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Nano-dispersing Lipophilic Antimicrobials for Improved Food Safety

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To the Graduate Council:

I am submitting herewith a dissertation written by Bhavini Dipak Shah entitled "Nano-dispersing Lipophilic Antimicrobials for Improved Food Safety." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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Nano-dispersing Lipophilic Antimicrobials for Improved Food Safety

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DEDICATION

This dissertation is dedicated to my loving parents Chandan and Dipak Shah,
and my beloved husband, Nihar Shah.

Mummy and Papa, you have given me the gift of life. Your selfless love, infinite sacrifices and flawless parenting have shaped me as a person and my experiences in life. No words are perfect to express my love and gratitude towards you.

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ABSTRACT

Naturally occurring food antimicrobials such as plant essential oils are receiving tremendous interest as intervention systems to enhance microbiological safety and quality. Poor water solubility of essential oils makes it difficult to incorporate them in foods, impacting visual appearance, antimicrobial effectiveness, and possibly organoleptic properties. Engineered nanoscale delivery systems can principally solve these challenges; but, those based on low-cost food ingredients and inexpensive and scalable processes are currently scarce. This dissertation presents a simple and scalable two-step technology to prepare nano-delivery systems. The first encapsulation step, based on emulsion-evaporation, involves preparing emulsions composed of an oil phase with thymol or eugenol, major compounds in extracts from thyme and clove respectively, in hexane and an aqueous phase with conjugates of whey protein isolate and maltodextrin, followed by evaporation of hexane by spray drying. The second step is to hydrate spray dried capsules to enable the formation of nanoscale particles. The encapsulation performance and dispersion characteristics were affected by amounts and types of conjugates (ratio of protein: maltodextrin and maltodextrin chain length), volume fraction and composition of the oil phase. The optimal conditions corresponded to 55.8 % encapsulation efficiency and 12.6 % loading for thymol and 47.9 % encapsulation efficiency and 7.9 % loading for eugenol. Dispersions prepared from the identified capsules contained particles smaller than 100 nm and were transparent at pH 3.0-7.0 and 0-50 mM before and after heating at 80°C for 15 min. Nano-dispersions and free oil were tested for antimicrobial activity against *Escherichia coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella typhimurium.* Nano-dispersed and free antimicrobials had similar effectiveness at various pH and temperatures in tryptic soy broth and apple cider, while in 2 % reduced fat milk, nano-dispersed antimicrobials were consistently more effective than unencapsulated ones. Therefore, the commercially viable nanoscale technology presented in this study enables the delivery of lipophilic antimicrobials for

enhanced microbial safety and quality, without compromising visual appearance of foods, especially clear beverages.

Keywords: lipophilic antimicrobial essential oils, pathogens, nanoscale delivery system, whey protein isolate – maltodextrin conjugate, spray drying, tryptic soy broth, apple cider, milk.

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1. Literature Review

1.1. Introduction

Preservation of food quality and ensuring consumer safety are two of the most significant concerns in the food industry. Food products, especially those that are perishable, can get easily contaminated by spoilage-causing microorganisms during their preparation, storage and distribution, and must be protected to avoid sensory deterioration and extend shelf life (Rasooli 2007). However, the more serious concern is that related to illness-causing foodborne pathogens. Several food preservation and pathogen control strategies including heating, refrigeration and addition of chemical antimicrobial compounds are being employed to make foods safe for consumption (Davidson and Harrison 2002) . However, the use of many of these methods is limited due to loss of nutrients, adverse effects on organoleptic characteristics of the foods, reduced consumer acceptance of the products or high cost (López-Malo and others 2000). Thus, there is growing interest in developing novel and economical food preservation and safety intervention technologies to replace traditional ones to meet industry and consumer needs.

One such approach involves the use of natural food grade antimicrobial compounds such as essential oils (Naidu 2000). Essential oils are highly aromatic and volatile liquids, usually extracted from the bark, leaves, stem or leaves of herbs and spices, with excellent antioxidant and antimicrobial properties against a broad spectrum of bacteria and fungi (Weiss and others 2009; Burt 2004; Branen and Davidson 2005). However, these antimicrobials are lipophilic, with marginal solubility in water, thus making it difficult to incorporate them in foods. Additionally, their efficacy in foods is reduced when compared to microbiological growth media due to interactions food components like proteins and lipids (Gutierrez and others 2009; Gutierrez and others 2008). There are on-going research efforts directed towards developing delivery systems for these antimicrobials to overcome these challenges, encapsulate target antimicrobials, protect them and control their release in foods (Donsì and others 2011a).

