive natural alternative to traditional preservatives.

Unfortunately, effective application of thymol often requires high concentrations, and this limits its use as a food preservative. In prior studies, thymol was added to liquid- or solid-phase medium and brought into direct contact with target microorganisms. The results shed light on the potential of using thymol as a natural antimicrobial in foods, by directly adding it into the food formulation, a delivery mode known as “instant addition” [17]. However, instant addition of liquid- or solid-phase thymol can have limited applications. In the food industry, it is common to use instant addition to control microbial growth. Although instant addition could achieve an immediate inhibition of undesired microorganisms, as the antimicrobial is being consumed in the reaction, the surviving microorganisms will resume growth, rendering food safety and quality at risk [18]. In addition, due to the complex interactions with food matrix, the efficacy of antimicrobials may be reduced [19, 20]. Consequently, instant addition of antimicrobials into the food formulation often requires excessive amount for effective inhibition [21]. In spite of its GRAS status, thymol exhibits moderate cytotoxicity [22, 23], thereby increasing its concentration in the formulation may not be permissible. Also, the intense aroma from high concentrations of thymol may cause negative organoleptic effects, causing the consumers to reject the products [24]. Lastly, as a natural antimicrobial, thymol is of higher cost than the common synthetic preservatives, thus adding high concentrations of thymol into foods is not economical to food manufacturers.

In order to broaden the applications of thymol, an immediate challenge is to reduce the concentration of thymol to below the safety, sensory and economical thresholds. This

challenge may be addressed by two approaches: one, exploiting novel modes of delivery instead of the traditional instant addition, and two, utilizing vapor-phase thymol instead of liquid- or solid-phase thymol.

Controlled release refers to the delivery of antimicrobials at a pre-determined rate over time. Compared to instant addition, it has been proposed to be a more effective delivery mode. In a previous study, controlled release of liquid-phase nisin has been shown to inhibit the growth of *Micrococcus luteus*, using only 15% of the amount used in instant addition [17]. It can be inferred from that study that by selecting the right mode of delivery, it may also be possible to reduce the amount of thymol to a level acceptable to both consumers and manufacturers, thereby increasing the potential of thymol as a food antimicrobial agent.

Since most research studies on the antimicrobial activity of liquid-phase thymol were conducted via instant addition, there is a lack of knowledge about how different modes of delivery affect the antimicrobial effect of thymol, especially when a combination of two or more delivery modes is used.

To fill this knowledge gap, the inhibitory effect of liquid-phase thymol against *Escherichia coli* DH5α via three modes of delivery – instant addition, controlled release, and combined mode (instant addition plus controlled release) – was investigated. The investigation involved the following tasks: (1) compare the minimum inhibitory concentration (MIC) of thymol via the three delivery modes, (2) study the growth kinetics of *E. coli* DH5α under the three modes, and (3) provide a rationale for the inhibitory effects via controlled release and combined mode.

Compared to liquid- and solid-phase, vapor-phase thymol has been less researched. Few previous studies have shown that via instant addition, thymol and thyme oil in vapor phase are effective against bacteria and fungi using less concentration, compared to those in liquid and solid phase [25-27]. However, current evidence is still insufficient, and more research is needed to substantiate the prominent antimicrobial activity of vapor-phase thymol. Moreover, prior studies failed to account for thymol's concentration profile over time. Although thymol is a volatile compound, a vapor pressure of 0.016 mm Hg at 25 °C [28] indicates that not all amount added will vaporize and the actual vapor concentration cannot be derived from simple calculation. In addition, in the headspace, vapor-phase thymol will be degraded by reactions with photochemically-produced hydroxyl radicals [29], or absorbed into the agar layer [30]. Therefore, the actual concentration in both the headspace and the agar layer should not be constant but should be reflected as a profile of concentration versus time. There is little knowledge of thymol's actual concentration profile upon its addition, and a lack of understanding of how the profile may affect microbial growth over time. It is plausible to hypothesize that concentration profiles of varying shapes will affect the inhibitory effect differently. Therefore, it is important to verify the antimicrobial activity of vapor-phase thymol, as well as to identify the actual concentration profile and correlate it with the antimicrobial behavior over time.

To further extend the applications of vapor-phase thymol, like the rationale of using controlled release to deliver liquid-phase thymol, controlled release can also be exploited to apply vapor-phase thymol instead of the traditional instant addition. Currently, there is a scarcity of knowledge concerning the antimicrobial activity of vapor-phase thymol in a

controlled-release manner. It can be hypothesized that by delivering vapor-phase thymol in a controlled manner, it may be possible to generate an effective concentration profile with less amount used, thereby enhancing the possibility of thymol as a food antimicrobial. A better understanding of how controlled release affects the antimicrobial activity of vapor-phase thymol can lay the foundation for controlled release packaging (CRP), where active compounds are incorporated into the packaging material and released in a controlled, timely manner, in order to enhance the quality and safety of foods [31-33].

Therefore, the objective of studying vapor-phase thymol is to investigate the inhibitory effect of vapor-phase thymol against *Escherichia coli* DH5α via instant addition and controlled release. The investigation involved the following tasks: (1) identify the minimum inhibitory dose of vapor-phase thymol via instant addition and compare it to the minimum inhibitory concentrations of liquid- and solid-phase thymol, (2) investigate the concentration profiles of thymol in the headspace and the agar layer to find correlations with the antimicrobial activity over time, (3) study the inhibitory effect of vapor-phase thymol via controlled release and compare it to the effect via instant addition, and (4) obtain the headspace concentration profile of the optimal release profile and identify the underlying reason for its inhibitory effect.

2. LITERATURE REVIEW

2.1. Foodborne Illnesses as a Global Concern

Foodborne diseases are a rising public health concern worldwide. In the United States alone, Centers for Disease Control and Prevention (CDC) estimated that 48 million cases of foodborne illnesses occur each year [1, 2]. Among them, 31 major pathogens cause 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths each year [2]. During the 10-year period from 1998 to 2008, out of the 7,998 outbreaks with a known etiology, bacteria caused 45% of the foodborne illnesses, virus (mostly norovirus) infection took up another 45%, chemicals and toxic agents contributed 9%, and the remaining 1% was caused by parasites (Figure 1) [34]. Therefore, to prevent foodborne diseases, microbial contamination is a serious issue that needs to be addressed throughout the entire food supply chain from production, transportation, storage and retail.

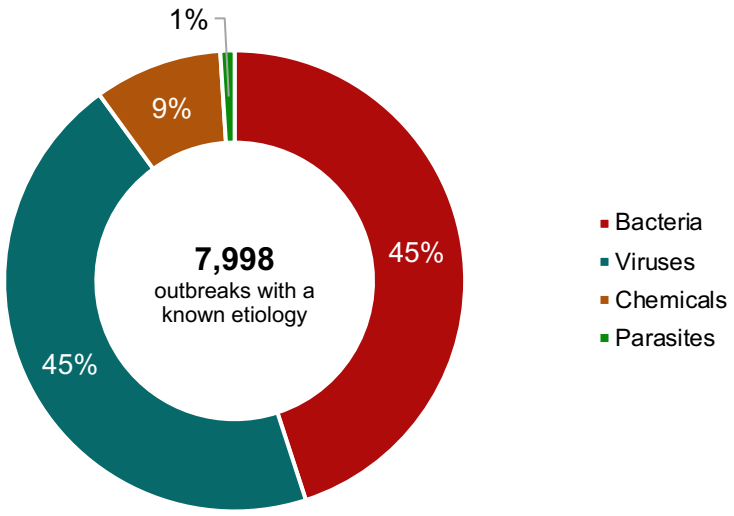

Figure 1 Percentages of foodborne illnesses caused by known etiologies. Data obtained from [34]

2.2. Current Food Preservation Technologies

Microbial contamination can occur at any point throughout the food supply chain, between harvest (or slaughter) of the raw material and consumption of the final product by the consumer [35]. Therefore, in order to successfully control foodborne pathogens, various preservation techniques are required in the manufacture and storage of food products [3]. Based on their modes of action, current food preservation techniques can be categorized into three major classes: (1) inhibition of microbial growth, (2) direct inactivation of microorganisms, and (3) restricting access of microorganisms to foods [35, 36]. Figure 2 demonstrates some of the major preservation technologies that are currently employed, separated by the above three classes.

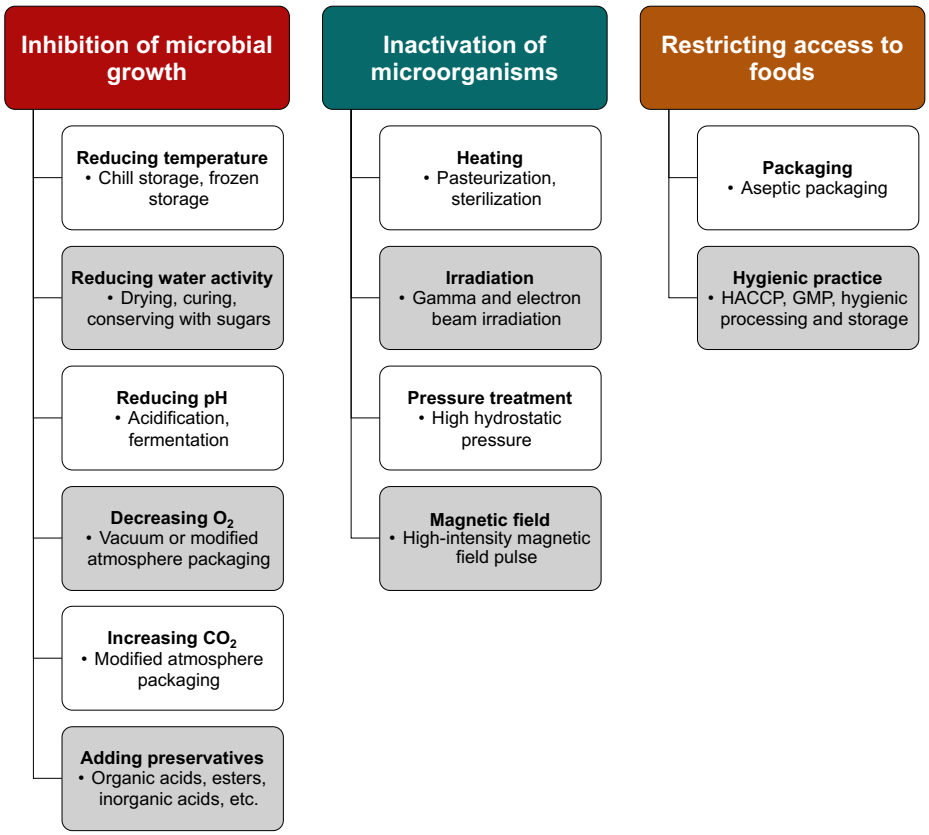

Figure 2 Major preservation techniques currently employed. Adapted from [35]

Although multiple preservation techniques can be utilized, currently food manufacturers rely heavily on traditional antimicrobial preservatives as the primary tools for controlling foodborne pathogens and extending shelf life of foods [4]. Some of the traditional food antimicrobials are summarized in Table 1. Most of the antimicrobials listed in Table 1 function to slow down, or in some instances completely inhibit, microbial growth [35]. However, the surviving microorganisms will resume growth once the antimicrobials are consumed/degraded or when the surviving cells are exposed to favorable conditions, such as temperature abuse during transportation, rendering food safety as risk. Therefore, with respect to ensuring food safety, direct inactivation is more appealing than inhibition.

Table 1 Traditional food antimicrobials approved by the Food and Drug Administration (FDA). Adapted from [4]

Group	Compounds	Microbial Target	Primary Food Applications
Acetates	Acetic acid, acetates, diacetates, dehydroacetatic acid	Yeasts, bacteria	Baked goods, condiments, confections, dairy products, fats/oils, meats, sauces
Benzoates	Benzoic acid, benzoates	Yeasts, molds	Beverages, fruit products, margarine
Carbonates	Dimethyl dicarbonate	Yeasts	Beverages
Lactates	Lactic acid, lactates	Bacteria	Meats, fermented foods
Nitrites/nitrates	Nitrites, nitrates	*Clostridium botulinum*	Cured meats
Parabens	Alkyl esters (propyl, methyl, heptyl) of p-hydroxybenzoic acid	Yeasts, molds, Gram-positive bacteria	Beverages, baked goods, syrups, dry sausage
Propionates	Propionic acid, propionates	Molds	Bakery products, dairy products
Sorbates	Sorbic acid, sorbates	Yeasts, molds, bacteria	Most foods, beverages, wines
Sulfites	Sulfites, metabisulfites, hydrogen sulfites	Yeasts, molds	Fruits, fruit products, potato products, wines

2.3. Drawbacks of Current Applications of Food Preservatives

Current applications of synthetic preservatives have drawbacks in two major aspects. Firstly, due to the long-running debate over the safety of traditional chemical preservatives, consumers have expressed the need to reduce the use of synthetic additives in foods. In fact, a survey performed by Nielsen [37] reported that more than 50% of consumers worldwide were avoiding artificial preservatives in food products, as of 2016 (Figure 3). Therefore, there is a clear necessity to discover novel preservation methods using natural antimicrobials [5].

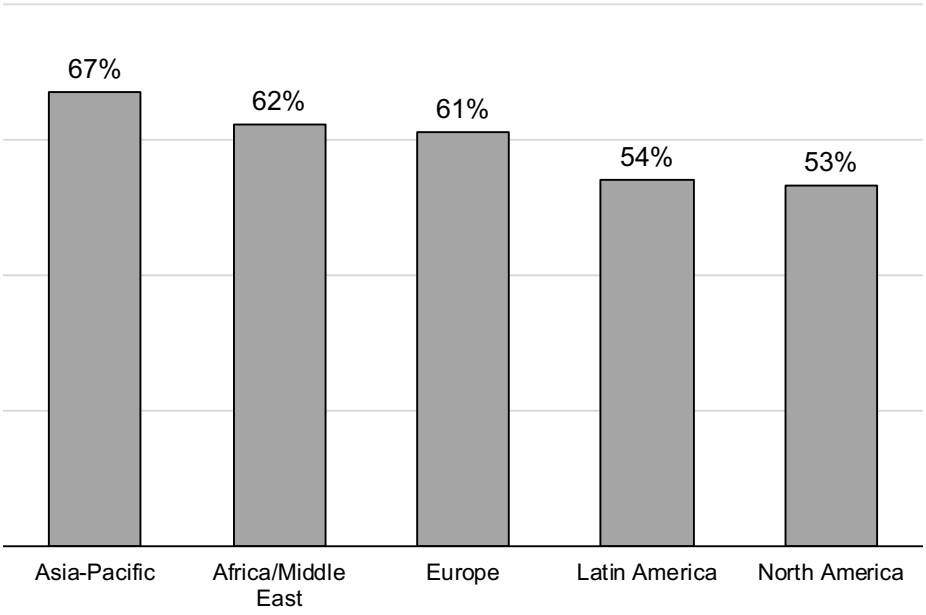

Figure 3 Share of consumers avoiding artificial preservatives in foods as of 2016, by region [37]

Secondly, the mode of delivery for antimicrobials is currently very limited. In the food industry, the common practice to control microbial growth is carried out via “instant addition”, a delivery mode where antimicrobials are directly added as an ingredient into

the food formulation [17]. The advantage of instant addition is obvious: via instant addition, undesired microorganisms can be inhibited immediately. However, as the antimicrobial is being consumed by the target microorganisms or degraded naturally, the surviving microorganisms will resume growth, leaving food safety and quality at risk [18]. In addition, due to the complex interactions with food matrix, the efficacy of antimicrobials may be reduced [19, 20]. Consequently, adding antimicrobials into food formulation often requires excessive amount for effective inhibition [21]. Although rarely reported, since a large quantity of antimicrobials is added instantly, there might be an unresolved risk of antimicrobial resistance developed in surviving microorganisms. Additionally, instant addition is unsuitable for some types of foods, such as fresh produce. These types of foods are thus often unprotected, making them potential risk factors of microbial contamination and foodborne illnesses.

Therefore, in order to address these two drawbacks, there is a clear necessity to search for new, more natural alternatives that can substitute the currently used chemical preservatives [36], as well as to uncover novel delivery modes to replace instant addition.

2.4. Overview of Natural Antimicrobials

Many natural antimicrobials have been discovered over the past decades, and several have already been applied in food preservation, in order to serve as an alternative to synthetic preservatives [35]. Based on their origin, natural antimicrobials can be classified into three categories: animal-derived, plant-derived and microorganism-derived.

2.4.1. Animal-Derived Antimicrobials

In animals, there's a wide range of antimicrobial systems and substances, which include the immune system in higher animals, various bacteriolytic enzymes, and non-enzymatic antimicrobial proteins or peptides [35].

Among the animal-derived antimicrobials, lysozyme perhaps possesses the greatest success in commercial applications. Lysozyme is a bacteriolytic enzyme, found in large quantities in body fluids, and commercially sourced from hen's egg white [38, 39]. It is effective against many Gram-positive bacteria in their vegetative forms, thus leading to its most successful application of inhibiting the outgrowth of *Clostridium tyrobutyricum* spores in certain types of cheeses [40]. However, when used alone, lysozyme is inactive against Gram-negative bacteria, as their outer membranes prevents lysozyme's access to the underlying peptidoglycan, which is the enzyme's substrate [35]. To extend lysozyme's potential applications, many methods have been proposed. To strengthen its biological properties, lysozyme can be modified by heat, chemicals or hydrolysis [41]. To enhance its effectiveness against Gram-negative bacteria, lysozyme can be combined with other agents, such as EDTA [42], dextran [43], lactoferrin [44], and nisin [45, 46].

Although most of the methods are still pending FDA approval, they have shown the prospective future to apply lysozyme in various food substrates, in order to improve the quality and safety of foods.

2.4.2. Plant-Derived Antimicrobials

Plants produce a vast variety of secondary metabolites that play an important role in defending plants against microbial infections, pests, predators and UV radiation, due to their biocidal effects against microorganisms and repellence to herbivores [47, 48]. Secondary metabolites are distinct from primary metabolites, as they are generally non-essential to the basic metabolism of plants [49]. To date, more than 100,000 plant secondary metabolites have been identified, most of which are believed to involve in the immune system of plants, exhibiting antimicrobial potentials [49].

Essential oils (EOs) are aromatic and volatile liquids extracted from plant materials, such as buds, flowers, leaves, stems, twigs, seeds, peel, fruits, roots, wood, bark, and whole plant [50-52]. Nowadays, EOs are commonly used in the food and cosmetic industry as natural flavorings and fragrances, and are well recognized for their antibacterial, antifungal and antiviral properties [53]. The major constituents of EOs can be classified into three groups: terpenes, terpenoids, and aromatic compounds, all with a key characteristic of low molecular weight [48, 53]. The antimicrobial activities of EOs are mainly attributed to the presence of oxygenated terpenoids, such as alcohols and phenolic terpenes [54, 55]. However, complex interactions between the bioactive components may lead to additive, synergistic, or antagonistic effects. Chemical structures of some representative EO constituents are shown in Figure 4.

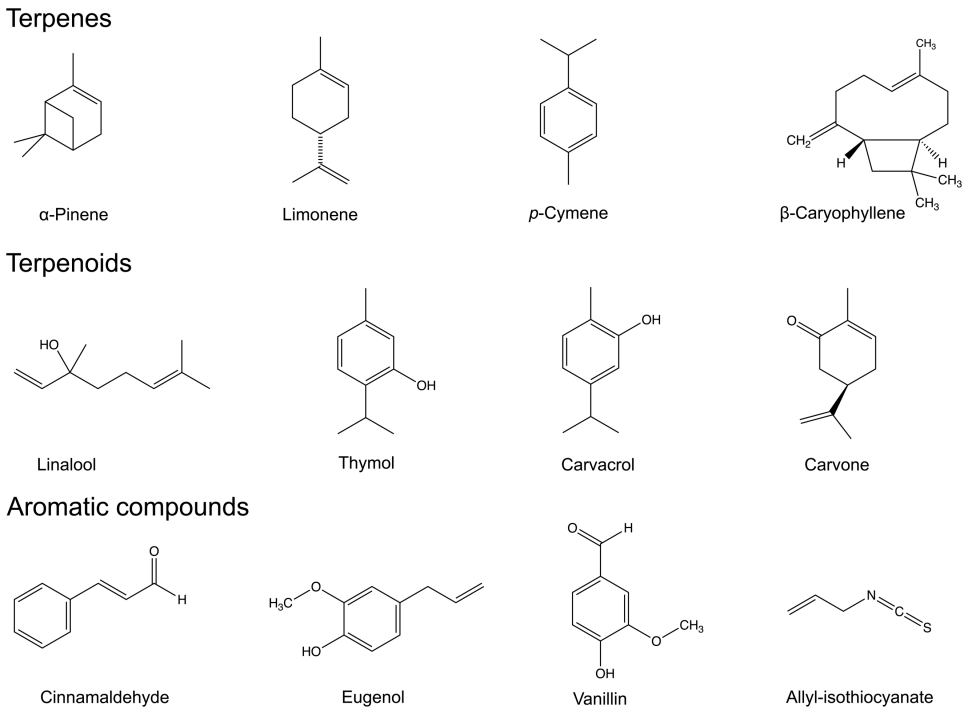

Figure 4 Chemical structures of common EO constituents

Terpenes are hydrocarbons generated from the combination of multiple base units of five carbons, known as the isoprene units (C_5H_8). Monoterpenes (two isoprene units, $C_{10}H_{16}$) and sesquiterpenes (three isoprene units, $C_{15}H_{24}$) are most commonly seen, but longer chains such as diterpenes (four isoprene units, $C_{20}H_{32}$) also exist [3]. Some examples of terpenes include *p*-cymene and limonene. In general, terpenes possess low or absent antimicrobial activity [8, 12, 56-58].

Terpenoids are terpenes that contain oxygen and move or remove methyl groups [3]. Some examples of terpenoids include thymol, carvacrol, menthol, and linalool. In general, terpenoids are active against a wide range of microorganisms, with thymol and carvacrol being the most active monoterpenoids identified so far [6, 8, 11, 12, 59].

Aromatic compounds commonly found in EOs are phenylpropenes, a subfamily of phenylpropanoids which are derived from phenylalanine. Phenylpropenes are characterized with a six-carbon aromatic phenol group, and a three-carbon propene tail [3]. Some examples of phenylpropenes include eugenol, vanillin and cinnamaldehyde, which appear to be potent antimicrobials [59-61]. Other EO constituents contain various degradation products, such as allicin and allyl isothiocyanate (AITC).

2.4.3. Microorganism-Derived Antimicrobials

Bacteria produce compounds to inhibit the growth of other bacteria. These compounds include organic acids, hydrogen peroxide, diacetyl, bacteriocins and other bactericidal proteins [62].

Bacteriocins are proteinaceous or peptidic antibacterial toxins, produced by both Gram-positive and -negative bacteria. They have been proposed to be classified into as many as five classes, including:

Class I: small, post-transcriptionally modified lantibiotics, such as nisin;

Class II: small, unmodified, heat-stable peptides, such as *pediocin* PA-1;

Class III: large, heat-labile proteins, such as lysostaphin [63].

Nisin, produced by some strains of *Lactococcus lactis*, is the only natural bacteriocin that has been approved by the FDA as a food preservative. Nisin was found to interact with the phospholipids in the bacterial cell membranes, thereby disrupting the membranes [38]. Its main targets are Gram-positive and spore-forming bacteria, such as *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus cereus* [64]. However, when used alone, its activity against Gram-negative bacteria is at least 100-fold lower, because

it’s unable to penetrate the lipopolysaccharide layer of the outer membrane, which restrains its access to the inner membrane [65].