In the present study, the overall goal was to develop food grade, nanoscale, transparent and heat stable antimicrobial dispersions to enhance microbial safety of foods, without adversely affecting sensory quality. Thymol and eugenol were chosen as model antimicrobials since they occur naturally in thyme and clove essential oils respectively, generally recognized as safe (GRAS) antimicrobials with a broad spectrum against gram-positive and gram-negative bacteria (Tajkarimi and others 2010). Conjugates of whey protein isolate and maltodextrin were chosen as encapsulation materials because they are commonly available, relatively inexpensive food materials with excellent emulsifying properties (Akhtar 2007). In addition, WPI-MD based matrices have been extensively used for the encapsulation of lipophilic food ingredients including fat-soluble nutrients (Choi and others 2010; Kagami and others 2003), flavors and (Bylaite and others 2001; Toure and others 2007) and essential oils (Baranauskiene and others 2006; Bae and Lee 2008). The method used to produce encapsulated antimicrobials was spray drying. It is one of the most commonly used encapsulation method in the food industry. It is simple, low-cost and scalable, and enables continuous production of free-flowing powders that are easy to handle, store and disperse in water for use in foods (Gharsallaoui and others 2007).

The objective of this chapter is to briefly review the topics relevant to this research, including the potential use of WPI-MD conjugates and spray drying to produce nanoscale dispersions of thymol and eugenol. The rationale of using natural antimicrobials to enhance food safety is first discussed, followed by a discussion on the challenges involved in incorporating essential oils in foods, the need for nano-dispersing antimicrobial for food applications, and finally the characteristics of the encapsulating materials and the encapsulation method.

1.2. Food safety concerns in the industry

Safety of consumer health has been a vital concern for many years, due to sporadic and continual cases of foodborne illnesses and outbreaks, caused by consumption of food contaminated by pathogenic

bacteria. The US Center for Disease Control and Prevention (CDC) estimates 9.4 million illnesses, 45,826 hospitalizations, and 1,351 deaths per year potentially caused by one of 31 known foodborne pathogenic bacteria (CDC 2011a). Although globalization of food products has made a wider variety of foods easily available to consumers all over the world, it has brought with it the intense need for supply chain and production control to make sure food supplies are safe. Foodborne pathogen contamination in one facility can cause broader outbreaks due to increased mass production, interstate trade and movement of products from one location to another. In March 2011, multistate outbreaks due to consumption of bologna contaminated with *E. coli* serotype O157:H7 got 14 persons infected in 5 states (CDC 2011b). Figure 1-1 shows the relative rates of infection with top five pathogens produced by the FoodNet surveillance program for the past decade (CDC 2010). It is evident that not only is there no drastic decreasing trend in the occurrence of foodborne outbreaks; but, there seems to be an increasing trend in the outbreaks associated with *Listeria monocytogenes*, despite the use of multiple control measures. Thus, there is an urgent need for effective pathogen control to reduce the number of microbial outbreaks, prevent health hazards, and preserve the keeping quality of foods. Traditionally, pasteurization, environmental control, and the use of chemical sanitizers are used for commercial sterilization and extension of shelf life of foods (Branen and Davidson 2005). Since a few decades, there has been interest in the 'hurdle' approach, which involves the use of multiple pathogen control methods, such as antimicrobials in adjunction with temperature treatments to improve food safety (CDC 2008; Jensen and others 2006; Olsen and others 2004).

1.2.1. Food antimicrobials

Food antimicrobials are chemical compounds, synthetically-derived compounds or naturally-occurring, present in or added to foods, food packaging, food contact surfaces, or food processing environments (López-Malo et al. 2000). They act as preservatives by preventing growth of spoilage organisms or

inactivating pathogenic microorganisms, thus retarding biological deterioration of foods and extending the shelf life of foods (Branen and Davidson 2005). Additionally, the presence of antimicrobial ingredients in foods may reduce the risk of microbial contamination, and resulting illnesses, post-processing, due to handling or storage abuse of products before consumption (López-Malo et al. 2000). In recent years, there is a growing consumer awareness and demand for minimally processed and 'natural' foods, while products containing synthetic antimicrobials have been increasingly rejected (Naidu 2000). Thus, there exists the need for development of new strategies to fulfill consumer demands and achieve food safety simultaneously.

1.2.2. Plant essential oils as 'natural' antimicrobials

Food antimicrobials can be synthetically-derived or occur naturally in plant and microbial sources. These generally include phenolic compounds and terpenes, flavonoids, etc., which may occur in many spices, herbs or their essential oils. Essential oils are highly volatile, hydrophobic liquids obtained from various parts of the plant, such as leaves, bark, seeds, root, leaves (Burt 2004). They can be sourced from over 1300 plants such as thyme (thymol), oregano (carvacrol), clove (eugenol), and cinnamon (cinnamaldehyde) (Holley and Patel 2005; Velluti and others 2003; Chao and others 2000). Chemically, they are mixtures of up to 100 different organic compounds such as aldehydes, alcohols, and esters (López-Malo et al. 2000). Traditionally used as culinary ingredients for food flavor, today approximately 300 essential oils are known and are of commercial value, are responsible for the aroma of food and pharmaceutical products, and can be expressed (cold), fermented, extracted or steam distilled for use (Burt 2004).

It has been identified that these compounds, especially those present in plant essential oils constitute the plant's defense mechanism against parasites, insects, and microorganisms (Kong and others 2007; Burt 2004). These compounds exhibit antimicrobial and antioxidant activity against a broad spectrum of

bacteria (Kim and others 1995), fungi or any combination thereof (Bennis and others 2004; Sridhar and others 2003). The potency of these antimicrobial compounds varies according to the type, genus, species, and strain of microorganism treated with them. One theory suggests that their chemical structure may influence their antimicrobial activity. These compounds have a molecular weight around 150-160 Da and contain a hydroxyl group in the molecule (Burt 2004), which may bind with bacterial proteins, impairing cellular functions. Another theory suggests that the hydrophobic nature of these oils enables them to alter cell membrane structure, leading to a disruption of the proton motive force, increased cell permeability leakage of cellular components and bacterial cell coagulation and collapse (Burt 2004), two of which are illustrated in Figure 1-2.

Phenolic compounds such as thymol, carvacrol, and eugenol are generally known as essential oils possessing the strongest antibacterial properties (García-García and others 2011). Thymol is an essential oil compound extracted from thyme and oregano, while eugenol, on the other hand, is an essential oil component extracted mainly from clove (Burt 2004). Both have shown to inhibit a wide range of bacteria including *E. coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus, Salmonella Typhymurium* (Oussalah and others 2007) and several fungi (De Martino L 2009). The minimal inhibitory concentration (MIC) of thymol for *Staphylococcus aureus* is found to range from 0.025 % (Karapinar and Esen Aktug 1987) to 0.05 % (Walsh and others 2003), and around 0.033 % and 0.14 % (Rhayour and others 2003) for *E. coli* in growth media. Several studies have investigated eugenol's antimicrobial activity in growth media. For example, 0.1 % (Kim et al. 1995) eugenol was shown to inhibit *Salmonella* strains, while the MIC for *E. coli* O157:H7 ranged from 0.05 % to 0.1 % (Walsh et al. 2003), *Staphylococcus aureus* was inhibited by 0.1 % eugenol, while 0.05 % eugenol was sufficient to inhibit *Listeria monocytogenes* (Gill and Holley 2006; Kim et al. 1995), and *Bacillus subtilis* was susceptible to 0.033 % eugenol (Rhayour et al. 2003).

1.2.3. Challenges in applying lipophilic antimicrobials in foods

Essential oils and their components are classified by the US FDA as 'Generally Recognized as Safe' (GRAS), which is why there is a tremendous interest in their use as antimicrobials in the development of 'natural' foods. However, essential oils are soluble in ethanol but, only sparingly soluble in water up to 0.01 % or 1.0 g/l (Burt 2004). This provides a challenge when applying these compounds as antimicrobials in complex food products. Antimicrobial efficiency of essential oils such as thymol and eugenol may be reduced or lost due to possible binding with food constituents such as fat or proteins, and >1 % eugenol may be required for same efficacy in systems such as cheese and meat (Tassou and others 1995). Finally, higher levels of essential oil compounds required to inhibit or inactivate microorganisms may exceed regulatory levels. While different effective concentrations result in flavor, color and appearance impacts on foods, one of the biggest challenges includes sensory threshold levels. Being highly volatile and aromatic, introduction of essential oil compounds in food products will therefore alter the flavor profile of the food, thus making compatibility with the food matrix and the desired sensory characteristics of the end product essential. (Gutierrez et al. 2008) reported that lettuce Samples treated with 1.0 g/l thymol and lemon balm were rejected by panelists upon sensory evaluation. Thus, there is interest in inclusion of essential oil compounds in protective matrices as a solution.