2.4.4. Prospect of Natural Antimicrobials

It can be clearly seen that in nature, there is an immense number of effective antimicrobials. However, with respect to their exploitation, there’s only a few that have been approved and commercialized for use in foods. It may be partly due to the absence of economic incentives to replace the current chemical preservatives – natural antimicrobials are generally less efficient but more expensive than synthetic chemicals. The financial burden to embark on the enduring but necessary testing before introducing a new antimicrobial may also prevent manufacturers from the research and development of new ingredients and procedures.

But this will change soon, in response to consumers’ needs for natural alternatives. The widening market opportunities will sooner or later spark a revolution in the food industry. There’s no doubt that natural antimicrobials will play an increasingly important role in the future, particularly in combination with other factors and techniques [35]. To accelerate the process, researchers are obligated to undertake more studies, both *in vitro* and in *vivo*, to identify tailored, more economical methods for applying effective natural antimicrobials in certain types of foods.

2.5. Thymol

Thymol, also known as 5-methyl-2-(propan-2-yl)phenol or 2-isopropyl-5-methylphenol, is a natural phenolic monoterpenoid, and a major constituent of the EOs extracted from plants in the *Lamiaceae* family, such as those of the genera *Thymus*, *Ocimum*, *Origanum*, *Satureja*, and *Monarda* [66-70]. These genera include several important groups of culinary herbs, such as thyme, basil, oregano and savory (Figure 5a). In plants, thymol is synthesized by the aromatization of γ-terpinene to *p*-cymene, followed by hydroxylation of *p*-cymene (Figure 5b) [71].

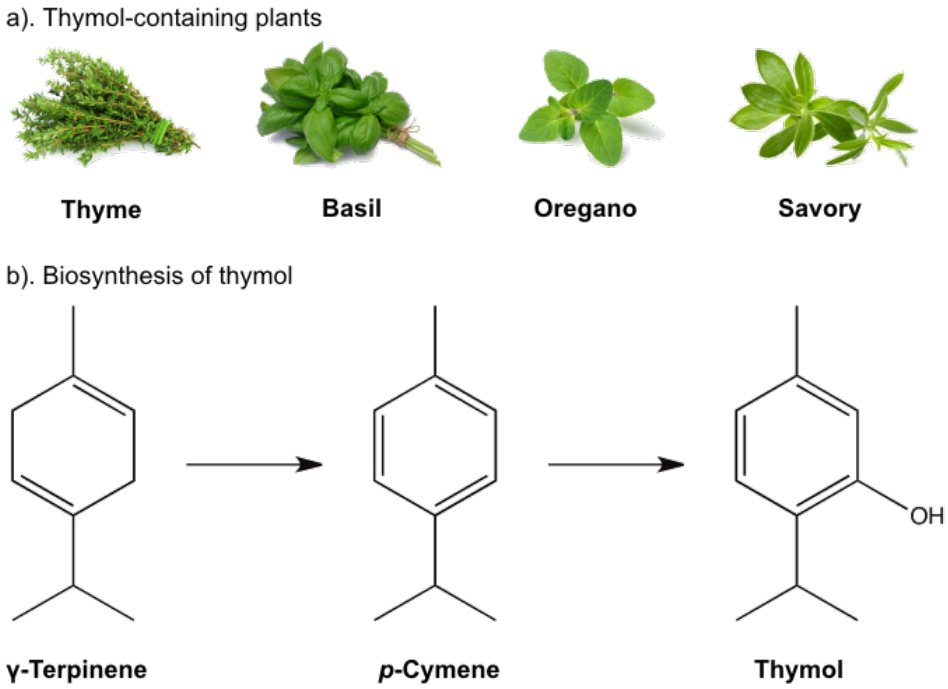

Figure 5 a) Representative thymol-containing plants, and b) biosynthesis of thymol

2.5.1. Physical and Chemical Properties of Thymol

Pure thymol exists as colorless, translucent crystals at room temperature with an aromatic, thyme-like odor and a sweet, medicinal, spicy flavor. It is a phenolic compound

with a molecular formula of $C_{10}H_{14}O$ and a molecular weight of 150.22 g/mol. Thymol and carvacrol are isomeric, with a change in the position of the hydroxyl group with respect to the methyl group [72]. Thymol is slightly soluble in water (0.9 g/1000 mL at 20 °C, 1 g/1000 mL at 25 °C), but very soluble in organic solvents such as chloroform (1 g/0.7 mL at 25 °C), ethanol (1 g/1 mL at 25 °C) and diethyl ether. Thymol has a melting point of 49 to 51 °C and a boiling point of 232 °C. Odor threshold of thymol is reported to be 86 to 790 ppb [73].

2.5.2. Commonly Used Antimicrobial Testing Methods

Over the past decades, numerous *in vitro* studies have revealed the broad-range antimicrobial activities of thymol. In these studies, thymol was prepared in one of the three forms: liquid-phase, solid-phase and vapor-phase.

The antimicrobial activity of liquid-phase thymol has been extensively studied using broth dilution methods, where serial dilutions of thymol solution are added into cultured liquid-phase medium. After incubation under optimal conditions, the end point is determined by selected methods, such as optical density (OD), plate count, visual observation, color change, conductivity, etc. Although there's no standardized test, the Clinical and Laboratory Standards Institute (CLIS, also formerly known as NCCLS) published methods for antibacterial and antifungal susceptibility testing that have been modified to test EOs and their constituents [74, 75].

The antimicrobial activity of solid-phase thymol can be studied using either agar dilution or various diffusion methods. Agar disk diffusion assay is the most commonly used method for routine antimicrobial susceptibility screening and testing [76]. CLSI has

published accepted and approved standards for bacteria and yeasts testing [77, 78]. In the agar disk diffusion assay, solid agar plates are inoculated with a standardized culture of the target microorganism. Then, filter paper disks, impregnated with the test compound of certain concentrations, are placed onto the agar surface. After incubation under optimal conditions, the diameters of the inhibition zones, where no growth of the test microorganism can be observed, are measured to reflect the inhibitory effect.

The antimicrobial activity of vapor-phase thymol is probably the least studied among the three forms. No standard assay exists, while many researchers have attempted different methods. One frequently used method was known as vapor diffusion assay or disk volatilization assay, modified from the agar disk diffusion assay [5]. The experimental procedure is similar, but instead of placing the impregnated paper disk on the agar surface, it is attached to the lid of the petri dish. This ensures no direct contact between the microorganisms and the test compound. After incubation, the diameters of the inhibition zones are measured to reflect the inhibitory effect.

The minimum inhibitory concentration (MIC) is quoted by most researchers as an indicator of the antimicrobial performance of EOs and their constituents [54]. But there's no uniform definition of MIC, and the definition of MIC differs between publications. Occasionally, the minimum bactericidal concentration (MBC) is stated, and is either close to or far apart from the identified MIC, depending on its definition. A list of commonly cited definitions of MIC and MBC is shown in Table 2. It can be seen that there's much variance, creating man-made discrepancies and complicating the already difficult task of comparing MICs and MBCs between studies.

Table 2 Definitions of MIC and MBC in antimicrobial testing

Term	Definition	Reference
MIC	The lowest concentration above which no growth is observed relative to control.	[6, 79]
	The lowest concentration resulting in maintenance or reduction of inoculums viability.	[80, 81]
	The lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth.	[52, 55, 82]
	The lowest concentration which resulted in a >90% decrease in inoculum viability.	[8]
MBC	The concentration where 99.9% or more of the initial inoculums are killed.	[8]
	The lowest concentration at which no growth is observed after sub-culturing into fresh broth.	[83]

MIC is not necessarily the most appropriate measure for the antimicrobial susceptibility. In broth dilution assays, solvents or emulsifiers (e.g. DMSO, methanol, Tween) are added to overcome the low solubility issue, which may alter the antimicrobial activity [84]. In diffusion assays, MIC is rarely used. Instead, inhibition zones where no sign of growth is observed are used as an indicator. But still, partitioning through agar layer is determined by the affinity with water, thus hard to quantify the amount of antimicrobial absorbed into the agar medium [76, 84]. In volatilization assays, vaporization takes place in accordance to the compound's volatility, hence the added amount does not represent the actual vapor concentration, and the calculated MIC does not reflect the lowest vapor

concentration to be effective. Taking all into consideration, researchers need to be aware of the flaws in MIC and take caution when using it to make conclusions and comparisons.

2.5.3. Antimicrobial Activities of Thymol

The effects of liquid-phase thymol against Gram-positive bacteria, Gram-negative bacteria, and fungi are summarized in Table 3, Table 4, and Table 5, respectively. As can be seen from the tables, the antimicrobial activity is highly variable. For different microorganisms, it could be explained by the distinct cell structure and resistance mechanisms. But even for the same species, the MICs can differ by up to 100-fold. The discrepancy might be due to the difference in the definition of MIC, cultured strains, experimental method, culture medium, volume of inoculum, culture temperature, incubation time, enumeration method, etc. It also reflects the fact that there's still a long way to go before liquid-phase thymol can be applied in real-life situations.

The effects of solid-phase thymol and EOs containing thymol as a major constituent are summarized in Table 6. It's noteworthy that although disk diffusion assay is widely used, the experimental details may vary and many external factors can affect the outcome, such as the diameter of the petri dish, the diameter of the filter disk, the volume and concentration of antimicrobials added, etc. Therefore, across different studies, it's far from reliable to directly compare the resulting inhibition zones as an indicator of the antimicrobial activity.

Compared to the other two phases, vapor-phase thymol is least studied. The effects of vapor-phase thymol and EOs containing thymol as a major constituent are summarized in Table 7. A few previous studies have shown that via instant addition, thymol and thyme

oil in vapor phase are effective against bacteria and fungi using less concentration than those in liquid phase and solid phase [25-27, 85]. However, it should be noted that such conclusion was drawn by directly comparing the MICs of vapor-phase thymol with those of liquid- or solid-phase thymol, while they might not be comparable. Despite the difference in the definition of MIC, it is typically calculated based on the amount added and the system volume. Across all three forms, the quantity of antimicrobials added is usually in the same order of magnitude. However, the system volume is less consistent. In liquid-phase and solid-phase testing, the system volume is the volume of cultured medium, which is generally totaled around 50 μL to 20 mL. In vapor-phase testing, the system volume is the headspace volume, which could be much greater, varying from tens to thousands of milliliters. Therefore, mathematically speaking, the vapor-phase MIC should be smaller in nature. Equaling a smaller MIC to higher effectiveness, without accounting for the factors affecting the MIC, is not valid. However, vapor-phase thymol is still worth investigating. Unlike liquid- and solid-phase thymol, whose MIC somewhat represents the actual concentration, vapor-phase thymol has a MIC that should be much greater than the actual vapor concentration in the headspace, due to the low volatility. When comparing the actual concentration present, it is plausible that vapor-phase thymol has lower concentration and hence to an extent more effective in microbial inhibition than liquid- and solid-phase thymol. Furthermore, the actual concentration, instead of the calculated MIC, is the portion that affects the flavor of the foods. As long as the actual vapor concentration is below the odor threshold, it can have the potential to be applied in foods. Thus, the activity of vapor-phase thymol is a research gap that remains to be filled.

It should also be mentioned that despite the difference in experimental procedures, thymol is usually added into the system via instant addition. The effects of other delivery modes on thymol's antimicrobial activity have not been explored, leaving yet another research gap to be filled.

Table 3 Antibacterial activities of liquid-phase thymol against Gram-positive bacteria

Microorganism	Strain	MIC (mg/L)	Reference
Staphylococcus aureus	MTCC 96	62.5	[86]
	ATCC 6538	25	[87]
		140	[6]
	ATCC 25923	156	[88]
		225	[8]
	ATCC 29213	250	[89]
	ATCC 6538P	310	[7, 90]
	blaZ	375.6	[91]
	ATCC 126000	1000	[12]
S. epidermidis	MTCC 435	125	[86]
	ATCC 12228	225	[8]
Enterococcus faecalis	ATCC 29212	225	[8]
		500	[12]
Listeria monocytogenes	NCTC 7973	100	[87]
	Scott A	200	[92]
	ATCC 7644	450	[8]
	CMCC 54004	1024	[93]
Bacillus cereus	ATCC 11778	450	[8]
		500	[12]
B. subtilis	MTCC 121	125	[86]
Streptococcus mutans	MTCC 890	125	[86]
	ATCC 25175	250	[94]
	ss-980	5000	[95]
S. pyogenes	ermB	94.64	[91]
S. sanguis, S. milleri, S. mitis	N/A	125	[94]
Clostridium perfringens	CVCC2027	375	[9]
	CVCC2030	375	[9]
Lactobacillus acidophilus	GIM 1.730	1500	[9]
L. reuteri, L. salivarius	N/A	1500	[9]
L. fermentum	ATCC 8289	500	[10]
L. brevis	ATCC 367	500	[10]
L. fructivorans	ATCC 8288	500	[10]
L. plantarum	ATCC 4008	500	[10]
Pediococcus acidilactici	ATCC 8042	500	[10]
P. damnosus	ATCC 11308	500	[10]
Leuconostoc mesenteroides	ATCC 8293	500	[10]
L. citrovorum	ATCC 23065	500	[10]

Table 4 Antibacterial activities of liquid-phase thymol against Gram-negative bacteria

Microorganism	Strain	MIC (mg/L)	Reference
Escherichia coli	ATCC 25922	10	[88]
	EC 100	61.5902	[15]
	ATCC 35210	100	[87]
	N/A	180.264	[96]
	CVCC1553	187.5	[9]
	CVCC1490	187.5	[9]
	AS1·90	200	[16]
	ATCC 35150 (O157:H7)	225	[8]
		450.66	[11]
	MTCC 723	250	[86]
	N00-666	375.55	[91]
	ATCC 25922	450	[8]
	ATCC 8739	1000	[12]
	ATCC 15221	5000	[7, 90]
Salmonella Typhimurium	ATCC 13311	50	[87]
	ATCC 14028	56.25	[8]
	ATCC 13311	150.22	[11]
	LT2	150.22	[96]
	SL 1344	256	[93]
	CVCC541	375	[9]
	SGI1 (tetA)	375.55	[91]
	ATCC 14028	750	[97]
S. Enteritidis	CVCC 2184	750	[9]
	ATCC 13076	750	[9]
S. Pullorum	C79-13	375	[9]
Pseudomonas aeruginosa	ATCC 9027	39	[88]
	ATCC 27853	100	[87]
	ATCC 2730	385	[6]
	ATCC 27853	> 900	[8]
Moraxella catarrhalis	ATCC 23246	1000	[12]
Yersinia enterocolitica	ATCC 9610	225	[8]
Erwinia amylovora	ATCC 29850	1600	[98]
E. carotovora	ICMP 9017	1600	[98]

Table 5 Antifungal activities of liquid-phase thymol against yeasts and molds

Microorganism	Strain	MIC (mg/L)	Reference
Candida albicans	CBS 562	39	[99]
	N/A	50	[87]
	ATCC 10231	112.5	[8]
	ATCC 10231	125	[12]
	ATCC 11006	350	[100]
	ATCC 10239	625	[95]
C. tropicalis	CBS 94	78	[99]
	ATCC 201380	125	[12]
C. krusei	CBS 573	39	[99]
Aspergillus niger	ATCC 6275	10	[87]
	PTCC 5154	200	[101]
A. fumigatus	N/A	25	[87]
	PTCC 5009	175	[101]
A. flavus	ATCC 9643	10	[87]
	PTCC 5004	125	[101]
A. ochraceus	PTCC 5017	125	[101]
	ATCC 12066	10	[87]
Alternaria alternata	PTCC 5224	400	[101]
Botrytis cinerea	PTCC 12481	300	[101]
Cladosporium spp.	PTCC 5202	100	[101]
Penicillium citrinum	PTCC 5304	250	[101]
P. chrysogenum	PTCC 5271	500	[101]
P. funiculosum	ATCC 36839	12.5	[87]
P. ochrochloron	ATCC 9112	25	[87]
Fusarium oxysporum	PTCC 5115	300	[101]
Rhizopus oryzae	PTCC 5174	450	[101]
Trichoderma viride	AM 5061	10	[87]
Saccharomyces cerevisiae	ATCC 9763	112.5	[8]
	SB36-85	225.33	[102]

Table 6 Antibacterial and antifungal activities of solid-phase thymol and EOs containing thymol

Microorganism	Antimicrobial	Dose[a]	IZ[b]	Reference
Bacillus subtilis	Thymol	50	9	[103]
	Thymus kotschyanus EO	~ 5	64	[104]
Enterococcus faecalis	Thymol	50	9	[103]
L. monocytogenes 1/2c	*T. zygis* EO	~ 15	38	[105]
	T. vulgaris EO	~ 15	29	
L. monocytogenes 4b	*T. zygis* EO	~ 15	30.6	[105]
	T. vulgaris EO	~ 15	37	
Mycobacterium smegmatis	Thymol	50	12	[103]
Staphylococcus aureus	*T. capitatus* EO	20	12	[106]
	Thymol	20	20	
	T. kotschyanus EO	~ 5	58	[104]
	T. zygis EO	~ 15	19	[105]
	T. vulgaris EO	~ 15	39	
S. epidermis	Thymol	50	12	[103]
Streptococcus mutans	Thymol	50	15	[103]
		500	1.8	[95]
Enterobacter aerogenes	Thymol	50	5	[103]
	Thymol	20	17	[106]
	T. capitatus EO	20	9	
Escherichia coli	Thymol	50	7	[103]
	Thymol	20	13	[106]
	T. capitatus EO	20	8	
	T. kotschyanus EO	~ 5	36	[104]
	T. zygis EO	~ 15	10	[105]
	T. vulgaris EO	~ 15	22.3	
E. coli O157:H7	*T. zygis* EO	~ 15	14.3	[105]
	T. vulgaris EO	~ 15	13.6	
Klebsiella pneumoniae	Thymol	50	7	[103]
	T. kotschyanus EO	~ 5	54	[104]
Pseudomonas aeruginosa	*T. kotschyanus* EO	~ 5	2	[104]
	Thymol	20	22	[106]
	T. capitatus EO	20	16	

Microorganism	Antimicrobial	Dose[a]	IZ[b]	Reference
Salmonella Enteritidis	*T. kotschyanus* EO	~ 5	26.2	[104]
	T. zygis EO	~ 15	26	[105]
S. Typhimurium	*T. zygis* EO	~ 15	24.3	[105]
	T. vulgaris EO	~ 15	30	
	Thymol	50	4	[103]
S. Typhi	Thymol	50	7	[103]
Shigella flexneri	*T. zygis* EO	~ 15	30	[105]
	T. vulgaris EO	~ 15	23.6	
S. sonnei	*T. zygis* EO	~ 15	24	[105]
	T. vulgaris EO	~ 15	23.6	
Yersinia enterocolitica	*T. zygis* EO	~ 15	40.6	[105]
	T. vulgaris EO	~ 15	25.3	
	Thymol	50	15	[103]
Candida albicans	Thymol	500	4.6	[95]
		50	9	[103]

a. Dose (μg/disk) indicates the amount added onto the paper disk on the medium.
b. Inhibition zone (IZ, mm) in this table excludes the diameter of the paper disk and equals to the diameter of the measured inhibition zone less the diameter of the paper disk

Table 7 Antibacterial and antifungal activities of vapor-phase thymol and EOs containing thymol

Microorganism	Strain	Antimicrobial	MIC (μL/L)	Reference
Staphylococcus aureus	ATCC 25923	*Origanum vulgare* EO	17	[107]
		Thymus vulgaris EO	17	
	N/A	*O. compactum* Benth. EO	125	[84]
	209P	Thymol	3260	[30]
Streptococcus pneumoniae	IP-692	Thymol	3260	[30]
	Prc-53	Thymol	3260	
S. pyogenes	ATCC 12344	Thymol	3260	[30]
Listeria monocytogenes	ATCC 7644	Thymol	21.8	[108]
		O. vulgare EO	66	[107]
		T. vulgaris EO	260	
Escherichia coli	ATCC 25922	*O. vulgare* EO	66	[107]
		T. vulgaris EO	33	
	NIHJ	Thymol	> 3260	[30]
Salmonella Enteritidis	N/A	*O. compactum* Benth. EO	125	[84]
	ATCC 27853	*O. vulgare* EO	130	[107]
		T. vulgaris EO	33	
S. enterica	CECT 4000	Thymol	10.9	[108]
H. influenzae	ATCC 33391	Thymol	3260	[30]
Aspergillus niger	N/A	*O. compactum* Benth. EO	125	[84]
	N/A	*T. vulgaris* L. EO	250	
Aspergillus flavus	CECT 2687	Thymol	43.6	[108]
Candida albicans	ATCC 64550	Thymol	10.9	[108]
	N/A	Thymol	38	[27]
C. glabrata	N/A	Thymol	19	[27]
C. tropicalis	N/A	Thymol	< 19	[27]
Alternaria alternate	N/A	*O. compactum* Benth. EO	31.25	[84]
	N/A	*T. vulgaris* L. EO	250	
Penicillium digitatum	N/A	*O. compactum* Benth. EO	125	[84]
	N/A	*T. vulgaris* L. EO	250	

2.5.4. Mode of Antimicrobial Action of Thymol

Although the mode of action of thymol is not fully understood, it is believed to be involved in the disruption of inner and outer membrane, as well as in the interaction with membrane proteins and intracellular targets, as summarized in Figure 6 [3].

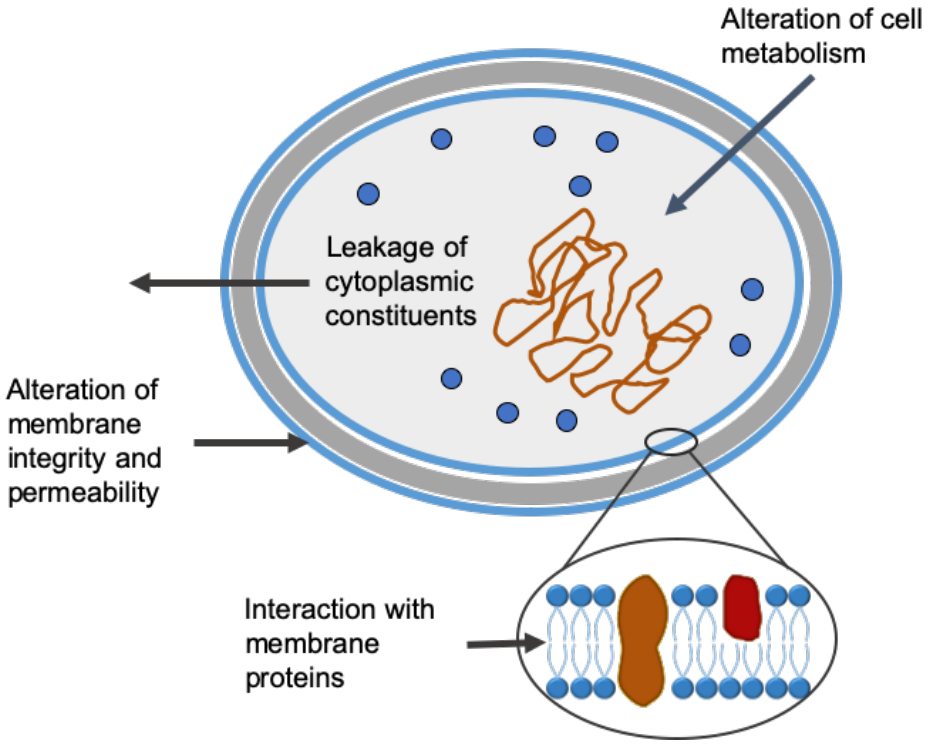

Figure 6 Summary of the antimicrobial modes of action of thymol

Several studies have shown that thymol impairs the inner membrane of bacteria. For both Gram-positive and -negative bacteria, thymol affects the integrity and permeability of cytoplasmic membrane, which has been well documented by the leakage of intracellular ATP, inorganic ions, cellular materials and carboxyfluorescein (cF), the uptake of nuclear stain ethidium bromide (EB), the dissipation of pH gradient, the loss of membrane potential and the alteration of membrane fatty acid profile [6, 11, 14, 16, 97, 109]. This

effect is believed to be dependent on the lipid composition and net surface charge of the membrane [7, 90]. In 2014, Chauhan and Kang [97] investigated the mode of action of thymol against *Salmonella* Typhimurium by scanning electron microscopy (SEM), which further confirmed that the disruption of membrane integrity is, at least, one of the main mechanisms of action of thymol.