1.3. Encapsulation of lipophilic antimicrobials

Encapsulation of volatile, hard to disperse antimicrobial ingredients is an efficient method to (i) protect encapsulated ingredients from environmental factors or processing conditions such as heat, oxygen or light, (ii) reduce interaction with degradative environment and allow for better control over mass transport and chemical reactions, (iii) control release encapsulated ingredient by means of pH, mechanical agitation and other stimuli in the environment, (iv) mask undesirable properties, such as

intense aroma, to a certain degree and (v) enable high loading and delivery of poorly soluble ingredients in aqueous phases, thus potentially increasing bioactivity or effectiveness by improving adsorption and uptake of ingredients in areas of food, such as water-rich phases or solid-liquid interfaces, where target microorganisms are preferentially located (Weiss et al. 2009; McClements and others 2007).

1.3.1. 'Nano-' versus Microencapsulation

A significant part of research has been conducted on the use of emulsion based delivery systems for encapsulation of lipopohilic ingredients such as flavors, fat-soluble nutrients and antimicrobial essential oils (McClements et al. 2007). Micrometer sized capsules by spray drying conventional oil-in-water emulsions made using modified starch (Glenn and others 2010) milk proteins, (Baranauskiene et al. 2006) and different polysaccharides (Adamiec and Kalemba 2006). More recently, there is interest in using structured emulsion systems for specific functional applications. Different biopolymer materials such as chitosan and Ca-alginate (Wang and Lucey 2003; Perez and others 2009; Hu and others 2004) have been used to create multilayered emulsions for additional protection of essential oils and volatiles. (ElShafei and others 2010) produced environmental friendly formulation for pest control using water-in-oil-in-water (W/O/W) type multiple emulsions of Eucalyptus and marjoram essential oils stabilized by xanthan gum. (Gavini and others 2005) created solid lipid microparticles to reduce volatility of the antimicrobial agent from juniper berries.

Although microencapsulation may provide excellent protection of essential oils from harsh, deteriorative conditions, there are some limitations due to size of capsules in the systems. Nanoscale systems generally produce particles ranging from 1 to 100 nanometers (nm). This is the size at which many of the fundamental biological structures are formed, composite materials assume their distinctive characteristics, and many important physical phenomena occur (NNI 2011). Since it is known most nanoscale materials usually have properties different from their conventional counterparts, it can be

understood that nano-encapsulation systems with small sized oil droplets provide unique physicochemical and biological properties. For example, when compared with systems containing larger capsules, nano-emulsions are more stable to phenomena such as gravitational separation, flocculation, and coalescence, thus providing longer physical stability, which is of relevance to food applications considering harsh processing conditions and need for extended shelf life during storage (Augustin and Hemar 2009; Farhang 2009). More recent and exciting applications of nanoscale systems are related to the fact that with small oil droplet size, they scatter light less efficiently and so may be transparent or only slightly turbid. This means that they can be applied to foods to evenly disperse lipophilic functional ingredients in aqueous environments with no significant change in their visual appearance or texture. (Donsì and others 2011b) developed nano-emulsions for a mixture of essential oil compounds including carvacrol to improve antimicrobial efficacy in fruit juices, without negatively impacting their organoleptic properties.

Additionally, nano-structured delivery systems such as liposomes containing antimicrobial compounds may be engineered to interact with biological surfaces with increased contact from large surface area, which is important in the case of encapsulated antimicrobials, since it may affect their bioactivity and efficacy (Were and others 2004). (Gaysinsky and others 2005) found enhanced antimicrobial efficacy of eugenol and carvacrol dissolved in microemulsions (or nano-metric surfactant micelles) against *E. coli* O157:H7 strain H1730 and E0019) in tryptic soy broth (TSB). Similarly, (Kriegel and others 2008) developed eugenol nano-fibers by electrospinning microemulsions using Surfynol 465 and poly(vinyl alcohol) solution, which were inhibitory against *Salmonella typhimurium* strain 2576 and *Listeria monocytogenes* strains Scott A and 101 in TSB.

However, there are two major limitations in practical applications of these systems. First, the need for high energy methods (which increases costs), and second, the need for high concentrations of

surfactants and co-surfactants (which may not be 'GRAS' and have toxicity concerns). There is a need to develop novel food safety intervention strategies, to utilize food grade, naturally occurring ingredients in foods as emulsifiers and lower energy and cost-effective methods, while achieving the advantages of nanoscale systems in antimicrobial applications in the industry.

1.3.2. Milk proteins and carbohydrates as emulsifying agents

Encapsulation of various lipophilic ingredients including flavor volatiles and essential oils has been traditionally done using different carbohydrate-based matrices such as starch, maltodextrins and gum Arabic (Gouin 2004; Reineccius 1989; Reineccius 1991; Shahidi and Han 1993) and milk protein-based matrices (Dickinson 1997). More recently, whey proteins have been used to encapsulate lipophilic compounds (Bylaite et al. 2001); (Lee and Rosenberg 2000). Whey proteins are amphiphilic and show a strong tendency to be adsorbed at fluid interfaces (air–water and oil–water), thus serving as excellent emulsifiers (Sánchez and Patino 2005; Dickinson 2001; Mellema and Isenbart 2004).

They are a class of milk proteins recovered from whey, the liquid that remains soluble after precipitation of caseins by enzymes or acids. β-lactoglobulin, α-lactalbumin, and bovine serum albumin together account for approximately 60 % of total whey proteins, while immunoglobulins, lactoferrine are some of the minor components (Farrell Jr and others 2004; Zhang and Zhong 2010). There is growing interest in the use of whey proteins in the form of whey protein isolate and whey protein concentrate due to their nutritional value and low cost.

However, their functional properties are affected by environmental conditions, such as pH, ionic strength, thermal processing, thus limiting their application in foods. These globular proteins undergo denaturation when heated above 60˚C, unfold and aggregate. Proteins carry charge due to the presence of negatively charged carboxyl and positively charged amine groups. These charged groups play an

important role in inter-particle repulsion. The effects are more pronounced near pH 5.0, which is near their isoelectric point, and in the presence of high ionic concentrations (Akhtar and Dickinson 2003; Wang and Lucey 2003). At the pI, the net charge is neutralized, minimizing electrostatic repulsion and increasing inter-particle attraction. This results in increased precipitation of proteins, causing the system to become turbid or form solid gels. These phenomena commonly occur during storage and impart undesirable properties to products such as non-flowability and opacity, which are unacceptable to consumers and present challenges to food manufacturers. Moreover, these negative effects are amplified at protein concentrations greater than 5 % (Zhang and Zhong 2009), which is a relatively low concentration considering that proteins are one of the macronutrients in foods.

It has been observed that when whey proteins are processed in conjunction with other additives, such as polysaccharides, they exhibit some unique advantages contributing to the functional properties in food such as improved emulsifying properties and stability to heat (Schmidt and Smith 1992; Schorsch and others 1999). Polysaccharides, on the other hand, do not have strong surface-active properties; but, are strongly hydrophilic, thus contributing to thickening, gelling, stabilizing and rheology of the aqueous phase (Dickinson 2003).

Heat-induced protein-polysaccharide conjugates have been suggested for dispersion and foam stabilizing applications (Akhtar and Dickinson 2003). Conjugation of proteins with carbohydrates is achieved by dry heating mixtures to induce covalent coupling of proteins and carbohydrates also known as Maillard reaction (Kato 2002). During the early stage of Maillard reaction, condenstation between an available, unprotonated amine group from lysine residues of proteins and the carbonyl group of carbohydrates occurs, without the requirement of chemical catalysis (Jiménez-Castaño and others 2007). During intermediate stage of the reaction, the Amadori compound undergoes degradation to produce a large number of compounds, which in the advanced stages form colored, insoluble, polymeric

compounds called Melanoidins. In order to use Maillard-type conjugates in food emulsions, the reaction must be controlled to prevent advancing to the later stages. Numerous studies have optimized the conditions for Maillaird-type conjugate formlation, including rate of heating, heating temperature, heating time, protein and polysaccharide types and concentrations (Zhu and others 2010; Akhtar 2007) The resulting conjugates combine the characteristic properties of proteins to adsorb to the oil-water interface, and that of polysaccharide to solvate by the aqueous phase in the emulsion (Chevalier and others 2001). This is of significance at pI, which favors maximum protein aggregation; but, the carbohydrate moieties covalently linked to proteins provide steric hindrance and promote inter-particle repulsion. Additionally, sugars bind with proteins upon conjugate formation and shift the pI of proteins towards more acidic pH, thus broadening the pH range at which proteins aggregate upon heating.

Conjugation of whey proteins with alginates, carrageenans, xanthan, and pectins promote protein functionalities including solubility, formation of soluble aggregates, and interfacial properties (Dickinson 2003; Mishra and others 2001; Syrbe and others 1998). When β-lactoglubulin or whey protein based conjugates are formed, final functional properties of conjugates depend on characteristics like polysaccharide hydrophilicity, viscosity and binding number (Dickinson 2008; Jiménez-Castaño et al. 2007; Dunlap and Cote 2004). Conjugates with suitable properties can be prepared by optimizing the protein:polysaccharide ratio (Akhtar and Dickinson 2003), size (Matemu and others 2009) and conformation (Doublier and others 2000) of both proteins and polysaccharides, depending on target application in foods.