For Gram-negative bacteria, thymol also disintegrates the outer membrane. Helander, Alakomi [11] evaluated the effects of thymol on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium and suggested that thymol disintegrates the outer membrane and releases outer membrane-associated materials, such as lipopolysaccharide (LPS). The mechanism of action might not be associated with chelation of cations or intercalation into the outer membrane, as magnesium ions (supplied as $MgCl_2$) could not prevent the disruption of outer membrane [11].

In addition to the interaction with cell membranes, increasing evidence has revealed that thymol interacts with membrane proteins and affects different cell metabolic pathways. Juven, Kanner [110] discovered that addition of bovine serum albumin (BSA) or Desferal neutralized the antibacterial activity of thymol, hence suggesting that complexation reactions take place between thymol and bacterial membrane proteins via hydrogen bonds and hydrophobic interaction, which are inhibited by BSA and Desferal due to competitive binding to thymol through their amino and hydroxylamine groups. Di Pasqua, Mauriello [14] exposed *Salmonella enterica* to a sublethal concentration of thymol and discovered that thymol plays a part in altering various metabolic pathways. Proteins in different functional groups were either upregulated or downregulated by the presence of thymol. In particular, key proteins involved in the protection against thermal

stress, such as GroEL and DnaK, were upregulated or synthesized *de novo*, while outer membrane proteins OmpX and OmpA, were also upregulated [14]. On the contrary, in *Erwinia amylovora*, thymol caused a significant decrease in the levels of all outer membrane proteins [98]. Thymol is also suggested to impair the citrate metabolic pathway, as well as enzymes involved in the synthesis of ATP [14].

The mode of action of thymol against yeasts and molds has been investigated to a lesser extent, but a few studies indicate that thymol interacts with fungal cell envelope and intracellular targets. Bennis, Chami [102] found that thymol induced lysis in *Saccharomyces cerevisiae* cells and hypothesized that the antifungal activity of thymol involves alteration of the envelope, including cellular membrane and cell wall of fungal cells. Interestingly, thymol generated deformity in budding cells, but only introduced cracks on the surface of simple cells, which implies that the effect of thymol depends on the proliferation stage [102]. Ahmad, Khan [111] further confirmed that thymol disrupts membrane integrity of fungal cells. Moreover, thymol was suggested to penetrate into the cell and target the ergosterol biosynthesis pathway, thereby impairing the synthesis of ergosterol [111]. Thus, its antifungal activity results from a combination of effects on the two events. Contrarily, Rao, Zhang [112] proposed that activation of specific signaling pathways, rather than a nonspecific lesion of membranes, accounts for the antifungal activity of thymol.

2.5.5. Biological Activities of Thymol

Aside from its antimicrobial activity, thymol, as well as its natural source thyme (*Thymus vulgaris* L.), has been employed for numerous indications, based on its antitussive, spasmolytic, antioxidant, and anti-inflammatory activity.

Spasmolytic/Antitussive Effects:

Flavonoids from thyme inhibited responses to agonists which stimulate acetylcholine and histamine receptors, or inhibited the availability of Ca^{2+} for muscle contraction, inducing relaxation of the smooth muscles of guinea-pig ileum and trachea [113]. *In vitro*, liquid thyme extracts exerted relaxing effect on carbachol-induced guinea-pig tracheal strip [114]. Although phenol concentration of the extracts was too low to be responsible for the activity, thymol in sufficient dosage was found to possess tracheal relaxant properties [114].

Antioxidant Effects:

In vitro studies in foods or cell lines have indicated the antioxidant properties of thymol. In 1999, Yanishlieva, Marinova [115] studied the autoxidation of purified triacylglycerols of lard (TGL) and of sunflower oil (TGSO) containing thymol ranging from 0.02% to 0.20%. The results showed that thymol is able to effectively inhibit the oxidation of both TGL and TGSO. In the presence of Fe^{3+} and ascorbate, thymol was found to decrease peroxidation of phospholipid liposomes [116]. In V79 Chinese hamster lung fibroblast cells, thymol displayed a concentration-dependent antioxidant capacity to decrease the production of reactive oxygen species (ROS) [117]. Pre-treatment of 25 μg/mL thymol was able to scavenge free radicals and modulate oxidative stress, thus suppressing genotoxicity, apoptosis and necrosis in gamma radiation-induced V79 cells [118]. *In vivo*

studies including animal models have also reported thymol's antioxidant nature, highlighting the possible benefit of thyme oil as a natural dietary antioxidant. Rats supplemented with thyme oil in their diets maintained higher activities of various antioxidant parameters, suggesting that they maintained a more favorable antioxidant capacity during their life time [119]. Aging rats fed with thyme oil or thymol retained substantially higher antioxidant enzyme activities and total antioxidant status in brain, [120]. Supplementation of thymol to broiler chicken significantly decreased the breast and thigh malondialdehyde (MDA) levels, indicating reduced lipid peroxidation by enhancing antioxidative action [121].

Anti-inflammatory Effects:

The release of human neutrophil elastase, which was induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP), could be inhibited by thymol [122]. Since elastase is a biomarker of inflammatory diseases, thymol is credited with anti-inflammatory activity by inhibiting excessive elastase activity. Prostaglandins (PGs) are important pro-inflammatory mediators whose biosynthesis is catalyzed by cyclooxygenases (i.e. COX-1 and COX-2) [123]. Thymol has been reported to be active against COX-1 with an IC_{50} value of 0.2 μM, but show a limited COX-2-specific inhibition [124]. *In vivo*, rodents were treated with thymol to assess its anti-inflammatory and wound healing potential [125]. Thymol showed significant reduction of the oedema, and diminished influx of leukocytes to the injured area [125]. Besides, wounds treated with collagen-based thymol films demonstrated bigger wound retraction rates and enhanced granulation reaction, while improved collagenization density and arrangement was also provided during the process of wound healing [125]. In another study, thymol habited inflammatory edema,

but did not inhibit leukocyte migration [123]. However, in an *in vivo* test on the chorioallantoic membrane of the fertilized hen's egg (CAM assay), no pronounced anti-inflammatory effect was found in thymol at 10 – 250 μg/pellet, but a dose-dependent strong irritation was still showing at 10 μg/pellet [126].

2.5.6. Safety Aspects of Thymol

Thymol, thyme oil and thyme have been classified as generally regarded as safe (GRAS) by the FDA as foods or food additives, and registered by the European Commission for the use as flavoring in foods, because no health risk to the consumer has been found [3, 13]. The United States Environmental Protection Agency (EPA) determined that the use of thymol as an active ingredient in pesticides should not cause adverse effects to either human health or the environment [127].

However, people still need to consider potential health risk before exposed to thymol via physical contact or in diet. *In vitro*, thymol likely possesses moderate cytotoxicity [23]. In V79 cells, 25 μM thymol caused an increased in DNA damage [117]. In Caco-2 cells, exposure to thymol has been associated with cellular damage, such as lipid degradation, mitochondrial damage, nucleolar segregation and apoptosis [22]. On acute oral administration, thymol exhibits slight toxicity, with an LD_{50} of 980 mg/kg body weight in rats [128], but is essentially non-toxic on acute dermal application, with an LD_{50} of > 2000 mg/kg body weight in rats [129]. In animal models, thymol could cause primary dermal and eye irritation. When applied to the ears of mice, thymol induced inflammatory ear edema, similar to the effect observed with croton oil, a known irritant agent [123]. The irritative effect is likely to involve histamine, prostanoids and other

inflammatory mediators [123]. In rabbits, thymol is corrosive to the skin and eye, but in guinea pigs, thymol shows no skin sensitization potential [129-131]. In humans, some case reports have described allergic contact dermatitis due to thymol [132, 133].

2.5.7. Current Uses and Limitations of Thymol

Thymol has many uses, such as in pesticides, cosmetic formulations, food flavorings, mouthwashes and pharmaceutical preparations.

Pesticides:

Thymol is an active ingredient in many pesticide products which are used indoor and outdoor, to control pests such as bacteria, fungi, viruses and animals, but some concerns have been raised due to the lack of some toxicological data [127, 134]. Thymol is also used in products applied to beehives to control *Varroa*.

Cosmetic formulations:

Thymol is used as a top note in men's fragrances [135].

Mouthwash:

Thymol had been found to be effective against oral bacteria, such as *Porphyromonas gingivalis*, *Selenomonas artemidis*, *Streptococcus sobrinus* and *Streptococcus mutans* [94, 136, 137]. Thymol is one of the active ingredients in antiseptic mouthwashes such as Listerine, which contains 0.064% of thymol [138].

Food preservative:

The use of thymol as a food preservative has been limited. As mentioned in the previous section, the common mode of delivery, instant addition, required high concentrations for effective inhibition. The necessity of high concentrations limits the use of thymol. On one

hand, because of its potential toxicity on human cells, increasing the concentration in the formulation may not be permissible. Studies are still needed to verify if this high concentration will impose a health risk on consumers. On the other hand, the intense aroma may cause negative organoleptic effects, making consumers reject the products [24]. In addition, being a natural antimicrobial, thymol incurs higher cost than the commonly used synthetic preservatives. Thus, a higher dosage could significantly increase the manufacturers' cost of goods sold and largely reduce their margins. It creates an economical barrier and disincentivizes the food manufacturers to adopt this novel antimicrobial. Therefore, although thymol may be a desirable antimicrobial due to its plant-derived nature, the high concentration requirement must be circumvented before it can be applied in foods.

2.6. Controlled Release Packaging

Food packaging provides four major functions: containment, communication, convenience and protection [139]. Containment is the most basic function of packaging – to enclose food. Communication is an important function to create product identity and influence consumer buying decisions. It also facilitates distribution and retail through the barcode and radio frequency identification (RFID), for example [140]. Convenience is a function that serves to satisfy consumer's busy lifestyle, including easy opening, resealability, and microwavablity [140].

Protection, the most essential function of food packaging, is also most relevant in this thesis. Ideally, food packaging should be able to protect the food product from physical damage, physiochemical deterioration, microbial spoilage and product tampering, thereby extending the shelf lives of foods [139, 140]. However, generally, the protection provided by food packaging is limited. If the shelf life of a food product is mostly affected by external environmental factors, such as physical damages, humidity, oxygen, light and temperature, packaging can serve as an effective solution [140]. When it comes to foods whose shelf lives are controlled by internal factors, traditional packaging is of little help. Therefore, innovative ideas are needed to broaden and redefine the protection function. Recently, a concept known as "active packaging" has emerged, switching the protection function of packaging from "passive" to "active".

2.6.1. Concept of Active Packaging

The protection function of traditional food packaging is considered “passive”, as it only serves to passively enclose the food and protect it from only the external environmental factors, but is unable to protect the food from internal factors, such as moisture migration inside the food (Figure 7).

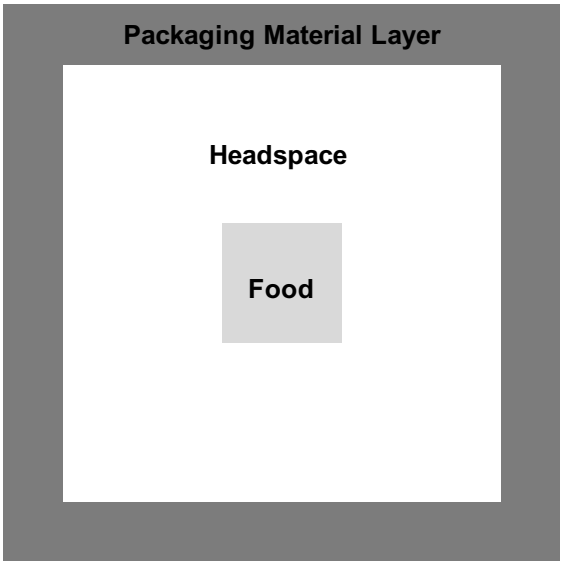

Figure 7 Illustration of passive packaging

Recently, a concept called active packaging has emerged. The definition of active packaging evolves over time. Theoretically, packaging is termed “active” when it performs some role other than only functioning as an inert barrier to the external environment [141]. The key idea behind is that the condition of the packaged food is actively and deliberately changed [142], in order to extend shelf life, improve food safety and/or enhance sensory properties, while still maintaining the quality of the packaged food [143].

Active packaging systems can be classified into three categories: absorbing, releasing, and others [143]. Absorbing systems utilize packages or sachets to remove unwanted substances, such as O_2, CO_2, ethylene, moisture and taints, so that a favorable internal environment and/or food condition can be achieved [139, 142]. Releasing systems releases wanted substances, such as CO_2, water, flavorings, antioxidants and preservatives, with the aim of maintaining, sometimes even improving, the quality, safety and sensory aspects of the food [139, 142, 144]. Other systems include self-heating, self-cooling, microwave susceptor, and selective permeable films [139]. An illustration of the active packaging concept is shown in Figure 8.

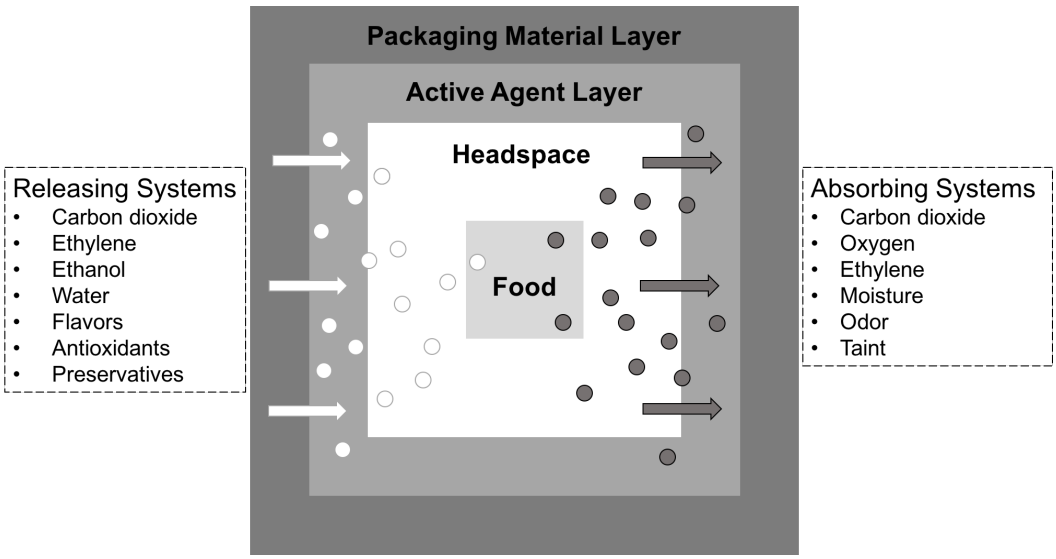

Figure 8 Concept of active packaging. Adapted from [145]

2.6.2. Concept of Controlled Release Packaging

Controlled release packaging (CRP) is a type of active packaging, which delivers active compounds from packaging material at targeted rates in a controlled manner over extended storage, to enhance the quality and safety of foods [146, 147]. The concept of controlled release has been used in the field of pharmaceutical for decades, where CRP has demonstrated great potential for drug delivery applications [148-150]. However, in the field of food packaging, CRP is relatively new, and yet to be raised to a quantitative level.

The uniqueness of CRP lies in its ability to provide a continuous supply of active compounds by controlling the release rate from the packaging material to the food system [140]. The word "controlled" underlines that the release rate of the active compounds is regulated based on the targeted reaction (e.g. food deterioration, lipid oxidation, microbial growth, etc.) [151]. Release profiles, the function of released amount over time, are specifically designed and could vary case to case. For instance, in the case of lipid oxidation, there exists an intrinsic induction period, where the level of primary oxidation product is stable. Dumping antioxidants during this phase not only result in wastage of antioxidants, but may even lead to undesirable pro-oxidation [152]. In contrast, for microbial growth, the initial lag phase is short, followed by a log phase, when microorganisms grow exponentially. Therefore, CRP should be designed to release lesser amounts of antioxidants during the inherent induction period of lipid oxidation, while higher amounts of antimicrobials is favorable during the lag phase of microbial growth. Thus, it is critical to understand the deterioration kinetics, in order to determine the optimum condition and target release rate.

Key advantages of CRP are summarized into below points:

(1) CRP is able to replenish active compounds continuously.

Traditionally, active compounds are mixed into the food formulation, a delivery mode known as instant addition (IA). As the active compounds are continuously consumed and/or degraded in the reaction, protective effect continues to decreased until it no longer exists [146]. Once all active compounds are gone, food deterioration will take place rapidly.

Controlled release packaging, in contrast, can overcome this limitation by continuously releasing active compounds at pre-determined rates to replenish the molecules that are consumed and/or degraded [140]. In this way, a pre-determined desirable amount is always present, ensuring a longer term of protection.

(2) The quantity of active compounds required in CRP is much less than instant addition for the same or even better protection.

Take food microbial problems as an example.

As a traditional delivery mode, instant addition has one direct advantage: an instant effect upon addition. Since large quantities of active compounds, in this case antimicrobials, are added into the food formulation, protection takes effect immediately. However, most of the antimicrobials are mixed into the inside of the food, where microbial growth is of less concern [140]. Contrarily, most spoilage reactions occur on the food surface, where instant addition is unable to selectively target. In addition, once antimicrobials are added, they are subject to rapid degradation and/or interaction with other food ingredients, resulting in substantial loss and diminished inhibitory effect at later time [140].

Therefore, excessive amount needs to be loaded, both inside and on the surface of the food product [146].

CRP can overcome this limitation by releasing only onto the surface of the food, thereby reducing the quantity of antimicrobials used. It also protects the unused antimicrobials from degradation by storing them in the package until they are released to the food. Reducing the amount may also help improve food quality and consumer perspective, since many additives give an off-flavor [146], and consumers have already expressed the aversion to too much additive.

(3) CRP is the promising solution for special foods [153].

CRP can be applied in some special foods. For example, Meal Ready-to-Eat (MRE) or combat meal have a shelf life of three years and a reputation of being unpalatable. If CRP is successfully applied in MRE packaging, it's possible that the food quality can be improved, since the strict requirements on food formulation can be loosened, given that the shelf life is guaranteed by CRP, not by the food itself. Another example is fresh produce, in which antimicrobials cannot be directly added.

2.6.3. Evidence Supporting the Use of CRP for Active Compounds

Previously in our lab, the soundness of CRP is supported by researches using antioxidants and antimicrobials [17, 21, 153, 154].

For example, in one study, the effects of instant addition and slow release of the antioxidant tocopherol on the oxidation of linoleic acid were compared (Figure 9) [154]. Lipid oxidation is one of the key chemical deterioration reactions which determines the shelf life of lipid-rich products [155]. In the initial stages of lipid oxidation, a steady state

called the induction period, when no rancidity can be detected, is a standard indicator of shelf life: a longer induction period means a longer shelf life [155, 156]. To extent the induction period, antioxidants are commonly added as food ingredients. It was observed that slow release of tocopherol, following a series of release rates, provided maximal extension of induction period.

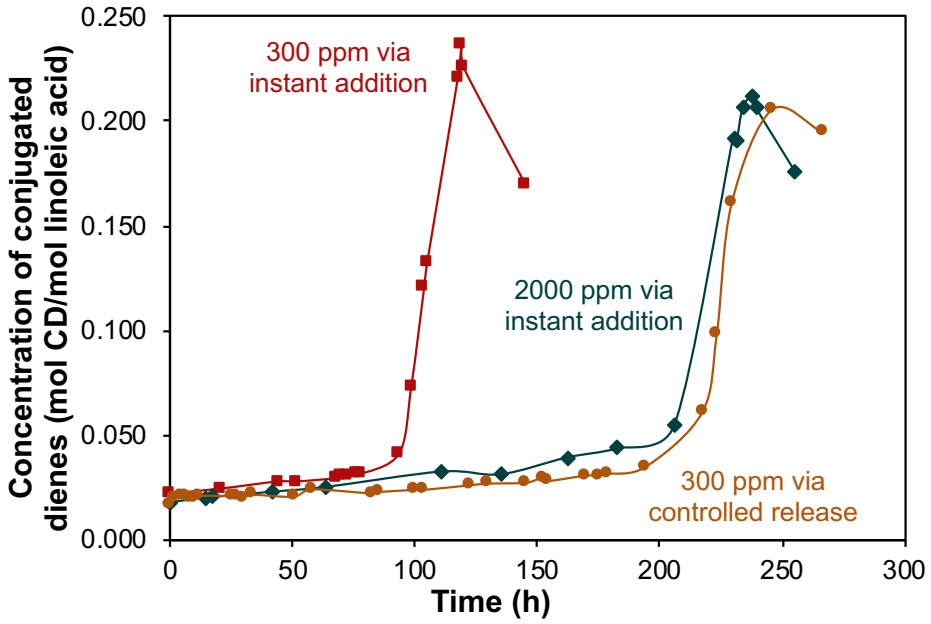

Figure 9 Effect of instant addition and controlled release of tocopherol on lipid oxidation

Tocopherol was also incorporated into low density polyethylene (LDPE) and polypropylene (PP) films for its slow release into linoleic acid [153]. Slow release from LDPE and PP effectively delayed the onset of lipid oxidation to 23 and 31 days respectively, compared to 19 days via instant addition. One plausible explanation is that tocopherol is unstable in linoleic acid, thus the film protects unused tocopherol from rapid degradation, resulting in a much higher antioxidant effectiveness [153].

As another example, the effects of the antimicrobial nisin on the growth of *Micrococcus luteus* were compared via instant addition and controlled release [17]. Controlled release of nisin was more effective than instant addition in inhibiting the growth of *M. luteus*, using as little as 15% of the amount compared to instant addition (Figure 10) [17]. One possible explanation for the striking results is that the initial fast release was able to kill or injure the microorganisms, while the subsequent slow release was sufficient to continuously suppress the injured surviving cells. Therefore, controlled release is able to utilize lesser amount to achieve the same or even better results than instant addition [17].

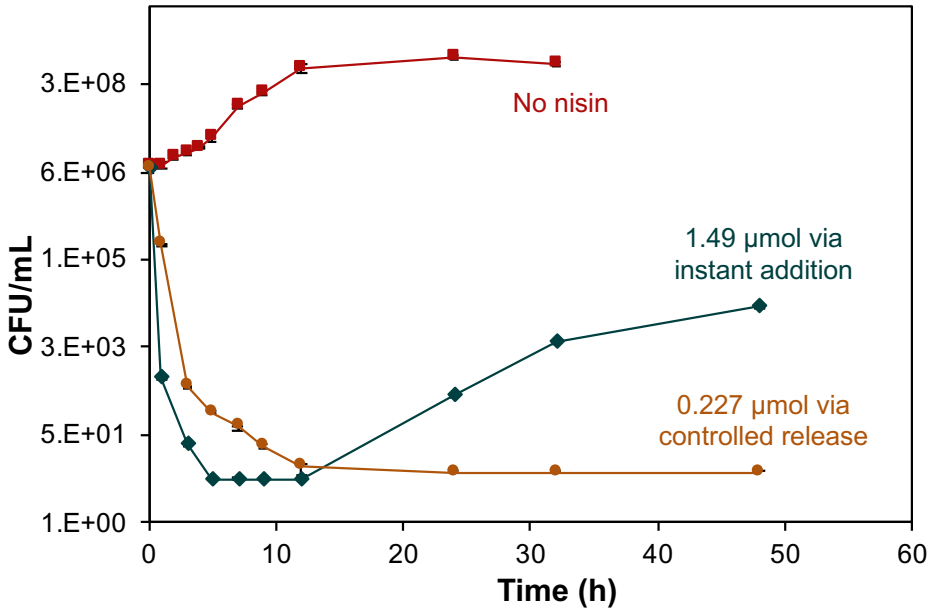

Figure 10 Inhibition of *M. luteus* by instant addition and controlled release of nisin

2.6.4. Mechanism of Releasing Active Compounds from CRP Films

The release of active compounds from polymer films involves three steps [139]:

(1) Diffusion within the polymer film toward the package-food interface;

(2) Partitioning at the package-food interface;

(3) Dispersion into the food matrix.