Whey protein isolate has been conjugated with hydrolysed polysaccharides such as maltodextrin and used in the encapsulation of various lipophilic ingredients including orange oil (Akhtar and Dickinson 2003), ginger oil (Toure et al. 2007), avocado oil (Bae and Lee 2008) and conjugated linoleic acid (Choi et al. 2010) to protect them from oxidative stress, volatilization and to enhance their bioactivity. Although

the reports on use of these conjugates for encapsulation of antimicrobial essential oils are scarce, studies on the use of whey protein-based matrices for encapsulation of many antimicrobial essential oils such as oregano essential oil (Zinoviadou and others 2009), rosemary and garlic essential oils (Seydim and Sarikus 2006), caraway essential oil (Bylaite et al. 2001) confirm the suitability of these ingredients in the current technology for food safety applications.

1.3.3. Spray drying as a low cost, low energy encapsulation method

Spray drying is one of the most common methods used for encapsulation of ingredients in the food industry due to its simplicity and cost effectiveness. It produces spray dried capsules in the form of micrometer sized powders, which are shelf stable, easy to handle and convenient to use. The principle process involves pumping a solution, emulsion or suspension, also called 'feed' through a nozzle into hot air or inert gas under pressure in a 'drying chamber', facilitating atomization into multiple fine droplets and subsequent evaporation of the solvent to form dried particles of micrometer size, which are then separated from the drying air in a separate unit operation called 'cyclone' (Wang and others 2011; Gharsallaoui et al. 2007). Droplets formed during atomization have large surface area, causing the solvent to evaporate quickly, thus minimizing contact time with high inlet temperatures and forming a dry 'crust' on the droplet surface, characteristic of shell structure of microcapsules (Augustin and Hemar 2009; de Vos and others 2010).

For encapsulation of essential oils, emulsion properties heavily determine the quality attributes of spray dried powders, such as surface free oil content, encapsulation efficiency and most importantly, microstructure, which may affect release and thus, end-functionality (Wang et al. 2011). Shape, size, and surface characteristics of capsules may be affected by distribution of solutes in dried particles. Different solutes adsorb preferentially to the droplet surfaces depending on their surface activity, molecular size, and physicochemical interactions. Poorly soluble solutes may precipitate quicker on the surface of

droplets, while molecules with higher surface activity and smaller size adsorb on to the droplet surface. Internal structure and composition of spray dried capsules is usually determined by the way solutes diffuse inside the crust (Vehring 2008; Masters 1991). Whey proteins, being amphiphilic, stabilize emulsions and have a stronger tendency to adsorb at the surface of lipophilic oil droplets during spray drying, while maltodextrins enhance the formation of the dry crust around drying droplets, thus improving the shell structure and composition (Sheu and Rosenberg 1995; Kagami et al. 2003).

Spray drying conditions also affect powder properties. Higher temperatures can cause quicker evaporation of solvent within the formed protein-maltodextrin shell structures, resulting in the build-up of pressure that may cause bursting effects on the shell, forming particles with porous structures, cracked crusts, other surface morphological defects or even ruptured capsules (Oakley 1997). With essential oils easily volatilized at mildly high temperatures, spray drying temperatures, feed rate, and compressed air pressure also affect the encapsulation efficiency and loading of capsules. Thus, careful emulsion formulation and optimization of spray drying conditions play an important role in developing delivery systems with controlled properties and targeted functionalities.

Overview of Dissertation Research

Exisiting preservation and pathogen control methods to deliver antimicrobials include microemulsions, nanoemulsions and use of liposomes. Although these technologies may provide small sized droplets and improved efficacy against microorganisms, they employ the use of high energy methods, which increases costs, and large amounts of surfactants, which may not be GRAS and may raise toxicity concerns, thus reducing consumer acceptance of foods. The current work is focused on developing novel food safety intervention strategies, based on an emulsion-evaporation, by employing low cost spray drying method, excellent emulsifying food materials such as WPI-MD conjugates and potent, GRAS antimicrobials naturally occurring in thme and clove essential oils.