In volatile systems, direct contact between the food and the package is not required. Active volatile compounds that have relatively high vapor pressures will be desorbed from the package into the headspace, and then be absorbed by the food (Figure 11, left chart) [139].

In leaching systems, direct contact between the food and the package is required. Non-volatile compounds will disperse or diffuse into the foods after dissolution at the package-food interface (Figure 11, right chart) [139].

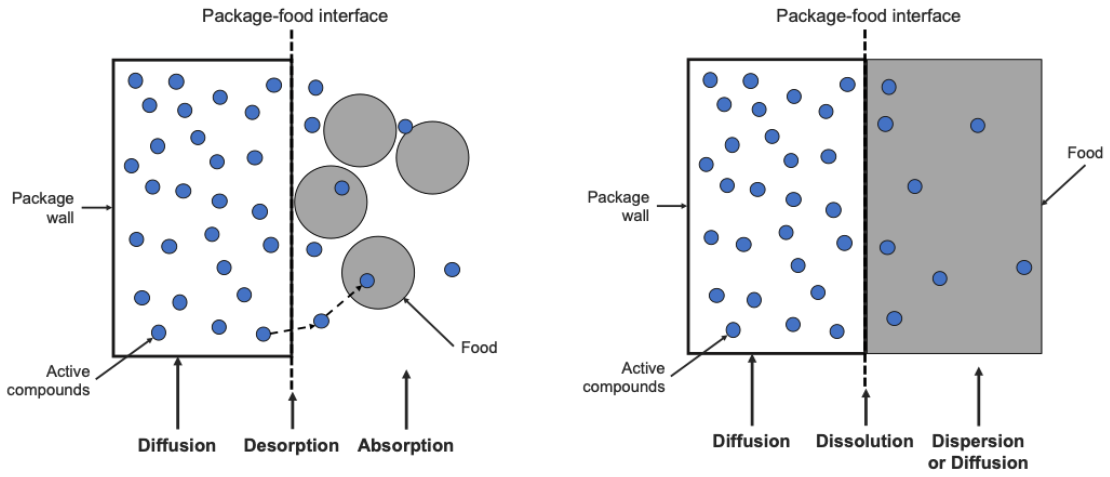

Figure 11 Migration of active compounds from package to food. Left chart: in the case of poor package-food contact. Right chart: in the case of good food-package contact. Adapted from [139].

Very often, the diffusion of active compounds is the rate-determining step, which can be described by Fick's First Law of diffusion:

$$J = -D_p \frac{dC}{dx} \quad \text{Equation 1}$$

Where J is the diffusion flux (mg m^{-2} s^{-1}), D_p is the diffusion coefficient or diffusivity of active compounds in packaging material (m^2 s^{-1}), C is compound concentration (mg m^{-3}), and x is the distance in the package film (m).

Fick's First Law described an ideal steady state where the concentration of active compounds in the package is considered infinite and unchanged over time. However, in reality, the diffusion of active compounds is dynamic. Fick's Second Law of diffusion takes into account the rate of change in concentration:

$$\frac{\partial C}{\partial t} = D_p \frac{\partial^2 C}{\partial x^2} \quad \text{Equation 2}$$

Where C is the concentration of active compounds as function of time (t) and location (x). Fick's Second Law is suitable for conditions where the active compounds are uniformly distributed in the packaging film.

Simplified solution forms of Fick's Second Law are often applied for easier analysis of diffusion within polymer films. When several assumptions are made, Equation 2 of simplified forms, also known as Crank's diffusion model, can be used with reasonable accuracy [157]:

$$\frac{M_{f,t}}{M_{f,\infty}} = \frac{2}{L}\left(\frac{D_p t}{\pi}\right)^{0.5} \quad \text{Equation 3}$$

and

$$\frac{M_{f,t}}{M_{p,0}} = \frac{2}{L}\left(\frac{D_p t}{\pi}\right)^{0.5}$$ Equation 4

Where $M_{f,t}$ is the amount of active compounds in food at time t, $M_{f,\infty}$ is the total amount of active compounds in food at equilibrium, $M_{p,0}$ is the initial amount of active compounds in the packaging film, and L is the thickness of the package film (m). It can be seen that diffusion of active compounds through the packaging film is proportional to the square root of time. Therefore, the diffusion coefficient or diffusivity of the active compound is usually determined by plotting $M_{f,t}$ versus square root of time.

The assumptions for Equations 3 and 4 include: the release of active compounds is from one side of the film; the time is short; and the ratio of the amount released at time t to the initial amount ($M_{f,t}$ / $M_{p,0}$) is less than 2/3.

3. PROBLEM STATEMENT

3.1. The Importance of Identifying Optimum Release Profile

As stated in the literature review, CRP has been proven to possess the potential of replacing the traditional instant addition as a novel alternative delivery mode. Current work on antimicrobial CRP mainly centers on developing antimicrobial films using various antimicrobials and packaging materials, and then testing their inhibitory effects against various microorganisms. This is merely a trial-and-error process with no guiding principles. The release profile of antimicrobials from film is rarely a deliberate design but a parameter to be measured after the film is prepared. However, the release of antimicrobials from packaging film over time does not necessarily guarantee greater effectiveness than instant addition, especially when the release rate does not match the microbial growth kinetics.

Based on previous studies, there may exist an optimum release profile, which releases antimicrobials (or other active compounds) at a targeted rate over time, to replenish the portion that's consumed/degraded and to maintain the inhibitory effect until the end of shelf life. Only when the optimum release profile is followed, can we exploit CRP most effectively and efficiently, by using the least amount of antimicrobial while still maintaining adequate microbial inhibition to meet the shelf life requirement of the food. The optimum can be a range of release rates suitable for the sustained microbial inhibition. Correlating varying release profiles to microbial growth and then quantifying the optimum is a necessary step before CRP can be taken into real-life applications.

3.2. Research Gaps

3.2.1. Application of Controlled Release in Natural EO Constituents

To the best of our knowledge, EOs and EO constituents have not been applied in a sophisticated controlled-release manner. Although in many studies, films incorporated with EOs or EO constituents have been developed and tested for their antimicrobial activity, the selection of antimicrobials and films lacked deliberation. It is still unknown how the selected antimicrobial is released from the packaging film, or how the release is linked to its antimicrobial activity. Without understanding the relationship between release profile and microbial inhibition, the development of antimicrobial package will be a laborious process with a lot of trial and error.

Given their plant-based origin and antimicrobial nature, as well as their current limitations such as strong odor and high dosage requirement, EOs and EO components are ideal candidates for CRP. In CRP, the release of antimicrobials follows a pre-determined profile over time. When antimicrobials are released in a more sophisticated controlled manner, it will be easier to connect the dots between release profile and microbial inhibition, thereby identifying the optimum release profile. If the development of CRP is proven successful, the current limitations of EOs and EO constituents will be circumvented, and their future potential as food antimicrobials will be greatly enhanced. Since little preliminary work has been done in the field of EOs, it is reasonable to start from the most prominent candidate. Since the antimicrobial activity of thymol has been reported as one of the most potent among EO constituents, it can serve as a starting point of the investigation.

3.2.2. Possibility of Combined Mode as an Alternative Delivery Mode

In previous work [151], an effective release profile against microbial growth was determined as initially fast rate, followed by slow rate. From the release profile, an optimum diffusivity of antimicrobial from packaging can be calculated based on Equation 3. If such diffusivity is achievable, it should be attempted for the sake of simplicity. However, controlled release alone might not always be able to generate the required release profile due to technical constraints. If controlled release fails to meet the optimum, another delivery mode should be attempted.

Combined mode of delivery, i.e. instant addition plus controlled release, has the potential to be a new delivery mode to achieve targeted optimum release profile. The advantage of combined mode of delivery lies in its potential to be highly flexible. By combining two delivery modes, it's able to create release profiles that cannot be achieved by either delivery mode alone. As a result, combined mode of delivery suits various situations where a targeted release profile cannot be generated due to technical limitations.

The antimicrobial effect of combined mode has not been studied. It is still unknown if combined mode can match, if not exceed, the effectiveness of controlled release. Yet this leaves an opportunity for further exploration.

3.2.3. Limitation of Previous Work Regarding Optimum Release Profile

In previous work [151], it was proposed that the inherent lag phase of microbial growth is critical to microbial inhibition, and thus the release of antimicrobials during this time period must be equal or more than its MIC to be effective. The importance of lag phase is well-founded, and there shall exist a minimum concentration that is required to be

released during the lag period. However, the requirement of releasing MIC could be a sign of overreach. One of the distinctive advantages of CRP is that it's able to utilize lesser amount, in order to achieve the same, or even better, inhibitory effect. Since the MIC represents the minimum concentration for effective inhibition via instant addition, it should pose as an upper bound for the antimicrobial to be released via controlled release. Otherwise, the benefit of CRP will be diminished.

Therefore, the range of the optimum release profile still remains unsettled. The required minimum concentration during the lag phase is worth more investigation, in order to facilitate the design of CRP, and to reduce the amount used to as little as possible.

3.2.4. Antimicrobial Activity of Vapor-Phase Thymol

Thymol, as well as other antimicrobials, is most extensively studied when it is added to liquid- or solid-phase medium. Conventionally, the antimicrobial activity of any compound is first screened using agar diffusion assay, where the compound in its "solid" phase is tested against various microorganisms. The test is followed by broth dilution assay, where the MIC of the compound in its "liquid" phase is determined. In contrast, vapor phase has received little attention, partially due to the lack of standardized test methods and the complexity of the test system.

However, it is of interest to explore the use of vapor-phase thymol instead of liquid- or solid-phase, in order to broaden the applications of thymol as a food antimicrobial. As mentioned in the literature review, vapor-phase thymol and thyme oil have been reported to be effective against bacteria and fungi, using less concentration than those in liquid phase and solid phase [25-27, 85]. Additionally, the use in vapor phase does not require

direct contact with the food, thus avoiding the interaction with food matrix and making it a more appealing option. However, a problem lies within these studies. The authors claimed that vapor-phase thymol had lower MICs, thus more effective in microbial inhibition than liquid- and solid-phase thymol. Although the calculation of MIC, which is the amount added divided by the system volume, is the same for thymol in all three forms, the discrepancy in the system volume may create an artificial difference, making the results incomparable. Whether the claim is reasonable needs to be further researched. As a result, current evidence is still insufficient, and more research is needed to substantiate the prominent antimicrobial activity of vapor-phase thymol.

3.2.5. Correlations between Concentration Profile and Microbial Inhibition

Previous studies [25-27] also failed to account for the change in concentration over time. Although thymol is a volatile compound, its low vapor pressure (0.016 mmHg at 25 °C) indicates that not all amount added will be present as vapor [28]. The calculated MIC does not necessarily represent the actual vapor concentration. Moreover, in the headspace, vapor-phase thymol will be degraded by reactions with photochemically-produced hydroxyl radicals [29] or absorbed into the agar layer [30]. Therefore, unlike MIC, the actual concentration in both the headspace and the agar layer should not be constant but a profile of concentration versus time. There is little knowledge of the actual concentration profile upon addition, and a lack of understanding of how the profile may affect microbial growth over time. It is plausible to hypothesize that concentration profiles of varying shapes will affect the inhibitory effect differently. Therefore, it is important to identify the actual concentration profile of vapor-phase thymol and correlate

it with the antimicrobial behavior over time. Only when understanding this correlation, are we able to design an effective release profile that gives an effective concentration profile.

3.2.6. Feasibility of Controlled Release of Vapor-Phase Thymol

Vapor-phase antimicrobials have great hidden value to be applied in CRP. In previous studies [17, 151], liquid-phase antimicrobials were released into a broth system. This only mimics a real-life situation where the package and the food have direct contact. Although it's been proven effective, it does not represent the situation where the package and the food do not have direct contact, which is widely seen in real life. Controlled release of vapor-phase thymol can simulate the latter. Since there is a scarcity of knowledge concerning the antimicrobial activity of vapor-phase thymol via controlled release, it is of necessity to test its feasibility. It's reasonable to speculate that by controlling the release of vapor-phase thymol, it may be possible to generate an effective concentration profile with lower amount used, thereby enhancing the possibility of thymol as a food antimicrobial. A better understanding of how controlled release affects the concentration profile and antimicrobial activity of vapor-phase thymol can lay a solid foundation for the future applications of CRP.

4. OBJECTIVES

4.1. Assumptions

The assumption for controlled release of both liquid- and vapor-phase thymol is that an initially fast then progressively slow release rate may provide greater inhibitory effect than instant addition. The assumption is supported by previous work thus release profiles following other trends, such as initially slow than fast release rate, were not attempted in this study.

4.2. Hypotheses

In this study, the inhibitory effects of liquid- and vapor-phase thymol on the growth of *E. coli* DH5α were studied via different delivery modes. The research can be divided into two parts: (1) investigation of antimicrobial activity of liquid-phase thymol, and (2) exploration of antimicrobial activity of vapor-phase thymol.

4.2.1. Hypothesis 1

For the first part of the study, it is hypothesized that combined mode of delivery (instant addition plus controlled release) can be effective against microbial growth utilizing less amount than instant addition and/or controlled release. The rationale for proposing this hypothesis is that instant addition and controlled release retain some inflexibility, due to the governing principles, while combined mode possess high degree of freedom, allowing it to generate target release profiles in a wider range. Hence combined mode might be

able to achieve better inhibitory effect under conditions where instant addition and controlled release have limited options.

The hypothesis can be further extended into an underlying argument that a minimum concentration must be delivered during the lag phase to suppress microbial growth. Once this minimum dosage is determined, any mode of delivery that suffices it will have the potential to effectively inhibit microbial growth, may it be instant addition, controlled release or combined mode. After the lag phase, it is also implied that a slow release is required to replenish the antimicrobial that's been consumed/degraded, in order to sustain the continuous inhibition.

4.2.2. Hypothesis 2

For the second part of the study, it is hypothesized that thymol in vapor phase is effective against microbial growth using lower concentration than thymol in other forms, and that the vapor concentration profile is a key link between the release profile and the microbial inhibition.

The rationale behind this hypothesis lies within thymol's chemical characteristics. Firstly, thymol is lipophilic and hydrophobic. When thymol is added into liquid- or solid-phase medium, its poor water solubility reduces its activity, thus higher concentrations are needed to be effective. In contrast, when thymol is applied via vapor diffusion, the vapor can directly attack the cellular membranes of the microorganisms, without being repelled by water molecules, thus requiring lower concentration to be effective. Secondly, unlike the case of liquid- or solid-phase thymol, where direct contact takes place between the antimicrobial and the microorganisms, vaporization is required before

vapor-phase thymol can interact and inhibit the target microorganisms. Consequently, the release profile of liquid- and solid-phase thymol reflects the actual concentration that comes into contact with the target microorganisms, while the release profile of vapor-phase thymol does not. In the case of vapor-phase thymol, the release profile characterizes the rate of thymol coming out of the packaging film, but does not directly correlate with the microbial inhibition. The missing link between them is the vapor concentration profile. Since vaporization of thymol, or any volatile compound, cannot be manipulated by external forces in a controlled environment, the vapor concentration profile must be measured by gas chromatography and correlated with the microbial inhibition. Then in turn, suitable release profile can be designed to match the desired vapor concentration profile (Figure 12).

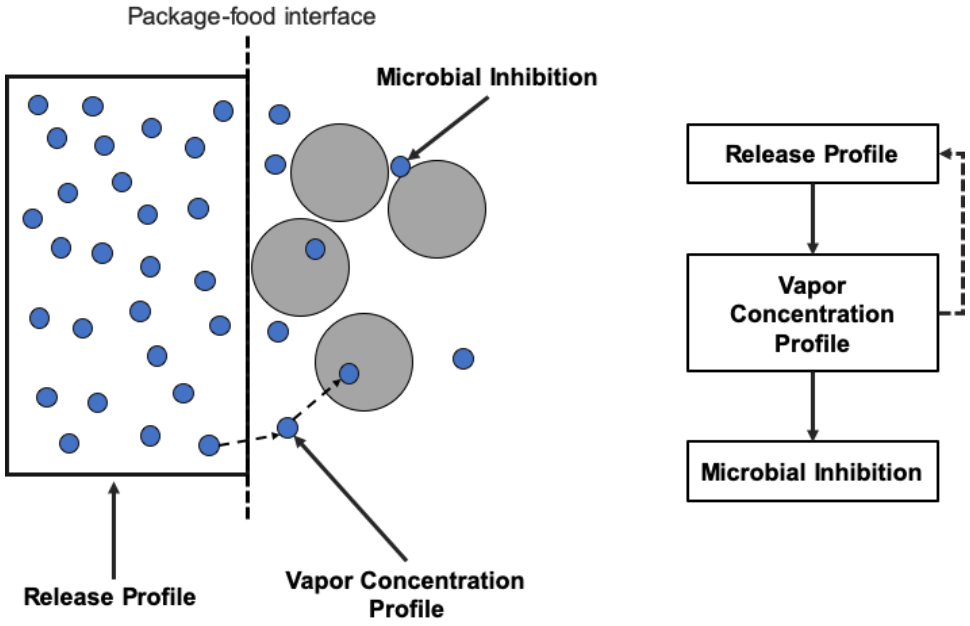

Figure 12 Linking release profile, vapor concentration profile and microbial inhibition

4.3. Objectives

The overall goal is to find the relationship between the release profile and the microbial growth kinetics, and to narrow down the range of target optimum release profiles.

4.3.1. Objective 1

For the first part of the study, liquid-phase thymol was investigated. Different modes of delivery were compared, and a rationale was provided to explain the different inhibitory effects against *Escherichia coli* DH5α. Accordingly, the range of optimum release profile was proposed.

To achieve this objective, several tasks were undertaken:

(1) To compare the inhibitory effect of liquid-phase thymol via three different modes of delivery, including instant addition, controlled release and combined mode of delivery (instant addition plus controlled release);

(2) To study the growth kinetics of *E. coli* DH5α under the three modes;

(3) To provide a rationale for the inhibitory effects via controlled release and combined mode of delivery;

(4) To propose and identify the range of optimum release profile.

4.3.2. Objective 2

For the second part of the study, vapor-phase thymol was investigated. The objective is to investigate the inhibitory effect of vapor-phase thymol against *Escherichia coli* DH5α via instant addition and controlled release. To address the objective, it is critical to correlate

the concentration profile and the antimicrobial activity of vapor-phase thymol, and to design a controlled release profile to meet the correlation.

The investigation involved the following tasks:

(1) To identify the minimum inhibitory dose of vapor-phase thymol via instant addition and compare it to instant addition of liquid- and solid-phase thymol;

(2) To investigate the concentration profile of thymol in the headspace and the agar layer to find correlations with the antimicrobial activity over time;

(3) To study the antimicrobial activity of vapor-phase thymol via controlled release and compare it to instant addition of vapor-phase thymol;

(4) To obtain the headspace concentration profile of the optimum release profile and identify the underlying reason for its inhibitory effect.

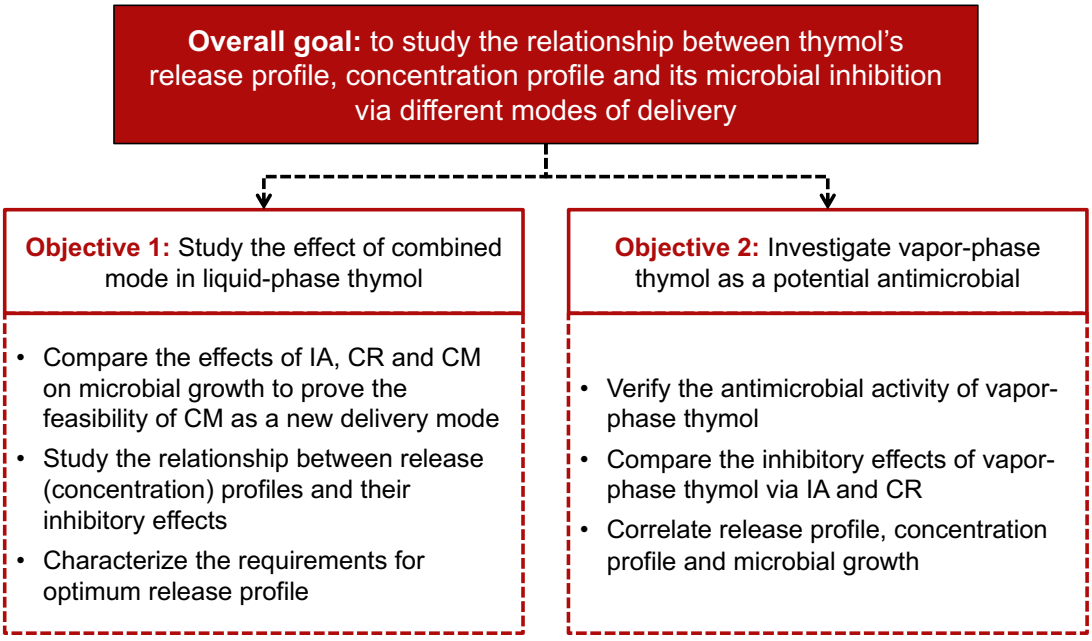

Figure 13 Objectives and tasks of the study

5. MATERIALS AND METHODS

5.1. Bacterial Strain

Bacterial strain *Escherichia coli* DH5α was obtained from *E. coli* Genetic Stock Center, New Haven, CT, USA. The strain was stored at -20 °C and a working culture was maintained at 4 °C on tryptic soy agar (TSA) plate. Upon use, subculture was grown in tryptic soy broth (TSB) at 37 °C overnight and then diluted to appropriate concentration.

5.2. Chemicals

Thymol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to obtain a stock solution of 100 mg/mL. Ethyl acetate was purchased from Fisher Scientific (Waltham, MA, USA). Other chemicals were purchased from VWR (Radnor, PA, USA).

5.3. Syringe Pump

An NE-1000 single syringe pump (New Era Pump System Inc., Wantagh, NY, USA) was used in this study (Figure 14). The syringe pump enables both injection and withdrawal from/to a syringe. Controlled by a microcontroller-based system, the syringe pump allows a wide range of pumping rates, varying from 0.73 μL/h to 2120 ml/h.

The syringe pump is programmable, allowing for complex pump programs. Up to 41 pumping phases can be programmed and stored in memory. In each program phase, the pump operates according to designated pumping rate and dispensing volume, or follows orders such as pause, stop, jump to phase, etc. The pump can also be controlled from a

computer using the RS-232 connection. New Era Pump System Inc. provides fee-for-service program SyringePumpPro, and free programs PumpTerm and WinPumpTerm, which are able to develop pump programs and send commands to a pump when connected to the computer.

The syringe pump is a useful tool to simulate the release of antimicrobials from package into the food. Release rates and dispensing volumes can be programmed into the syringe pump, according to the release profile generated by Equation 3.

Figure 14 NE-1000 syringe pump

5.4. Antimicrobial Activity of Liquid-Phase Thymol via Instant Addition

The antimicrobial activity of liquid-phase thymol via instant addition was studied using a broth macro-dilution assay modified from CLSI [74].

Serial twofold dilutions of thymol were prepared in TSB in a series of test tubes to achieve final concentrations of 1000, 500, 250, 125, and 62.5 mg/L [158]. Overnight culture of *E. coli* DH5α was diluted in TSB and added to each tube to achieve an initial inoculum of approximately 5×10^5 CFU/mL. The final volume in each tube was 2 mL. The concentration of DMSO in each tube did not exceed 1% (v/v). *E. coli* inoculum in TSB with 1% DMSO was used as positive control, in order to confirm that 1% DMSO did not possess antimicrobial activity. Tryptic soy broth with 1% DMSO was used as negative control. The tubes were incubated at 37°C for 24 h, and subsequent experiments were carried out.

The minimum inhibitory concentration (MIC) of liquid-phase thymol via instant addition was determined following 24 hours of incubation. To determine MIC, 10 µL of the broth was removed from each sample tube and inoculated on a TSA plate. Serial dilutions were carried out when necessary. After incubation, the number of surviving microorganisms was enumerated by plate count. The MIC is the lowest concentration that resulted in > 90% decrease in inoculum viability, equivalent to > 1-log reduction in viable count.

5.5. Antimicrobial Activity of Liquid-Phase Thymol via Controlled Release and Combined Mode of Delivery

5.5.1. Simulation of Controlled Release of Thymol

Dilutions of thymol were prepared to obtain stock solutions with concentrations of 20, 15, 10, and 8 mg/mL. To simulate controlled release from packaging, an NE-1000 single syringe pump was used to deliver thymol into the broth over time. Five milliliters of each thymol stock solution from an airtight syringe (VWR, Radnor, PA, USA) loaded on the syringe pump was injected into a sterile PET bottle (Fisher Scientific, Pittsburg, PA, USA) containing 200 mL of TSB inoculated with 5×10^5 CFU/mL *E. coli* culture. The entire system was placed in an incubator at 37°C and shaken at 200 rpm to ensure immediate mixing of thymol into the broth and uniform mixing of *E. coli* (Figure 15). In order to minimize cell dilution effect resulting from the addition of thymol solution, the amount of thymol solution pumped into the broth was no more than 2.5% volume of the broth.

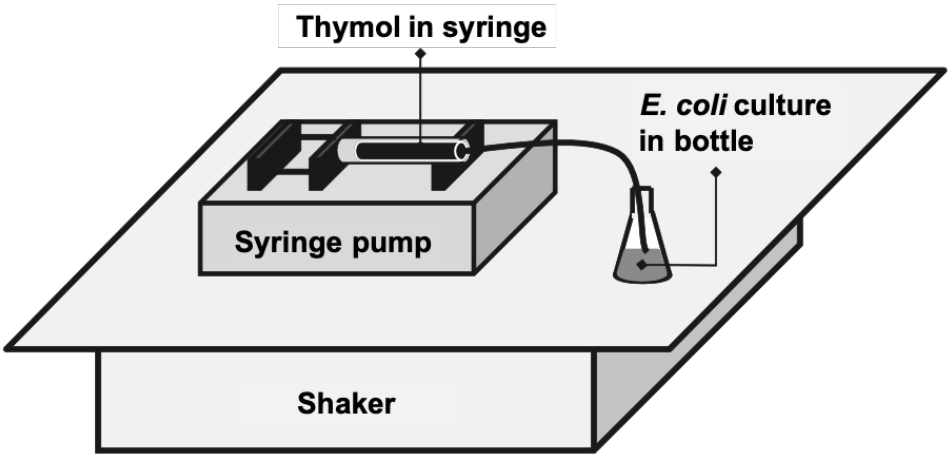

Figure 15 Illustration of syringe pump set up in experimentation of liquid-phase thymol via controlled release and combined mode of delivery

The syringe pump was programmed into 12 phases, with progressively slower injection rate of thymol solution for each subsequent phase. Figure 16 shows the cumulative volume added into the bottle as a function of time. At the end of 24 hours, 5 ml each of the stock solutions of 20, 15, 10, and 8 mg/L concentration was added, resulting in the final thymol concentrations in the broth to be 500, 375, 250, and 200 mg/L, respectively.

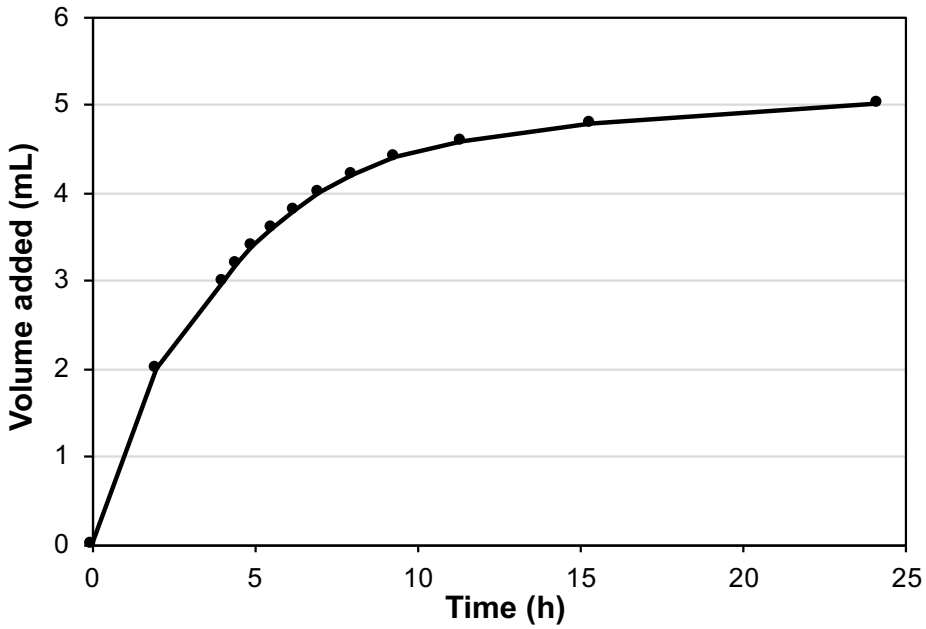

Figure 16 Volume of thymol solution added into broth within 24 hours

5.5.2. Simulation of Combined Mode of Delivery of Thymol

For combined mode, thymol was injected into the broth via a combination of instant addition and controlled release. Half of final target amount of thymol was injected via instant addition to obtain the desired initial concentration quickly, within one minute. The other half of the thymol was injected via controlled release as above using the syringe

pump system following the pre-set program to mimic the progressively slower release over time. The final concentrations of thymol in the broth were also 500, 375, 250 to 200 mg/L at the end of 24 hours.

The MIC of thymol via controlled release and combined mode was determined following the same procedure as in section 5.4.

5.6. Determination of Target Release Profile

5.6.1. Determination of Growth Kinetics for Instant Addition, Controlled Release and Combined Mode of Delivery

The same final concentration, 250 mg/L of thymol, was delivered into cultured broth via the three different delivery modes. At predetermined time intervals, samples were taken, and serial dilutions were carried out as needed, using TSB. Cells were enumerated by plate count method. The inhibitory effect of thymol was observed by plotting the growth curve of log CFU versus time. *E. coli* inoculum in TSB supplemented with 1% DMSO was used as positive control.

The logistic model [159] (Equation 5) was used to fit the growth of *E. coli* DH5α in the absence of thymol (positive control), and the lag phase was calculated using Equation 6.

$$\log N(t) = A + \frac{D}{1 + e^{-B(t-M)}} \quad \text{Equation 5}$$

$$t_{lag} = M - \frac{2}{B}\left(1 - \frac{2}{1 + e^{BM}}\right) \quad \text{Equation 6}$$

Where

M = time at which half the maximum cell concentration is reached

B = maximum specific growth rate

A = value of lower asymptote

D = difference between the lower and upper cell density

5.6.2. Determination of Required Minimum Concentration during Lag Phase

Based on results of above experiments, it was proposed that a certain minimum concentration should be released in order to achieve a log reduction of at least 0 at the end of the lag phase. This concentration serves as a minimum concentration in the lag phase for the design of controlled release profiles. To validate this argument, time kill assay was carried out.

Dilutions of thymol were prepared in TSB in a series of test tubes to achieve final concentrations of 500, 350, 300, 250, 200, 175, 150, 100, and 50 mg/L. Overnight culture of *E. coli* DH5α was diluted in TSB and added to each tube to achieve an initial inoculum of approximately 5×10^5 CFU/mL. The final volume in each tube was 10 mL. The concentration of DMSO in each tube did not exceed 1% (v/v). *E. coli* inoculum in TSB with 1% DMSO was used as positive control, in order to confirm that 1% DMSO did not possess antimicrobial activity. The tubes were incubated at 37°C for 1.14 hours, which was the determined inherent lag phase under normal conditions.

Subsequently, 10 μL of the broth was removed from each sample tube and inoculated on a tryptic soy agar (TSA) plate. Serial dilutions were carried out when necessary. After incubation, the number of surviving microorganisms was enumerated by plate count, and the log reduction for each concentration of thymol was determined.

A dose response model was used which reflected the relationship between the response (i.e., log CFU) and the logarithms of concentrations (i.e., log concentration) (Equation 7).

Data obtained from plate count was fitted to the model and the dose response curve was plotted using log reduction versus log concentration with R version 3.4.1 (The R Foundation for Statistical Computing, http://www.R-project.org), and the concentration at which log reduction equals zero was obtained from the curve.

$$\log \mathrm{CFU} = \mathrm{Bottom} + \frac{\mathrm{Top} - \mathrm{Bottom}}{1 + 10^{(\log \mathrm{IC50} - \log \mathrm{concentration}) \times \mathrm{HillSlope}}} \quad \text{Equation 7}$$

Where

Top = value of upper asymptote

Bottom = value of lower asymptote

IC50 = the concentration of inhibitor that gives a response that's exactly halfway between Bottom and Top.

HillSlope = the steepness of the family of curves [160].

5.6.3. Design of Release Profile Using Crank's Model

After obtaining the minimum concentration required to be released during the lag phase, Crank's diffusion model (Equation 4) can be used to generate target optimum release profiles. Two types of target release profiles were generated: one type is the release of thymol via controlled release, and the other type via combined mode.

For the release profile using controlled release, $M_{p,0}$ was selected to be 500 mg/L, the MIC of instant addition of thymol; L was assumed to be 3 mil (76.2 μm); $M_{f,t}$ was the derived minimum concentration from section 5.7, and t was the lag phase, 1.14 hours. The D value was then calculated from Equation 4, and the release profile was generated using the D value. Because the $M_{f,t}$ and t were fixed values, only one release profile could be derived from Equation 4, regardless of the L and $M_{p,0}$ selected.

For the release profiles using combined mode, since certain amounts of thymol were instantly added into the culture, the amounts in this study were selected to be 50, 75, and 100 mg/L. The $M_{p,0}$ and $M_{f,t}$ values were the same values in the target controlled-release profile, less the amount initially added. L was also assumed to be (76.2 μm), and t was 1.14 hours. Various D values were calculated from Equation 4, and the release profiles were then generated using the D values.

5.7. Antimicrobial Activity of Vapor-Phase Thymol via Instant Addition

5.7.1. Determination of Minimum Inhibitory Dose of Vapor-Phase Thymol

The antimicrobial activity of vapor-phase thymol was studied using a vapor diffusion assay adapted from Lopez, Sanchez [5] with modification.

Briefly, petri dishes (50 mm I.D.) containing 10 mL of TSA medium were inoculated with 100 μL of *E. coli* DH5α culture (10^6 CFU/mL). Thymol was dissolved in ethyl acetate and diluted in a two-fold series to achieve final concentrations ranging from 40 to 0.625 mg/mL. Two hundred microliters of each dilution were added to a 7-cm blank filter paper which was previously stuck on the bottom of a 260-mL sterile glass jar. The filter papers were let dry for 2 minutes to remove ethyl acetate. Next, the inoculated petri dishes were then placed onto the caps, which were then screwed back on the jars. A filter paper impregnated with ethyl acetate was used as the control, in order to confirm that ethyl acetate did not possess antimicrobial activity. Figure 17 provides a simple illustration of the assay set-up. The glass jars were inverted and incubated at 37 °C for 24 hours, and the diameter of the inhibition zone, where no growth of *E. coli* was detected, was measured.

The minimum inhibitory dose (MID) of vapor-phase thymol was determined as the lowest calculated headspace concentration to completely inhibit microbial growth. It was calculated based on the amount instantly added (in mg) and the headspace volume (in L), and expressed as weight per unit volume (in mg/L headspace). Conventionally, the antimicrobial activity of vapor-phase essential oils (EOs) and their constituents is represented by the minimum inhibitory concentration (MIC), a term also commonly used in the studies of liquid- and solid-phase EOs. Although MID is fundamentally equivalent

to MIC, the reason for using this new term in this work was to stress the fact that MID was a calculated value, and to distinguish it from the actual headspace concentration.

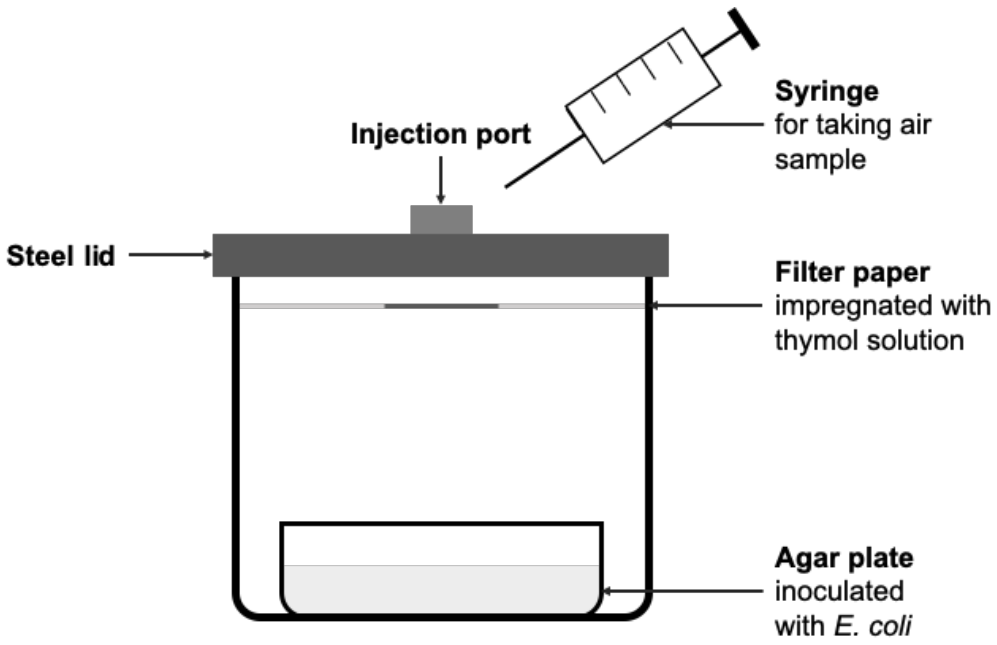

Figure 17 Illustration of vapor diffusion assay set-up

5.7.2. Time Kill Assay

Time kill assay was performed following similar procedure as previously described in the vapor diffusion assay. Inoculated petri dishes were exposed to thymol dilutions of 10 and 5 mg/mL, i.e. 8 and 4 mg/L headspace, for 1, 2, 4 and 6 hours. Inoculated petri dishes exposed to ethyl acetate for the same time periods were used as the control. The petri dishes were then removed from the glass jars and incubated normally until 24 hours. After the incubation period, the diameter of the inhibition zone was measured. Reduction in viability (%) was calculated based on the inhibition zones with respect to the control group [161].

5.7.3. Determination of MIC of Solid-Phase Thymol

The antimicrobial activity of solid-phase thymol was studied using a paper disk diffusion assay [5] with modification. Petri dishes containing 10 mL of TSA medium were inoculated with 100 μL of *E. coli* DH5α culture (10^6 CFU/mL). Thymol was dissolved in ethyl acetate and diluted in a two-fold series to achieve final concentrations ranging from 640 to 2.5 mg/mL. Ten microliters of each dilution were added to a 1-cm blank filter disk, which was then placed on top of the cultured medium. Ten microliters of ethyl acetate were used as the control. The petri dishes were incubated at 37 °C for 24 hours, and the diameter of the inhibition zone was measured. The MIC was defined as the lowest concentration to totally inhibit microbial growth. The MIC value was expressed as weight per unit volume (mg/L agar).

5.7.4. Headspace and Agar Layer Analyses

In the vapor diffusion assay, the amount of thymol added into the system was known, however, the concentration calculated based on the added amount could not reflect the actual concentration profile. To determine the actual concentration profile of thymol in both the headspace and the agar layer, a method was adapted from Inouye, Takizawa [30] with modification.

Briefly, a hole was bored on the steel cap of the glass jar and sealed with an injection port. Inoculated petri dishes were exposed to thymol dilutions of 10 and 5 mg/mL, i.e. 8 and 4 mg/L headspace. An air sample (1 mL) was taken at 1, 2, 4, 6, 8 and 24 hours using an airtight syringe (Figure 17). The air sample was injected into an airtight vial

containing 500 µL of ethyl acetate. The vial was shaken for 3 minutes to dissolve thymol vapor into the solvent. Five microliters of the extract were then subject to gas chromatography (GC) analysis.

Absorption of thymol vapor into the agar layer was determined by extraction. After the air sample was taken, the agar layer was removed from the glass jar and placed in another glass jar containing 20 mL of ethyl acetate. Extraction lasted 3 hours, followed by drying over anhydrous magnesium sulfate. Five microliters of the extract were then subject to GC analysis.

GC analysis was carried out using a GC system (Hewlett Packard HP 5890 Series) with a DB-5 column (30 m × 0.320 mm I.D., 0.25 µm film thickness) and an FID detector. Helium was used as the carrier gas. The column temperature was initially set at 55 °C for 2 minutes, then raised from 55 to 135 °C at a rate of 5 °C /min, and finally held at 135 °C for 2 minutes.

5.8. Antimicrobial Activity of Vapor-Phase Thymol via Controlled Release

5.8.1. Generation of Thymol's Release Profiles

The release of antimicrobials from packaging into food involves 3 steps [139]:

(1) diffusion within the polymeric film, (2) partitioning at the interface, and (3) dispersion into the food matrix. The diffusion of antimicrobials is usually the rate-determining step, which can be described by Crank's diffusion model [157], based on the assumptions that the release is from one side of the film, the time is short, and the ratio of the amount of antimicrobial released to the amount of antimicrobial in the film after time t is less than 2/3. Crank's diffusion model can be expressed by Equation 4.

In this study, M_0 was selected as 8 mg/L headspace, the MID obtained in the vapor diffusion assay (2.2.1). L was assumed to be 3 mil (76.2 μm). Various values of M_t and t were selected to calculate D. The release profiles were then generated using the various D values.

5.8.2. Simulation of Thymol's Controlled Release

The generated release profiles were simulated by a NE-1000 syringe pump system. Appropriate amount of thymol solution was loaded in an airtight syringe (VWR, Radnor, PA, USA) mounted on the syringe pump, and injected into a sterile glass jar containing a petri dish inoculated with 100 μL of *E. coli* DH5α culture (10^6 CFU/mL). The needle tip was attached to a blank filter paper inside the glass jar, to facilitate vaporization and avoid direct addition of liquid into the jar. The syringe pump was programmed into several phases, each of which was set to deliver thymol solution at a fixed rate for a period of time. The rates were calculated according to the release profiles generated previously, in order to mimic the release of thymol from packaging films. The syringe pump together with the glass jar was placed at 37 °C. After 24 hours, the petri dishes were taken out and the diameter of the inhibition zone was measured. The petri dishes were then continued to be incubated for another 48 hours to observe any sign of microbial growth. Figure 18 provides a simple illustration of the experiment set-up. The most effective release profile was identified as the optimum, and the headspace concentration profile was then measured. Following the optimum release profile, thymol was injected into a glass jar containing an uninoculated petri dish. At 1, 2, 4, 6, 8 and 24

hours, an air sample (1 mL) was taken using an airtight syringe. Following the same procedure as in 2.2.4, the air sample was subject to GC analysis.

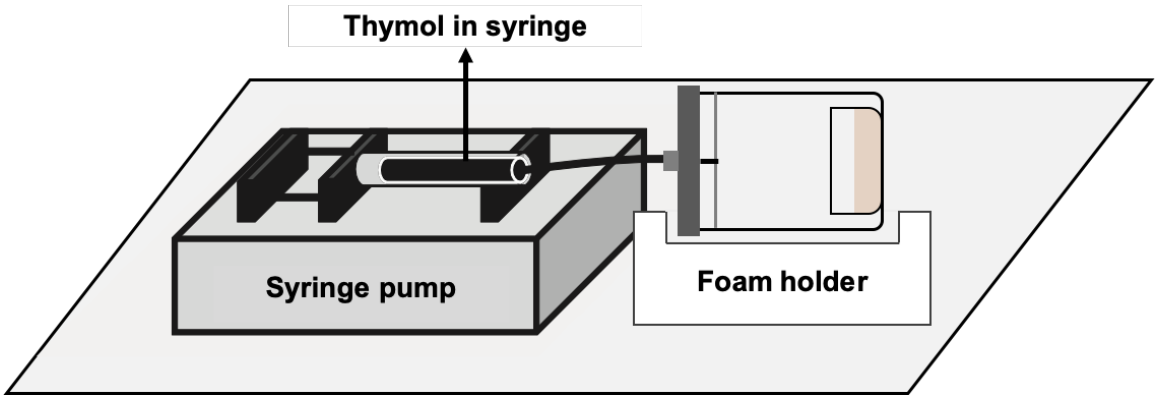

Figure 18 Illustration of set-up for controlled release of vapor-phase thymol

5.9. Data Reproducibility

To ensure reproducible results, each experiment was performed at least three times in triplicate.

6. RESULTS AND DISCUSSION

6.1. Antimicrobial Activity of Liquid-Phase Thymol

6.1.1. Inhibition of *E. coli* DH5α by Instant Addition of Thymol

The effect of liquid-phase thymol via instant addition on the growth of *E. coli* DH5α was studied to provide a basis for the design and experimentation of thymol release via controlled release and combined mode of delivery.

Figure 19 shows the antimicrobial activity of thymol against *E. coli* DH5α via instant addition. The final thymol concentrations of 1000, 500, 250, 125 and 62.5 mg/L resulted in log reductions of 5.74, 5.74, 0.32, -2.09 and -2.72, respectively. Total inhibition of *E. coli* DH5α was observed for 500 and 1000 mg/L.

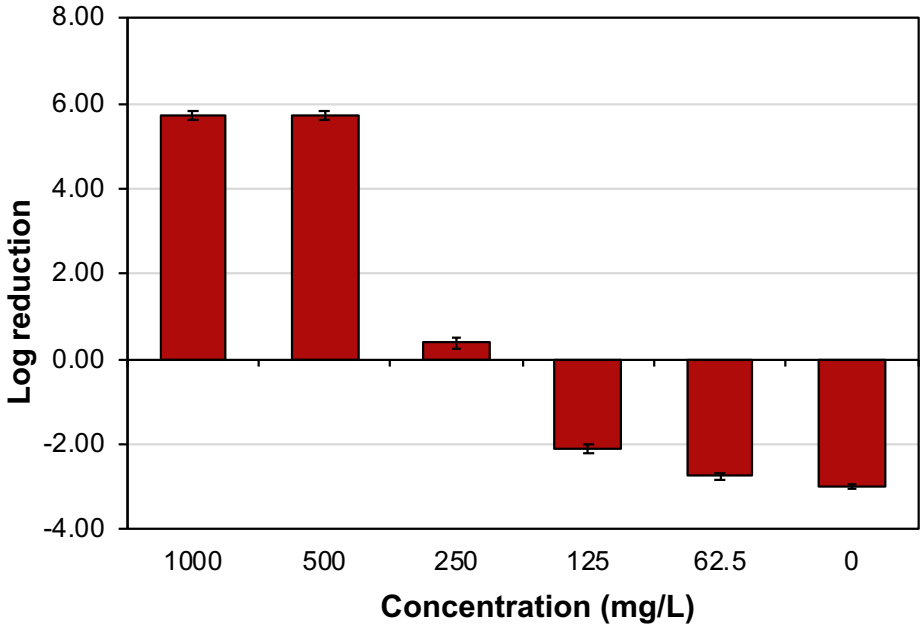

Figure 19 Inhibitory effect of thymol on growth of *E. coli* DH5α via instant addition

According to the definition of MIC and the above observation, the MIC of liquid-phase thymol was determined to be 500 mg/L, which is consistent with the 400 and 450 mg/L MIC values of thymol reported by Pei, Zhou [80] and Helander, Alakomi [11], respectively. However, based on the literature review, the MIC values of thymol on various strains of *E. coli* can be as low as 10 mg/L [88], and as high as 5000 mg/L [7, 90]. The discrepancy might be due to the differences in *E. coli* strains, definition of MIC, experimental method, culture medium, volume of inoculum, temperature, incubation time, and enumeration method.