Preparation, characterization and application of food grade nanoscale delivery systems of thymol (Chapters 2, 3, and 4) and eugenol (Chapters 5 and 6) using whey protein isolate -maltodextrin conjugates was conducted in this dissertation research. The principle of preparing nano-dispersions includes two steps. The first encapsulation step is based on emulsion-evaporation involving the preparation of emulsions by emulsifying an oil phase with thymol or eugenol in hexane into an aqueous phase with conjugates, followed by evaporation of hexane by spray drying. The second step is to hydrate spray dried powders to enable nanoscale particles. Chapter 2 focuses on optimizing emulsion compositions, i.e., amounts and types of conjugates, volume fraction of the oil phase, and the amount of thymol in the oil phase, for preparation of thymol-containing capsules by spray drying. The spray dried capsules were determined for encapsulation performance. Capsules were also hydrated in water and adjusted for pH and ionic strength, and the obtained dispersions were evaluated for optical clarity, particle size distribution, and heat stability. Extensive screening of emulsion preparation conditions enabled the identification of systems with good encapsulation performance and transparent and heat stable dispersions at pH 3-7 and 0-50 mM NaCl. The identified capsules were tested for its antimicrobial efficacy in growth media (Tryptic Soy Broth; TSB), presented in Chapter 3. Nano-dispersed thymol was tested at different concentrations against *Escherichia coli O157:H7, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella typhimurium* at various pH and temperatures and was compared with antimicrobial efficacy of free thymol. In Chapter 4, the antimicrobial tests were extended to two model food systems - apple cider, a clear and relatively simple system, and 2 % reduced fat milk, a more complex food matrix- for bacteria of *Escherichia coli O157:H7 and Listeria monocytogenes*. Since eugenol is slightly more soluble than thymol and is a better choice for food matrices containing lipids, Chapter 5 is focused on the optimization of conditions for nano-dispersing eugenol, with the assumption that a slight change in antimicrobial chemical structure (thymol vs. eugenol) impacts nano-dispersion properties. The identified capsules, prepared using a conjugate different from the one identified for

thymol, were tested for inhibition of *Escherichia coli O157:H7 and Listeria monocytogenes* in TSB and 2 % reduced fat milk, detailed in Chapter 6. The characterized physicochemical and antimicrobial properties of these novel GRAS antimicrobial delivery systems can be used to evenly disperse essential oils at concentrations higher than their solubility in food matrices to enhance food safety, without affecting visual appearance.

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Appendix

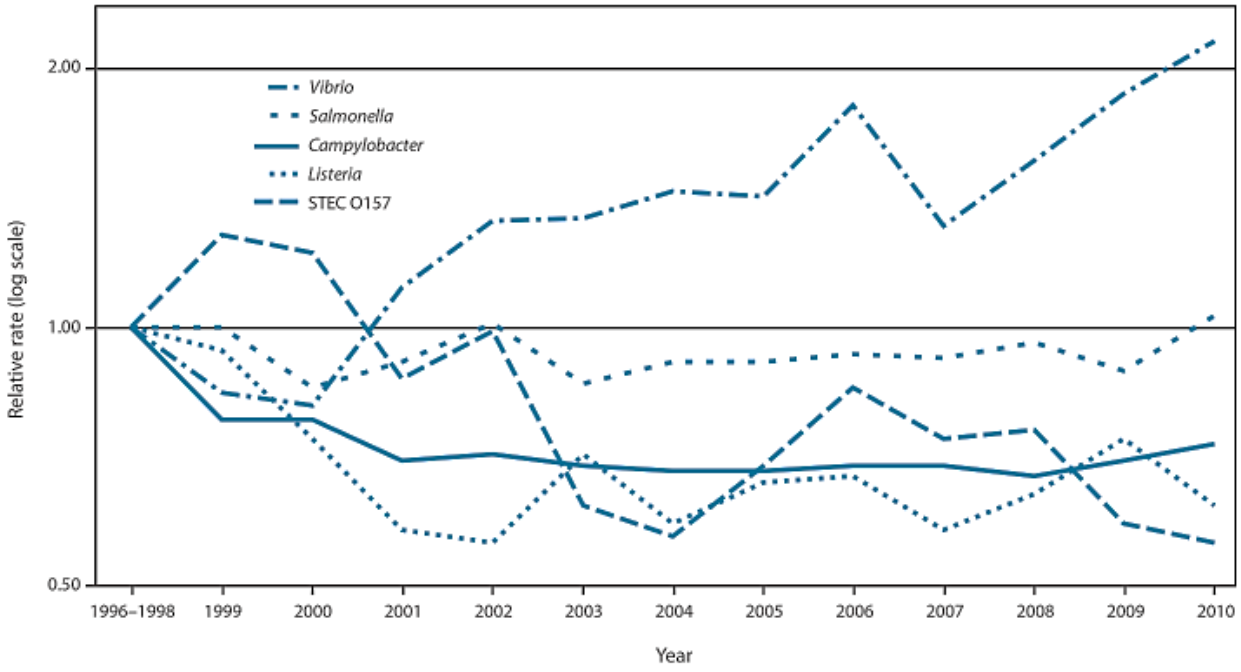

Figure 1-1. Relative rates of laboratory-confirmed infections with Campylobacter, STEC O157 (Shiga toxin-producing), *Listeria monocytogenes*, *Salmonella*, and Vibrio compared with 1996–1998 rates, by year — Foodborne Active Surveillance Network, United States, 1996–2010 (Source: CDC 2010)