6.1.2. Inhibition of *E. coli* DH5α via Controlled Release and Combined Mode

Figure 20 shows the release profiles generated by the syringe pump system.

For controlled release, four profiles of concentration in the broth versus time are shown by the grey lines in Figure 20, with starting concentration to be 0 and final concentrations of 500, 375, 250 and 200 mg/L achieved at the end of 24 hours.

For combined mode, four profiles of concentration in the broth versus time are shown by the black lines in Figure 20, with initial concentrations of 250, 187.5, 125 and 100 mg/L at time zero and same endpoints as in controlled release.

It should be mentioned that the release profiles were generated using a guiding principle of initially fast and progressively slower, but they did not particularly follow Crank's diffusion model.

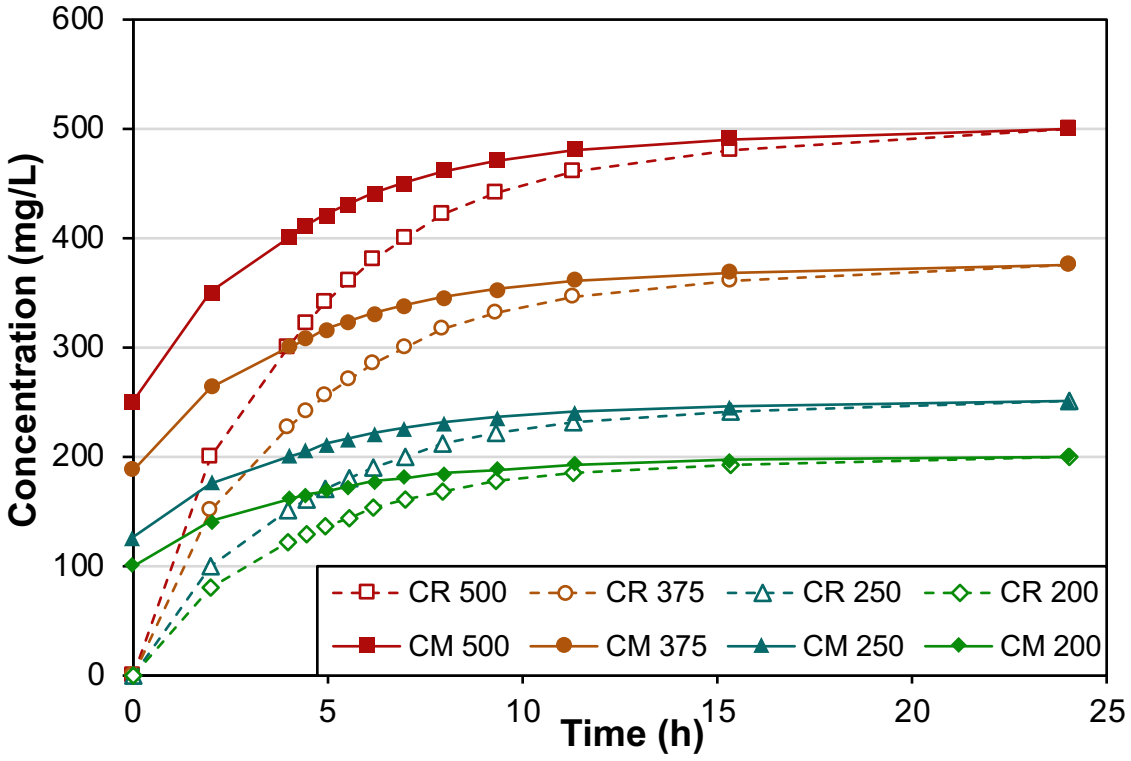

Figure 20 Release profiles generated by a syringe pump system

Note: Open square (□) represents releasing thymol solution via controlled release to reach a final concentration of 500 mg/L; closed square (■) represents releasing thymol solution via combined mode to reach a final concentration of 500 mg/L; open circle (○) represents releasing thymol solution via controlled release to reach a final concentration of 375 mg/L; closed circle (●) represents releasing thymol solution via combined mode to reach a final concentration of 375 mg/L; open triangle (△) represents releasing thymol solution via controlled release to reach a final concentration of 250 mg/L; closed triangle (▲) represents releasing thymol solution via combined mode to reach a final concentration of 250 mg/L; open diamond (◊) represents releasing thymol solution via controlled release to reach a final concentration of 200 mg/L; closed diamond (♦) represents releasing thymol solution via combined mode to reach a final concentration of 200 mg/L.

Figure 21 shows the antimicrobial activity of thymol against *E. coli* DH5α via controlled release and combined mode of delivery.

For controlled release, at the end of 24 hours, final concentrations of 500, 375, 250 and 200 mg/L resulted in log reductions of 5.62, 3.78, 0.29 and -0.29, respectively. Total inhibition of *E. coli* DH5α was achieved by a final concentration of 500 mg/L, while a final concentration of 375 mg/L resulted in a log reduction of more than 1-log. The MIC of thymol via controlled release is thus determined as 375 mg/L, which is 25% lower than the MIC of thymol via instant addition.

For combined mode, at the end of 24 hours, final concentrations of 500, 375, 250 and 200 mg/L resulted in log reductions of 5.56, 5.76, 2.86 and -1.29, respectively. Total inhibition of *E. coli* DH5α was achieved by final concentrations of 500 and 375 mg/L, while a final concentration of 250 mg/L resulted in a log reduction of more than 1-log. The MIC of thymol via combined mode is thus determined as 250 mg/L, which is 50% lower than the MIC via instant addition and is 33% lower than the MIC via controlled release.

The results suggest that a final concentration of 500 mg/L is able to completely inhibit the growth of *E. coli*, whether via instant addition, controlled release, or combined mode. It implies that the added amount is more than enough to kill off all the microorganisms and in the case the mode of delivery is less important. However, when the final concentration was reduced to 250 mg/L, the mode of delivery made a difference. Instant addition resulted in a 0.32-log reduction, controlled release resulted in a 0.29-log reduction – but combined mode (instantly adding 125 mg/L and slowly releasing 125 mg/L thymol) resulted in a 2.86-log reduction, which is significantly better than the

results obtained from the other two delivery modes. This indicates that different modes of delivery can result in significantly different inhibitory effects, even if the final concentrations or the amounts of thymol added to the broth are the same.

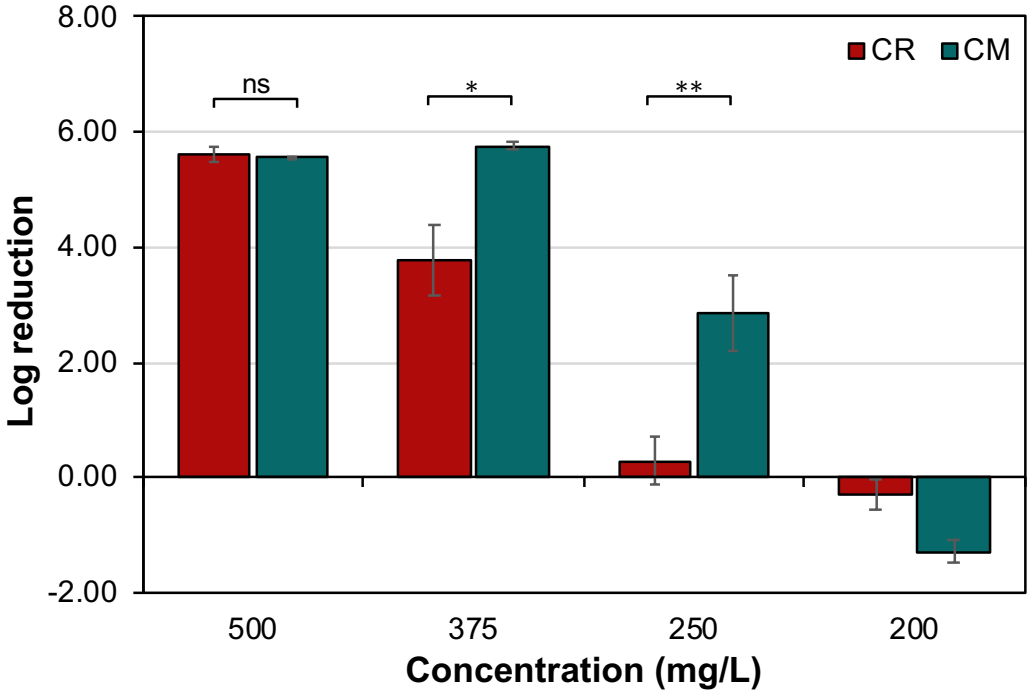

Figure 21 Inhibitory effect of thymol on growth of *E. coli* DH5α via controlled release and combined mode of delivery

Note: Releasing 375 and 250 mg/L thymol via different modes of delivery resulted in significantly different effects (t-test, CR versus CM, ns = not significant, *P<0.05, **P<0.01, ***P<0.001). Standard deviations are shown as error bars.

6.1.3. Growth Kinetics of *E. coli* DH5α for Instant Addition, Controlled Release, and Combined Mode of Delivery

The same final concentration, 250 mg/L of thymol, was added to inoculated broth, and growth curves of *E. coli* DH5α were drawn to investigate the underlying mechanism of

microbial inhibition. Figure 22 clearly shows that different modes of delivery can result in different patterns for inhibition, even when the final concentrations are the same. This is due to the multiple factors in play: microbial growth, release of thymol into the broth, and consumption/degradation of thymol.

For final concentration of 250 mg/L achieved via instant addition, a sharp decrease in bacterial count was observed in the first 2 hours of incubation. However, since 250 mg/L was below the MIC of instant addition, some *E. coli* cells (around 2-log CFU/mL) survived under the selective pressure. As thymol in the broth was being consumed or degraded, and no thymol was supplemented into the broth, the concentration of thymol in the broth decreased over time. The surviving *E. coli* cells slowly acquired adaptability to the environment and began to grow, eventually resulting in a less than 1-log reduction at the end of 24 hours.

For final concentration of 250 mg/L thymol achieved via controlled release, the growth curve overlapped with that of the control during the first 4 hours, indicating that the concentration of thymol in the broth was insufficient to inhibit *E. coli* growth. As thymol had accumulated over time to some effective concentration, a sudden 2.68-log reduction in bacterial count was observed at 6 hours, while 4.40-log CFU/mL of *E. coli* cells still remained. After 6 hours, although some thymol was slowly supplemented into the broth, it might be far from enough to make up for the portion that had been consumed/degraded during the microbial inhibition. As a result, the remaining thymol in the broth was unable to inhibit the growth of the surviving *E. coli* cells, which were able to multiply at a faster rate than the rate of being inhibited by thymol, thus the growth continued, eventually resulting a less than 1-log reduction at the end of 24 hours.

For final concentration of 250 mg/L achieved via combined mode, the growth of *E. coli* slowed down during the first 2 hours, because of the initial 125 mg/L concentration created via instant addition, exerting a selective pressure to delay the growth of *E. coli*. At 4 hours, bacteria count began to drop because thymol had accumulated to a point where more *E. coli* cells were killed than multiplied. As thymol was continuously supplemented into the broth, the selective pressure in the environment kept reducing the bacteria count, eventually resulting in a 2.86-log reduction at the end of 24 hours.

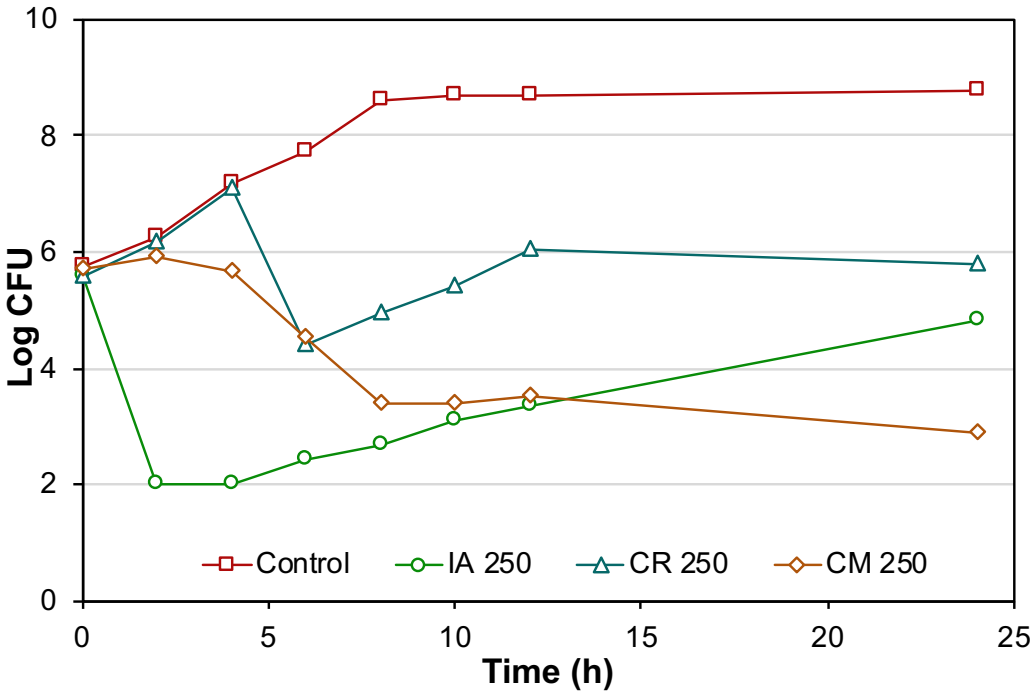

Figure 22 Growth kinetics of *E. coli* DH5α under different modes of delivery

Note: Open square (□) represents *E. coli* culture in the absence of thymol (control); open circle (○) represents *E. coli* culture treated with instant addition of 250 mg/L thymol; open triangle (△) represents *E. coli* culture treated with controlled release of 250 mg/L thymol; open diamond (◊) represents *E. coli* culture treated with 250 mg/L thymol via combined mode of delivery.

6.1.4. Proposed Requirements for Effective Microbial Inhibition

As suggested by Balasubramanian, Lee [17], the concentration achieved during the lag phase is critical to inhibit subsequent microbial growth. Based on the previous work and the results from this work, we propose that two requirements must be met to achieve effective microbial inhibition: (1) a fast release of antimicrobial during the lag phase to achieve a minimum concentration in the broth to inhibit microbial growth, and (2) a progressively slower release at later time to replenish antimicrobial that is consumed or degraded. An optimum release profile should be able to satisfy both requirements, using as little amount as possible.

As shown in Figure 22, instant addition met the first requirement, but failed to meet the second requirement due to the absence of sustained release. Controlled release met the second requirement but failed to meet the first requirement due to insufficient concentration achieved during the lag phase. Combined mode, being the most effective among the three modes, merely slowed down, rather than inhibited microbial growth during lag phase; therefore, it met the second requirement but did not completely meet the first requirement.

To further quantify the first requirement, the inherent lag phase was estimated by the logistic model as 1.14 hours (Figure 23). For a final concentration of 250 mg/L, at the end of this inherent lag period, instant addition, controlled release, and combined mode achieve concentrations of 250, 57, and 154 mg/L, corresponding to the estimated log reductions of 2.0, -0.34, and -0.12, respectively. It can be estimated from these data points that a concentration of approximately 165 mg/L is required to achieve a log reduction of 0 at the end of the lag phase. This concentration of 165 mg/L may serve as

the minimum concentration to satisfy the requirement for the first requirement, and a reference to design the optimum release profile. Depending on the shelf life requirement of the food product, concentrations higher than 165 mg/L may be used to provide positive values of log reduction and stronger microbial inhibition, so long as these higher concentrations do not exceed the sensory thresholds and regulatory requirements of the product.

After the lag phase, since the microorganisms are already inhibited, a subsequent slow release to replenish the consumed portion is needed, in order to maintain the inhibitory effect. The amount released at later time depends on the required shelf life. Based on the experiment results, for foods with relatively shorter shelf life such as fresh produce, only small quantities are required. The release rates should also follow the Crank's diffusion model, in order to best simulate the release from CRP.

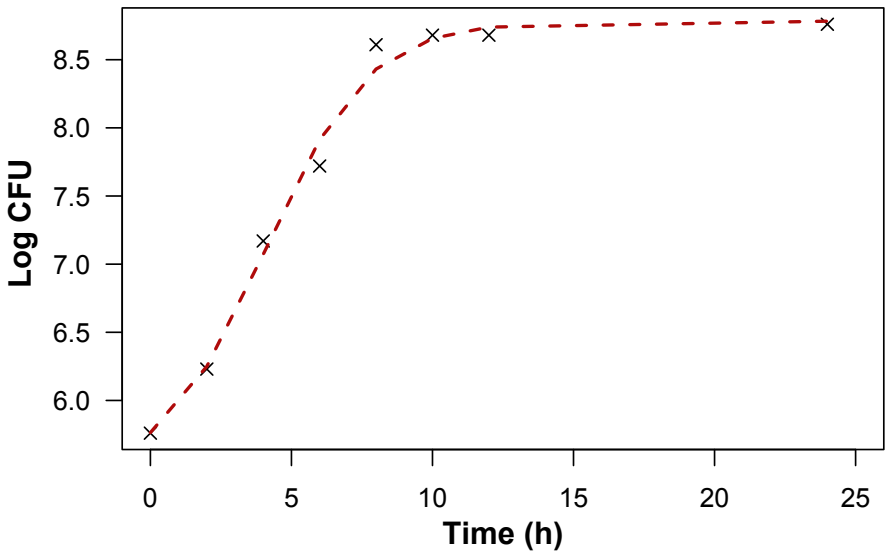

Figure 23 Curve-fitting of *E. coli* growth kinetics using logistic model

6.1.5. Validation of the Minimum Concentration Required during Lag Phase

As predicted in the previous section, a minimum concentration is required to be released during the lag phase, in order to serve as an immediate pressure to the microorganisms. In order to identify this minimum concentration, *E. coli* culture was exposed to varying concentrations of thymol for 1.14 hours, and the surviving microorganisms were enumerated using plate count. A dose response curve of log CFU versus log concentration was drawn using R (Figure 24). The parameters of the fitted curve are shown below in Table 8. The R squared value was 0.9890, indicating a good fit.

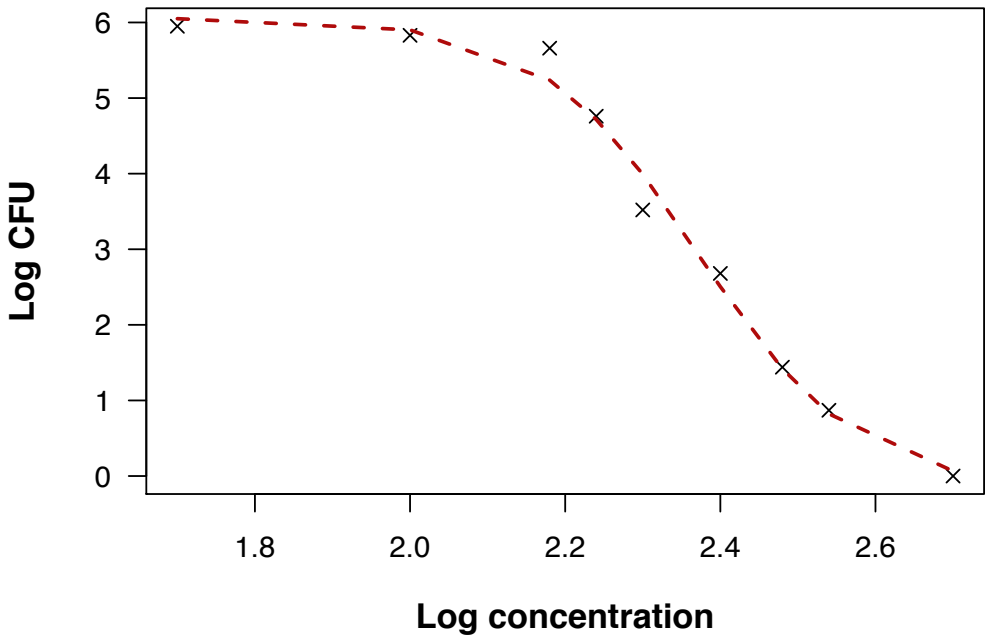

Figure 24 Dose response curve fitted to time kill data

Table 8 Four parameters generated from the dose response curve

Parameters	Best-fit values	Standard Error	t value	Pr(> |t|)
Bottom	6.05986	0.4137	-0.409	0.69949 ^{ns}
Top	-0.1692	0.2417	25.070	1.88×10^{-6} ***
logEC50	2.3711	0.0218	108.776	1.25×10^{-9} ***
HillSlope	-4.2806	0.7434	-5.758	0.0222 **

Note: t-test, ns = not significant, *P<0.05, **P<0.01, ***P<0.001

The relationship between log CFU and log concentration can be expressed by the following equation:

$$\log \mathrm{CFU} = -0.1692 + \frac{6.2282}{1 + 10^{(-4.281) \times (2.371 - \log \text{concentration})}} \qquad \text{Equation 8}$$

It can be derived from the equation that the minimum concentration required to achieve a 0-log reduction during the lag phase is 123 mg/L. Therefore, to design an optimum release profile for liquid-phase thymol to effectively inhibit *E. coli* growth, the release of thymol must reach approximately 123 mg/L during the lag phase.

The estimated value for the minimum concentration was 165 mg/L, which is somewhat close to the calculated 123 mg/L. Due to the inevitable system errors in the experiments, as long as the estimation and the calculation fall within an acceptable range, it's still a valid argument.

6.1.6. Target Release Profiles Generated Using Crank's Diffusion Model

After identifying the minimum concentration to satisfy the first requirement, Crank's diffusion model was used to generate target release profiles that meet this minimum

concentration. The parameters generated from Equation 4 were shown in Table 9. Using these parameters, four release profiles were generated and shown in Figure 25.

As was concluded in the previous section, there is a required minimum concentration, i.e. 123 mg/L, that needs to be released during the lag phase of 1.14 hours. Therefore, to generate target release profiles that meet these specific time and concentration values, there will only be one controlled release profile, regardless of the film thickness and diffusivity parameters. It demonstrates the inflexibility of using only one mode of delivery. When circumstances change and no actual packaging film is able to release antimicrobials following the designed release profile, the optimum release profile cannot be generated, and effective antimicrobial activity cannot be achieved.

Contrarily, combined mode provides higher flexibility in designing release profiles. As can be seen, in order to satisfy the required minimum concentration in the lag phase, by adjusting the amount of thymol instantly added, various release profiles can be generated, based on different diffusivities. Thus, in reality, combined mode provides more freedom in selecting packaging films and designing release profiles to meet the target.

It should be pointed out that instant addition and controlled release are the preferred delivery modes due to their simplicity, and they should be attempted first before seeking an alternative delivery mode. However, when simple delivery mode cannot satisfy the complex requirements, combined mode can serve as an effective alternative to circumvent the challenges.

Table 9 Target release profiles generated using Crank's diffusion model

Release Profile	Instant addition (mg/L)	$M_{f,t}$ (mg/L)	t (h)	$M_{p,0}$ (mg/L)	D (m^2/s)	Simulated time length (h)
CR-1	0	123	1.14	500	6.72×10^{-14}	3
CM-1	50			450	2.92×10^{-14}	6
CM-2	75			425	1.42×10^{-14}	12
CM-3	100			400	3.67×10^{-15}	24

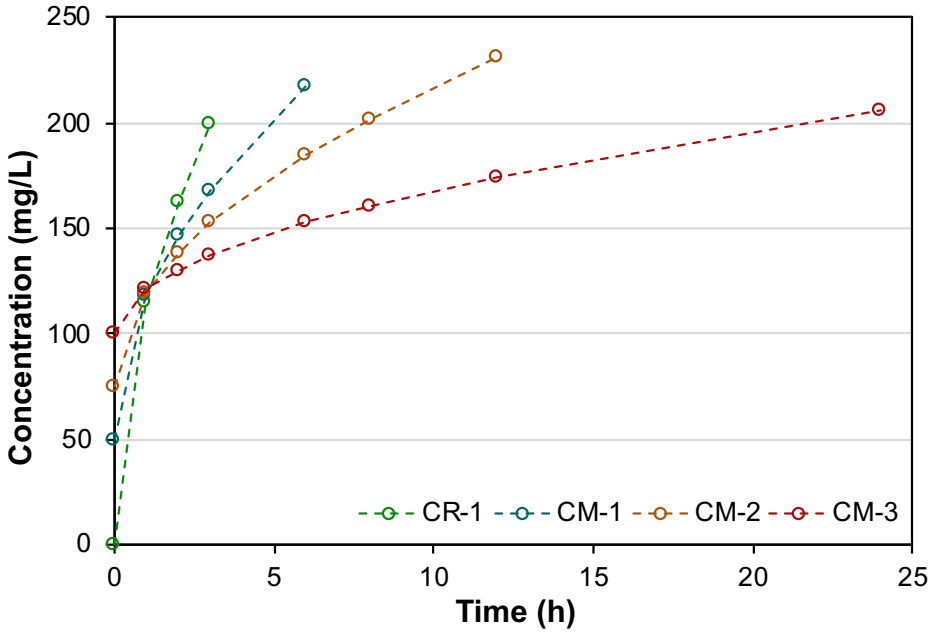

Figure 25 Target release profiles generated from Crank's diffusion model

Note: CR-1 corresponds to the target release profile generated using controlled release as the delivery mode, with a final concentration of 200 mg/L released in 3 hours. CM-1 to CM-3 correspond to the three target release profiles generated using combined mode as the delivery mode, with final concentrations of 217, 231 and 206 mg/L released in 6, 12 and 24 hours, respectively.

6.2. Antimicrobial Activity of Vapor-Phase Thymol

6.2.1. Antimicrobial Activity of Vapor-Phase Thymol via Instant Addition

The effect of thymol via instant addition on the growth of *E. coli* DH5α was studied to verify the antimicrobial activity of vapor-phase thymol and provide a basis for the design and experimentation of controlled release of thymol.

Figure 26 shows the antimicrobial activity of vapor-phase thymol. Instant addition of thymol between 4 and 32 mg/L headspace resulted in microbial inhibition, while 2 mg/L headspace and lower did not exhibit any inhibitory effect. Since the lowest concentration to show total inhibition was 8 mg/L headspace, it was determined as the MID. However, one thing worth noting is that the MID was calculated based on the amount of thymol added into the closed system and the headspace volume, and did not reflect the actual vapor concentration. Since thymol is not highly volatile, not all added was able to vaporize. The actual headspace concentration was determined by GC and described in later section.

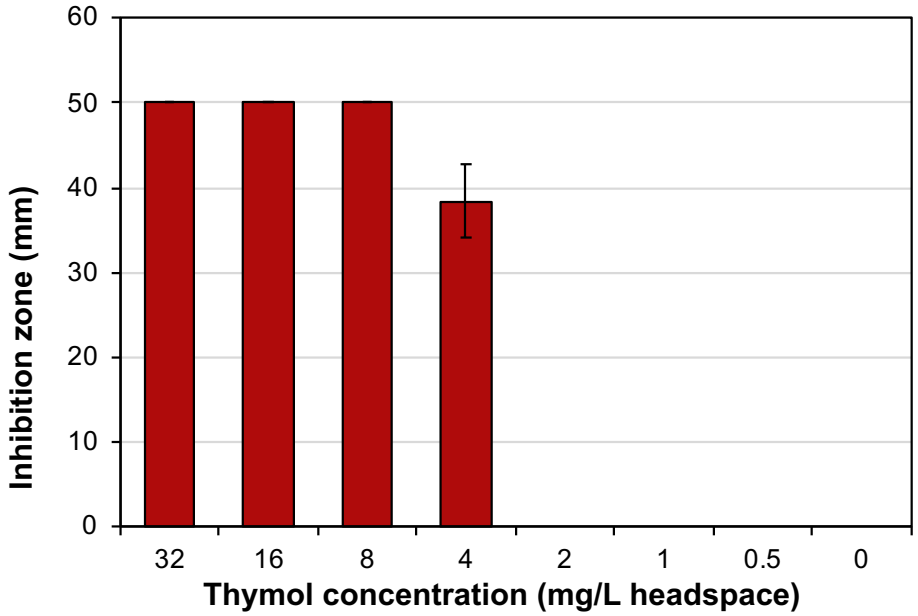

Figure 26 Inhibitory effect of vapor-phase thymol via instant addition, expressed as the diameter of inhibition zone (mm). Inhibition zone was determined by measuring the region where no microbial growth was observed after 24 hours of incubation at 37°C under each treatment condition.

Figure 27 shows the antimicrobial activity of solid-phase thymol. The inhibition zone increased gradually as higher concentration was initially added. Concentrations of 320 and 640 mg/L agar were nearly able to totally inhibit microbial growth. The MIC was thus determined as 320 mg/L agar. Because of thymol's low volatility, it's reasonable to assume that the added thymol will mostly remain on the agar surface or in the agar layer. Therefore, the calculated MIC is an appropriate representation of the actual agar layer concentration. The MIC of solid-phase thymol was consistent with the MIC obtained from liquid-phase macro-dilution assay, which was 500 mg/L.

From the results, it can be seen that instant addition of liquid- and solid-phase thymol requires high concentrations to be effective, while vapor-phase thymol was able to inhibit microbial growth utilizing lower concentration than liquid- and solid-phase thymol. The

results are consistent with previous studies [25-27, 85]. Thymol's lipophilic nature could account for the variation in the effective concentrations. When thymol is added into liquid- or solid-phase medium, its poor water solubility hinders its activity, thus higher concentrations are needed to be effective. In contrast, when thymol is applied via vapor diffusion, the vapor can directly attack the cellular membranes of the microorganisms without contacting the agar medium, thus lower concentrations are needed to be effective. There is practical meaning to the results: In real-life situations, microbial contamination mostly occurs on the surface of the food, instead of uniformly distributes within the food. As a result, adding liquid- or solid-phase thymol into the food formulation is an inefficient and ineffective way to inhibit microbial growth. Contrarily, vapor-phase thymol can be a potent alternative with less concentration used, as it mostly targets the surface of the food and avoids interaction with the food matrix.

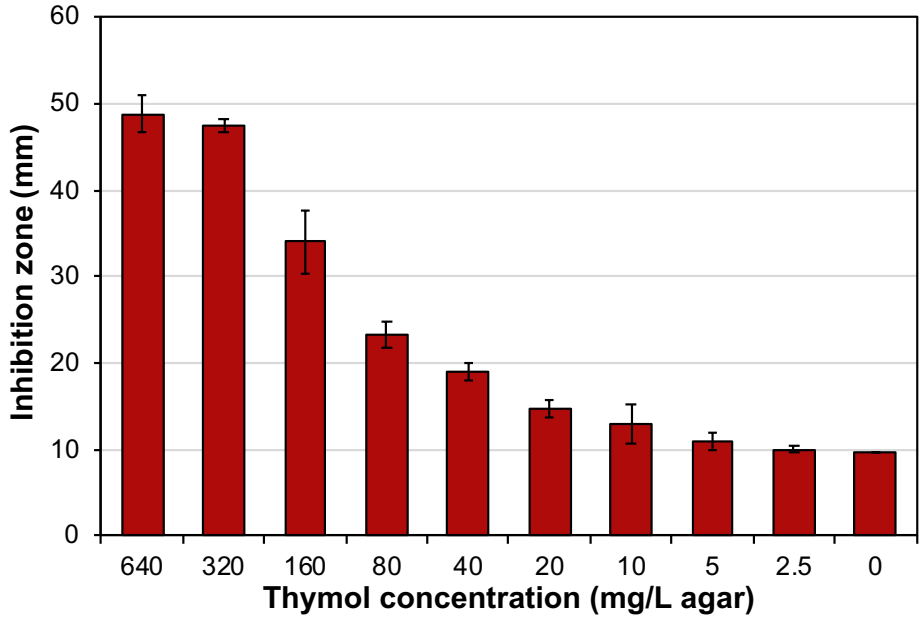

Figure 27 Inhibitory effect of solid-phase thymol via instant addition, expressed as the diameter of inhibition zone (mm)

6.2.2. Correlation Between Concentration Profile and Antimicrobial Activity

Appropriate amount of thymol was instantly added into glass jars to obtain calculated headspace concentrations of 8 and 4 mg/L, which were the determined MID and ½ MID. The concentration of thymol in the headspace (vapor-phase) and in the agar layer (solid-phase) was measured at pre-determined time intervals, and results are shown in Figure 28.

As can be seen in Figure 28a, the actual vapor concentration of thymol in the headspace was well below the calculated value and changed constantly during the incubation period. When MID was added via instant addition, the vapor concentration kept increasing until 4 hours, to reach a maximum of 0.11 mg/L headspace. It then declined to 0.034 mg/L headspace at 6 hours and maintained at a low level until 24 hours. When ½ MID was added instantly, the vapor concentration reached a maximum of 0.12 mg/L headspace at 1 hour, a faster time course than instant addition of MID. The vapor concentration then quickly declined to 0.021 mg/L at 6 hours and maintained at a low level until 24 hours. Several reasons could cause the actual vapor concentration to be lower than calculated: (1) Thymol's low volatility, thus not all added was able to vaporize immediately, and (2) Loss of vapor-phase thymol, due to microbial consumption, agar absorption, natural degradation and system leakage. Therefore, the MID value is not an accurate measure of the actual concentration in the headspace. While it does not represent the actual vapor concentration, the MID nonetheless denotes the amount added, therefore still can serve as a reference for horizontal comparison between different vapor-phase antimicrobials under the same condition and vertical comparison for the same antimicrobial under different conditions, such as instant addition versus controlled release.

The absorption of thymol into the agar layer is shown in Figure 28b. In contrast to the low vapor concentration, more than half of thymol added was absorbed into the agar layer. Initially, the two concentration profiles in Figure 28b overlapped, indicating that the absorption occurred at full speed. The profiles then diverged after 2 hours. Agar absorption slowed down in the ½ MID profile but maintained in the MID profile. The maximum concentrations in the agar layer were 53.2 and 106.7 mg/L agar, for ½ MID and MID respectively. It reflected a doubling relationship as the amount added was also double. However, if compared to results from paper disk diffusion assay, these maximum concentrations were well below the required MIC of solid-phase thymol, suggesting that thymol absorbed in the agar might not play a significant role in microbial inhibition. Moleyar and Narasimham [162] has reported that the growth inhibition of vapor-phase essential oils is a combined effect of volatile compounds and the compounds absorbed into the medium. Nevertheless, it is still uncertain as to which portion contributes more to the overall effect. Gocho [163] stressed the substantial contribution of the volatile compounds which were absorbed into the agar, while Inouye, Tsuruoka [164] pointed out the importance of vapor accumulation. Here, we hypothesize that the vapor-phase thymol in the headspace was the main contributor to the overall antimicrobial effect, while the solid-phase thymol absorbed into the agar layer did not play a major role in inhibiting microbial growth.

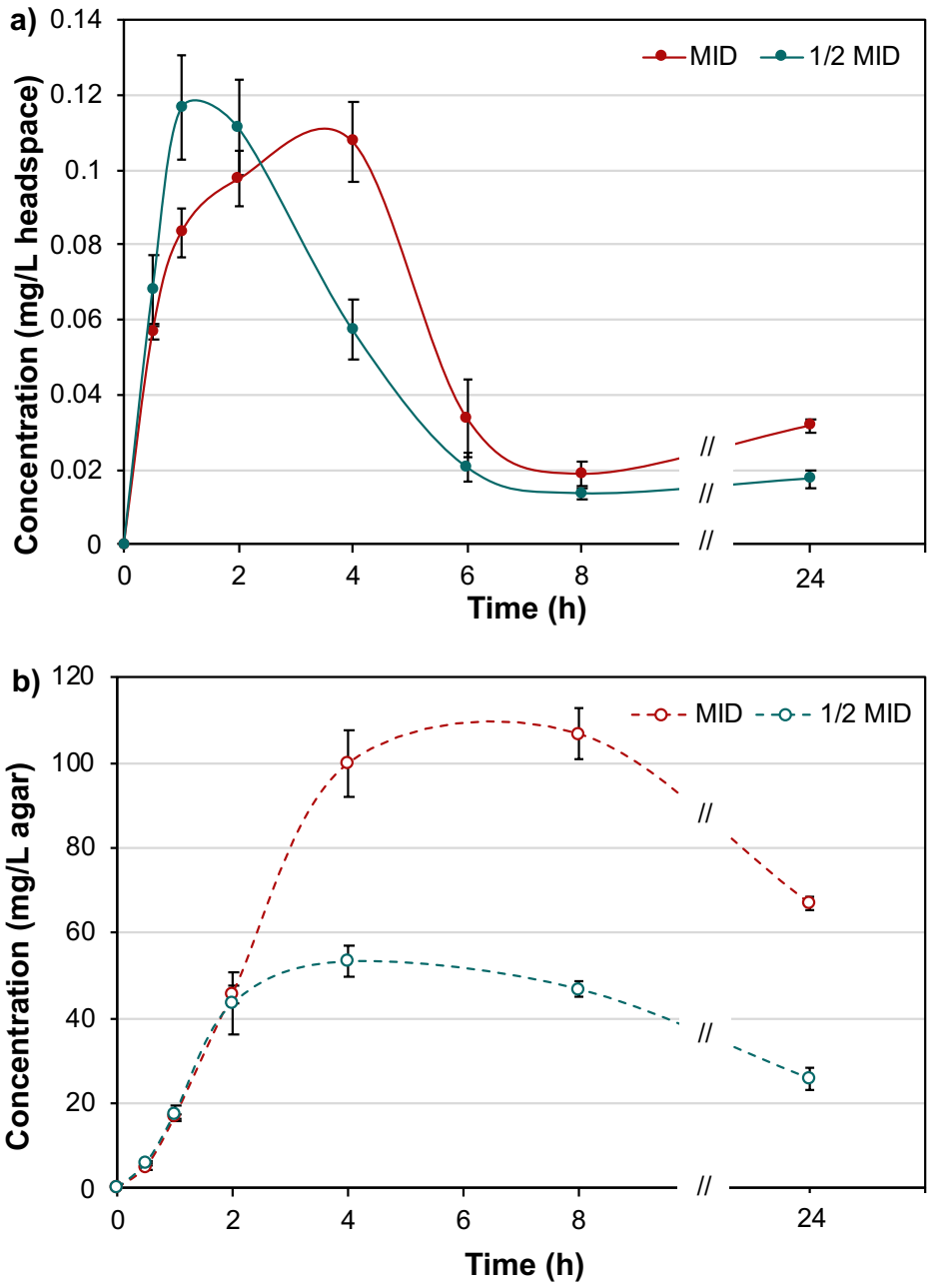

Figure 28 Concentration profiles of thymol via instant addition. Appropriate amount of thymol was instantly added to obtain 8 and 4 mg/L headspace, i.e. MID and ½ MID respectively. a) Headspace concentration profiles of vapor-phase thymol. b) Agar layer concentration profiles of solid-phase thymol.

The results of the time kill assay could validate our argument. Inoculated petri dishes in a glass jar were treated with instant addition of MID or ½ MID for 1, 2, 4 and 6 hours, and then removed from the glass jar and incubated normally until 24 hours. The reduction in viability at different time intervals was determined, which was calculated based on the inhibition zone at corresponding time points, with respect to the control. As shown in Figure 29, there's a positive correlation between the vapor concentration profile and the microbial inhibition. Since the instant addition of ½ MID resulted in a faster vaporization during the first hour, it also exhibited a greater inhibitory effect than instant addition of MID: 72% reduction in viability compared to 38% (t-test, $P = 0.018$). Therefore, a high initial vapor concentration was effective in inhibiting microbial growth. However, since the concentration profile of ½ MID showed a rapid decline after the first hour, its inhibitory effect did not increase but merely maintained afterwards (72% at 1 h versus 77% at 24 h, $P = 0.63$). The instant addition of MID had a lower vapor concentration during the first 2 hours, thus rendering less inhibitory effect. But at 4 hours, the vapor concentration increased to a maximum, therefore allowing its inhibitory effect to also increase significantly to 85% reduction in viability. At the end of the incubation period, the inhibitory effects of instant addition of MID and ½ MID were significantly different (100% versus 77%, respectively, $P = 0.0041$). The results support the correlation between the vapor concentration profile and the microbial inhibition. In contrast, the correlation between the agar concentration profile and the microbial inhibition is indistinct. As is shown in Figure 3 b), the two agar layer profiles overlapped in the first two hours, while the microbial inhibition for that time period significantly differed.

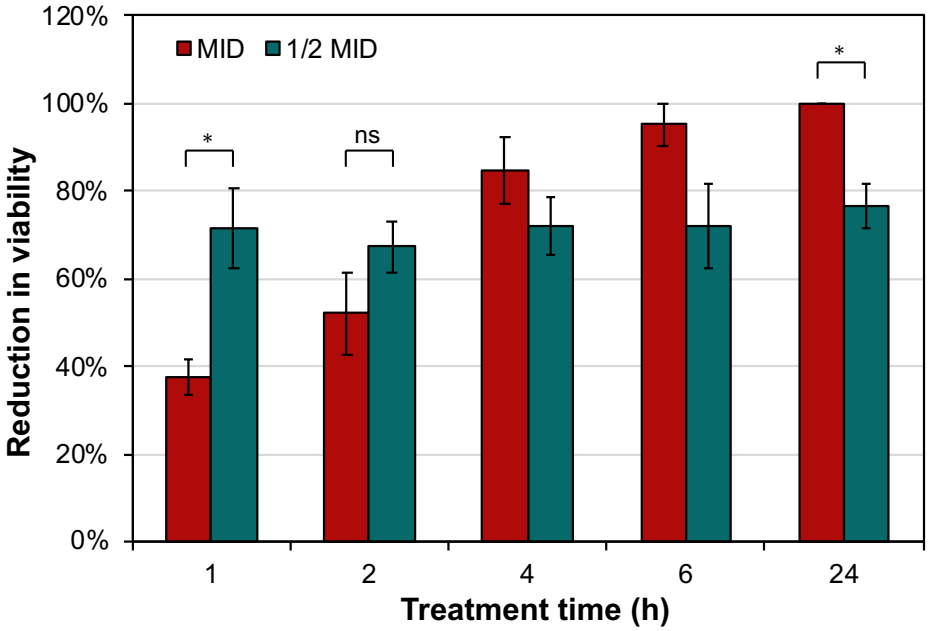

Figure 29 Inhibitory effect of vapor-phase thymol via instant addition at different time intervals. Reduction in viability (%) was calculated based on the corresponding inhibition zone with respect to the control.

Note: A 100% reduction in viability means no sign of growth was observed, while a 0% reduction in viability means the growth was identical to the control group.

Note: Instant addition of MID and ½ MID resulted in significantly different effects (t-test, MID versus ½ MID, ns = not significant, *P<0.05, **P<0.01). Standard errors are shown as error bars.

To summarize, when thymol is added into a closed system, it is present in both the headspace and the agar layer. Vapor-phase thymol in the headspace, although at a relatively low concentration, is the primary contributor to microbial inhibition. A high initial vapor concentration exerts immediate inhibition, however, if not sustained, low concentration at later time contributes little to the overall inhibitory effect. To achieve a better outcome, the concentration profile must maintain a growth trend for a longer

period, i.e. 4 hours in this case, allowing for continuous inactivation and inhibition of the microorganisms. Then later treatment of low concentration is able to sustain and slightly increase the antimicrobial effect. On the contrary, solid-phase thymol in the agar layer, although taking up half of the amount added, plays a minor role in microbial inhibition. It could be caused by thymol's hydrophobicity or immobility once absorbed into the agar. The results also explained why in the prior vapor diffusion assay, less added amount was unable to inhibit microbial growth. When a lower dose was added, thymol vaporized too quickly along with ethyl acetate. Peak headspace concentration was reached almost immediately but declined rapidly. The short time was insufficient to exert any antimicrobial activity.

This finding supports the use of controlled release packaging (CRP) to deliver vapor-phase thymol over time. A key advantage of CRP is that its continuous release is able to replenish the portion that's been consumed or degraded. With CRP, the vapor concentration profile is able to sustain a growth trend for desired time length, thereby ensuring the antimicrobial effect. At later time, as the microorganisms have already been inhibited, the release from packaging can cease and the vapor concentration gradually lowers, thereby still maintaining the inhibitory effect but avoiding the repellent odor to consumers.

6.2.3. Antimicrobial Activity of Thymol via Controlled Release

To mimic the release of antimicrobials from packaging, release profiles need to be generated using Equation 1. In this study, L was assumed to be 3 mil (76.2 μm) and M_0 was selected to be 8 mg/L headspace, the MID obtained in the vapor diffusion assay.

Table 10 shows the four D values calculated from assumed M_t and t values, varying from 4.22×10^{-13} to 5.28×10^{-14} m^2/s. Higher *D* value corresponded to faster release. Based on Table 10, four release profiles, CR-1 to CR-4, were generated, as shown Figure 30. Time was simulated from 0.5 to 8 hours respectively, because Equation 4 is limited to $M_t/M_0 \leq 2/3$. Compared to the MID in instant addition, the amounts used in these four release profiles corresponded to 41% to 58% of MID, respectively.

Table 10 Diffusivities predicted by varying the concentration of thymol released during the first 0.5 h

Release Profile	M_t (mg/L headspace)	*t* (h)	*D* (m^2/s)	Simulated time length (h)
CR-1	3.27	0.5	4.22×10^{-13}	0.5
CR-2	2.31	0.5	2.11×10^{-13}	1.25
CR-3	1.63	0.5	1.06×10^{-13}	3
CR-4	1.15	0.5	5.28×10^{-14}	8

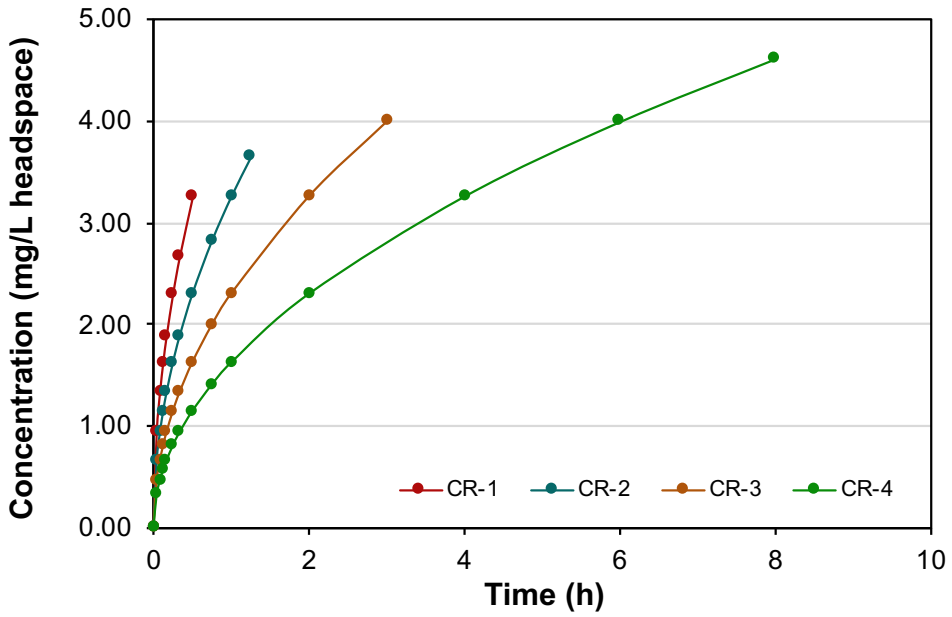

Figure 30 Controlled release profiles of thymol by varying diffusivities generated by Crank's diffusion model (Equation 4) and simulated by syringe pump system.

Note: Close square (■) represents release profile generated using diffusivity of 4.22×10^{-13} m^2/s. Close diamond (♦) represents release profile generated using diffusivity of 2.11×10^{-13} m^2/s. Close circle (●) represents release profile using diffusivity of 1.06×10^{-13} m^2/s. Close triangle (▲) represents release profile using diffusivity of 5.28×10^{-14} m^2/s.

Figure 31 shows the inhibition zones resulted from the four release profiles. After 24 hours of incubation, almost all four profiles showed a total microbial inhibition (an inhibition zone of ~50mm). The only exception was CR-3 with an inhibition zone of 42 mm. When the petri dishes continued to be incubated for another 24 hours, the antimicrobial effects declined in the order from 1 to 4. CR-1 still maintained an inhibition zone of 50 mm, while CR-2 reduced to 43 mm, CR-3 to 23 mm and CR-4 to 8.7 mm. The inhibitory effects stabilized afterwards, as after 72 hours of incubation, the inhibition zones were close to those at 48 hours. As can be seen, CR-1 possessed a bactericidal effect, while the other three possessed a combined effect of part bactericidal and part bacteriostatic. When thymol was released following CR-1, it effectively killed all the microorganisms thus no sign of growth was observed. When thymol was released following the latter three profiles, it was able to kill some microorganisms and delay the growth of the rest. The surviving microorganisms were able to resume growth after 24 hours. Among the four release profiles, CR-1 had the best inhibitory effect, with only 41% of the amount used compared to instant addition. Hence it was selected as the optimum release profile. The results again support the superiority of controlled release as a delivery mode over instant addition, as it's able to utilize less amount to achieve the same inhibitory effect.

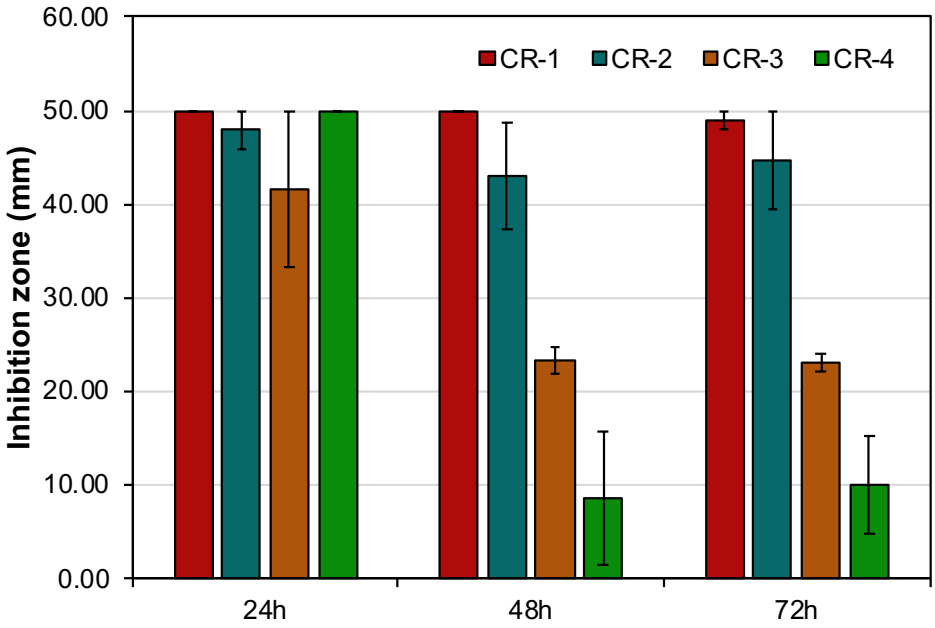

Figure 31 Inhibitory effect of vapor-phase thymol via controlled release, expressed as the diameter of inhibition zone (mm)

6.2.4. Headspace Concentration Profile via Controlled Release

Since the headspace concentration is critical to microbial inhibition, the vapor concentration of CR-1 was obtained by GC analysis. Figure 32 shows the profile, which appeared to have similar trends as in the instant addition of MID. The headspace concentration continued increasing until 4 hours. At 4 hours, the headspace concentration reached a maximum of 0.043 mg/L headspace, which was around 40% of the maximum concentration reached in instant addition of MID. Considering the amount released was only 41% of the amount used in instant addition, the lower concentration was reasonable. The vapor concentration then declined to 0.24 mg/L headspace and maintained at that level.

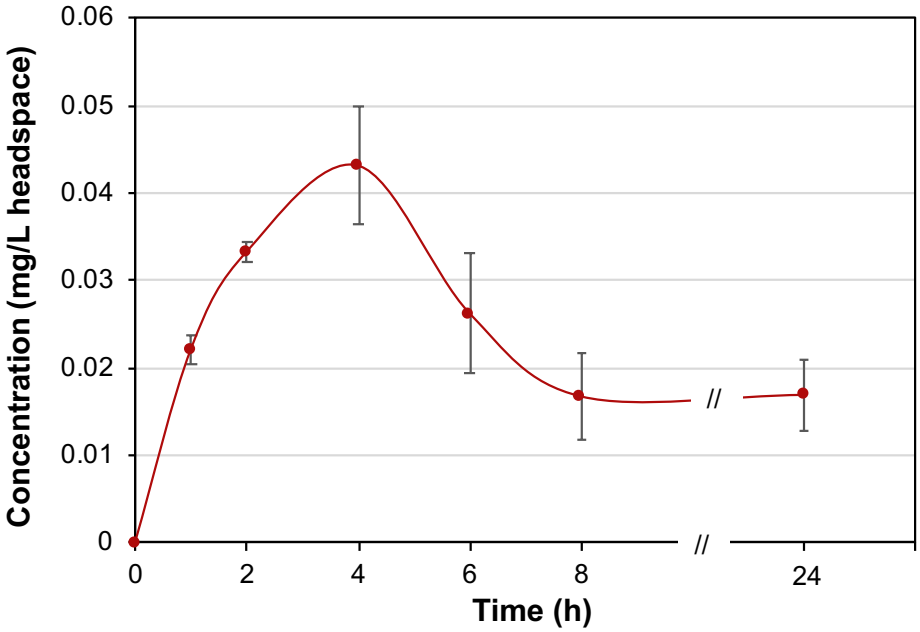

Figure 32 Headspace concentration profile of thymol via controlled release, following a release profile using diffusivity of 4.22×10^{-13} m^2/s (CR-1).

From the result, we can see that controlled release is a more effective method to deliver vapor-phase thymol than instant addition. In this study, thymol was prone to be absorbed into the agar layer than stay as vapor. Therefore, a high headspace concentration could not sustain but declined rapidly. Once the concentration started to decrease, it rendered limited additional inhibitory effect. Therefore, it would be ideal if the concentration profile had a prolonged period when the vapor concentration kept increasing. The time period must be at least 4 hours based on the results. In instant addition, high dose was required to maintain the increasing trend as thymol was subject to loss once added. In controlled release, the timely release of thymol was able to continuously supplement the lost portion, thus lower dose was required to maintain the increasing trend.

We could also propose that using vapor phase thymol is able to overcome the current disadvantages of high concentration requirement and undesirable odor. Since the effective concentration profile only had a maximal headspace concentration of 0.043 mg/L headspace, it was below the lowest odor threshold of 86 ppm [73]. Incorporating thymol into controlled release packaging and delivering it in a timely manner is a feasible idea to inhibit the growth of food microorganisms.

7. CONCLUSIONS

This study indicates that the mode of delivery can greatly affect the inhibitory effect of thymol against *E. coli* DH5α, both for liquid- and vapor-phase thymol.

In the first part of the study, the results further imply that combined mode is a new and effective option for the design of controlled release packaging. Based on the conditions used in this study, combined mode of delivery was more effective than the other two delivery modes in inhibiting the growth of *E. coli* DH5α. The MIC of thymol via instant addition, controlled release and combined mode of delivery were 500, 375 and 250 mg/L, respectively. Compared to instant addition, controlled release and combined mode were able to reduce the overall amounts of thymol used by 25% and 50%, respectively. The superior result of combined mode might be attributed to the instant inhibitory effect, which slowed down cell growth during the lag phase, followed by the sustained stress from controlled release of thymol, which continuously suppressed the cells.

Based on the results of this and two earlier studies, we propose two requirements to achieve effective microbial inhibition: (1) a minimum concentration of antimicrobial must be achieved during the lag phase, and (2) a subsequent continued release of antimicrobial must be maintained. In addition, the release should be fast during the lag phase and progressively slower afterward, since this will result in the use of least amount of antimicrobial to achieve microbial inhibition.

The first requirement was further investigated, and the minimum concentration was pin pointed to be around 123 mg/L. This target concentration must be reached during the inherent lag phase, 1.14 hours, in order to result in an at least 0-log reduction. Any

delivery mode that suffices this requirement has the possibility to effectively inhibit microbial growth, be it instant addition, controlled release or combined mode. However, a subsequent release to meet the second requirement is equally important, which instant addition fails to fulfill.

Based on the identified minimum concentration and Crank's diffusion model, four target release profiles were generated which conceptually satisfy both requirements. It can be seen that controlled release by itself is inflexible, while combined mode provides higher degree of freedom. Once a targeted (concentration, time) data point is settled, i.e. (123, 1.14) in this case, only one release profile can be generated via controlled release alone, while numerous release profiles can be produced via combined mode. It again verifies the ability of combined mode as an alternative when instant addition and controlled release are unable to achieve satisfactory microbial inhibition.

These two requirements are useful for food scientists to determine the required concentration profile of antimicrobial in the package, and this required concentration profile in turn is useful for the packaging engineer to determine the required release profile of antimicrobial for the design of controlled release packaging.

In the second part of the study, the antimicrobial activity of vapor-phase thymol against *E. coli* DH5α was studied via instant addition and controlled release. Instant addition of vapor-phase thymol was able to achieve total inhibition, with much less concentration used than instant addition of liquid- and solid-phase thymol, namely 8 mg/L headspace versus 500 mg/L and 320 mg/L agar, respectively. This might be due to thymol's lipophilic nature, which hinders its activity in hydrous medium, but allows its vapor to directly attack the bacterial membranes. Furthermore, controlled release of vapor-phase

thymol was able to achieve the same inhibitory effect, with only 41% of the amount used in instant addition of vapor-phase thymol.

Prior to this work, most studies on vapor-phase EOs assumed complete volatilization and constant vapor concentration, which might not be realistic. It was unknown that for vapor-phase thymol, how the actual concentration might differ from the calculated MID. We here report that in a closed system, only a fraction of thymol existed in the headspace in the form of vapor, with a concentration well below the calculated value. Contrarily, more than half of thymol was absorbed into the agar layer. This might result from thymol's low volatility and relatively high molecular weight, which makes it slow in vaporization but susceptible to sedimentation into the agar.

Previously, the knowledge gap also existed in the relationship between the actual concentration profile and the antimicrobial activity over time. We here identify the correlation between the headspace concentration and the inhibitory effect. Although most thymol was absorbed into the agar, the vapor was the main contributor to the overall microbial inhibition. A high initial headspace concentration was effective in inhibiting microbial growth, as it immediately killed many of the microorganisms. However, if not sustained, lower concentrations at later time were unable to further inhibit the surviving microorganisms. To achieve a better result, a prolonged phase of 4 hours, where the headspace concentration kept increasing, was critical. During this time period, microorganisms continued to be killed or damaged. At later time, as surviving microorganisms may have already been damaged, lower concentrations were more likely to kill the injured cells.

Controlled release is a novel delivery mode where antimicrobials are released in a controlled manner. We here report the effectiveness of vapor-phase thymol via controlled release, using only 41% of the amount to achieve the same inhibitory effect compared to instant addition. It was made possible because via controlled release, thymol was continuously supplemented to make up for the portion that had been lost. In this way, the vapor concentration profile was able to follow the trend, where the headspace concentration continued increasing until 4 hours. It again stressed that for vapor-phase thymol, a growth phase in the headspace concentration profile is required to be effective. Effective CRP design must account for this requirement, and should generate a release profile that gives the desired concentration profile.

We hereby propose the feasibility of using vapor-phase thymol as a food antimicrobial and its potential to be used in controlled release packaging. Future studies are needed to incorporate thymol into packaging material, observe its release from package, and test the antimicrobial effect. This study also provides a reference of the optimal release profile, which will help the design of packaging that can achieve the required concentration profile.

8. FUTURE STUDIES

8.1. Characterization of the Optimum Release Profile

In this study, two requirements were proposed for effective microbial inhibition: (1) a fast release of antimicrobial during the lag phase to achieve a minimum concentration in the broth to inhibit microbial growth, and (2) a progressively slower release at later time to replenish antimicrobial that is consumed or degraded. Although the requirements improved our understanding of controlled release, they are of a qualitative and descriptive nature. In order to guide the design and development of CRP, quantitative characterization of the two requirements is of great necessity.

This study has already taken the first step into quantifying the first requirement. It was proposed that during the lag phase, the minimum concentration released must be able to at least maintain a 0-log reduction. Based on the results, a more systematic approach can be taken to pinpoint this minimum concentration for any type of microorganism in combination with any type of antimicrobial: (1) For the target microorganism, its growth curve under optimal conditions can be measured and plotted, followed by fitting the data with a logistic model. Accordingly, the inherent lag phase can be calculated from the model. (2) Next, the MIC of the selected antimicrobial via instant addition can be measured by broth dilution methods. The MIC serves as a reference for subsequent experiments. (3) A time-kill assay can be carried out to observe the effect of varying concentrations of antimicrobial (between 0 and the MIC via instant addition) against microbial growth during this inherent lag period. The data can be fitted to a response curve. Following the three steps, the relationship between the added concentration and

the log-reduction can be quantified, and the target minimum concentration can be interpolated.

For the second requirement, it's notable that the purpose of characterization is to serve as a basis for the design of CRP, thus the sustained release at later time must follow the Crank's diffusion model, in order to mimic the release from package. Additionally, other principles to follow for the second requirement include: (1) the microorganisms must be inhibited until the desired shelf life, i.e. > 1-log reduction; (2) the release of antimicrobial does not necessarily need to be continued until the shelf life; and (3) the concentration used during this time period must be less than the MIC of instant addition minus the concentration already released during the lag phase. Under these guiding principles, various release profiles can be generated – while only one profile is produced via controlled release, numerous release profiles can be yielded via combined mode. The microbial growth kinetics under these release profiles can be measured, and then correlated with the release kinetics.

It will be ideal if a mathematical relationship between the release profile and the microbial growth could be found. When an empirical model could be established, it should be able to best serve future researches as a guideline.

8.2. Validation of Target Release Profile with Real Food Systems

8.2.1. Optimum Release Profile Validated with CRP

As in section 6.1.6, four optimum release profiles were generated using Crank's diffusion model. The proposed requirements for optimum release profile, and the generated release profiles need to be validated with real system.

To test the optimum release profile, several tasks need to be carried out:

(1) Identification of the test system:

Potential validation systems could include liquid food, where the food content is in close contact with the package, or solid food, where the food content is tightly wrapped with packaging film. Evaluation could also be carried out in model systems that closely simulate food composition. For example, lettuce model media can be prepared using exudate from shredded lettuce mixed with agar medium [165]. Similarly, mixing skim milk powder with agar medium can obtain a milk model medium [166]. Beef extract alone can act as a meat-based model medium [166]. Although in literature mostly solid-phase medium was tested, liquid-phase medium could also be attempted.

(2) Identification of the target microorganism:

Target microorganism must be carefully selected so that it represents a real threat to the safety of the selected food system. For example, CDC reports that leafy vegetables cause more foodborne illnesses than any other commodity, and are frequent sources of norovirus, *E. coli* O157 and *Salmonella* spp. outbreaks [167]. Therefore, *E. coli* and *Salmonella* spp. are great target microorganisms when verifying the controlled release of thymol to a lettuce leaf model. Likewise, land animal commodities account for the highest proportion of illnesses for *Campylobacter* spp., *Clostridium perfringens*, *Listeria* spp., *Salmonella* spp., and *Streptococcus* spp. group A, which are candidates if a milk- or meat-based model is in use.

(3) Establishing baseline for selected model system and microorganism:

The inhibitory effect of thymol via instant addition should be investigated to provide a basis for the following investigation of controlled release. Agar diffusion assay or broth

dilution assay can be performed to measure the inhibition zone upon addition of thymol, or to uncover the required MIC for effective microbial inhibition. The amounts used in the instant addition can serve as a reference for the design of controlled release profiles.

(4) Simulation of CRP using syringe pump:

Before a CRP is developed, controlled release of thymol using the syringe pump system should be examined for its effectiveness. Release profiles can be designed based on the guidelines in section 8.1. The syringe pump then executes the pre-determined profiles to release thymol onto the surface of the inoculated agar medium, or into the inoculated liquid broth, which are incubated and inspected for microbial inhibition. The expectation at this step is being able to achieve the same inhibitory effect using less amount than instant addition. If the expectation is met, an optimum release profile which utilizes least amount can be selected for next steps. If the expectation is not met, the assumptions must be checked, and the experiment design must be revised correspondingly.

(5) Development of CRP and validation of optimum release profile:

Polymeric material and film thickness, along with other parameters, need to be chosen based on the optimum release profile. Thymol is then incorporated into the film, and measured for its release from the film, in order to determine if the release profile matches the designed optimum release profile. If the target release profile cannot be met due to technical difficulties, a combined mode can be attempted as discussed in section 8.2.2.

(6) Experimentation of CRP on the food system:

The developed CRP could then be tested on the inoculated food model, in order to check the microbial inhibition, which is expected to be effective. If the expectation is met, then

it's fair to conclude that it is feasible to use predicted optimum release profile to design and develop CRPs.

8.2.2. Optimum Release Profile Achieved via Combined Mode

In section 6.1.6, it was also mentioned that using controlled release alone might not always meet the target release profile. As such, combined mode can be adopted. To validate the soundness of combined mode, real food systems are also be introduced. The proposed tasks are very similar to the tasks in section 8.2.1, except that part of the thymol will be added via instant addition. It is made possible by immediately mixing thymol into the medium or attaching a filter paper impregnated with thymol solution onto the agar surface. Next, controlled release of thymol is accomplished through the syringe pump system or the physical packaging film.

It is necessary to validate the model with different types of food systems, during which process, new parameters or factors will emerge and need to be taken into consideration based on the outcome. In this way, the knowledge boundary can be expanded, facilitating the real-life applications of CRP.

8.3. Characterization of the Correlation between Headspace Concentration Profile and Microbial Growth in Vapor-Phase Thymol

In the second part of the study, it was proposed that the headspace concentration has significant contributions to microbial growth. While a high initial vapor concentration is able to effectively kill the microorganisms, a prolonged growth phase may be able to achieve better inhibition, as it inhibits microbial growth in a more continuous manner.

The observation demonstrates the effectiveness and feasibility of controlled release in vapor-phase thymol. Two branches can be drawn from this conclusion:

8.3.1. Short-Term Treatment of Vapor-Phase Antimicrobial as an Environmental Application

As was observed, treatment of high vapor concentration for a short period of time is an effective method for microbial inhibition. Therefore, it can be of use as an industrial method for disinfection and sterilization. For example, vapor-phase thymol can be used as an easy environmental application in places such as hospitals. Exposing medical apparatus to vapor-phase thymol can be an alternative to the currently used methods, such as autoclave, UV, ozone, chlorine dioxide, etc.

To further verify the practicability of this idea, it is important to understand the killing effectiveness and efficiency of vapor-phase thymol (or any other vapor-phase antimicrobial). In fact, instead of thymol, it is preferable to select an antimicrobial with higher volatility so that high vapor concentration can be achieved. Examples of candidates include carvacrol and AITC. Being isomeric, both carvacrol and thymol have been greatly appraised for the antimicrobial activity. But carvacrol has a higher vapor pressure, as can be seen from its liquid state in room temperature, as opposed to the crystalline thymol. AITC is highly volatile and antimicrobial, but its pungent flavor makes it even less desirable to be applied in foods than thymol. Utilizing it as an environment application could take advantage of its strength whilst circumvent its weakness.

Different vapor concentrations of the selected antimicrobial need to be generated, by simply adding it into a closed system and monitoring its vapor concentration profile by

GC. The effect of short-time exposure on microbial growth can be identified by the time-kill assay – Inoculated agar medium is placed into the closed system, apart from the antimicrobial to avoid direct contact. The system is incubated for varying time periods, then the agar medium is removed and incubated normally. The microbial growth on the agar medium is observed for a few days, in order to confirm the bacteriostatic or bactericidal effect.

8.3.2. Release of Vapor-Phase Thymol from CRP

CRP can be utilized to ensure the quality and safety of foods in a prolonged shelf life. As was identified in this work, effective concentration profile requires a growth phase of at least 4 hours, where the vapor concentration continues increasing. At later time, the vapor concentration declines to below detection limit. It makes CRP a suitable vehicle for delivering antimicrobials. As is all known, one existing problem of natural antimicrobials is that excessive addition of nature antimicrobials such as EOs will alter the flavor and taste of foods, causing consumers to reject the food products. However, this problem can be easily resolved using CRP. Active release and higher concentrations in the package will mostly occur during the transportation and storage stage, where microorganisms are continuously inhibited. The release then stops and the vapor concentration decreases. When the food reaches the consumer, the strong odor already fades, and the food is ready for consumption.

To further push the development of CRP, it is critical to quantify the correlation between the headspace concentration profile and microbial inhibition. Although it is recognized that an inverted “V” shape is the generally preferred shape, we still need to understand

the requirement for the peak concentration, and the growth rate. Concentration profiles of varying shapes can be generated by the syringe pump, and their different effects on microbial growth could be measured to find an optimum. On top of that, a CRP that is able to meet the required concentration profile must be developed and tested.

8.4. Target Release Validation with Real Food System

The development of CRP must be followed by validation in a food system. The process is similar to that in section 8.2, while in this section, the developed CRP should be placed apart from the food, instead of directly contacting the food.

The inoculated food and the developed CRP can be placed into a closed system and tested under accelerated conditions. The microbial growth on the food will be observed at pre-determined time intervals, as well as the vapor concentration profile. At the end of the test period, a sensory test on the food can be carried out, in order to make sure that the food is still in edible condition with no apparent change in characteristics.

9. ACKNOWLEDGEMENTS OF PREVIOUS PUBLICATIONS

The thesis is composed in part of an independent article that has already been published in *Food Packaging and Shelf Life*: Wang and Yam [168]. This previously published article represents the original independent work of the PhD candidate, Yan Wang and shall be acknowledged.

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