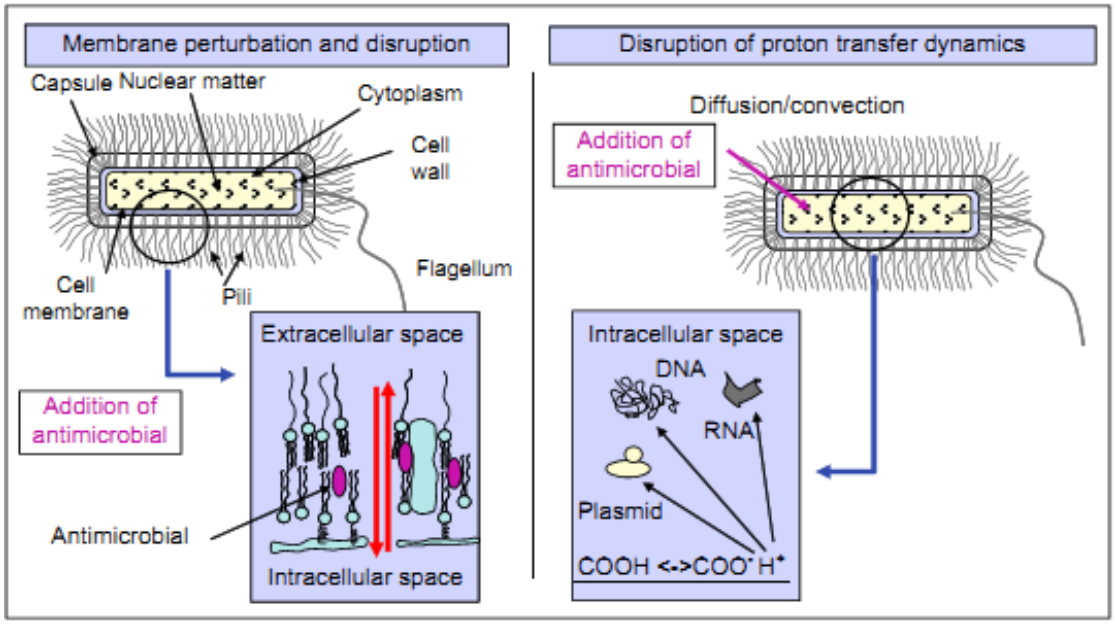

Figure 1-2. Illustration of two basic mechanisms of action of antimicrobials. (Left) addition of antimicrobial leads to insertion into the membrane of bacteria. (Right) Diffusion of an antimicrobial into the cell interior. Adapted from Weiss and others (2009).

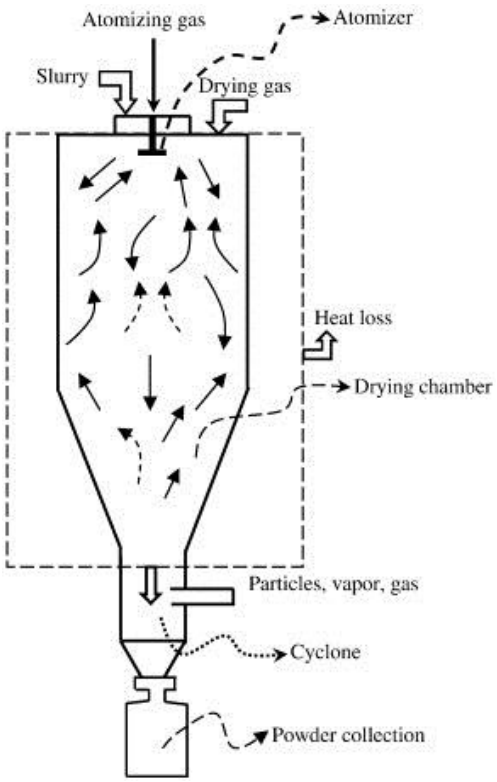

Figure 1-3. Schematic illustration of spray drying principle. Adapted from Wang and others, (2011).

2. Nano-dispersing thymol for enhanced dispersibility and antimicrobial effectiveness. Part 1. Physicochemical properties

Abstract

The food industry is interested in using plant essential oils such as thymol, as novel and efficient food safety intervention strategies against a broad spectrum of pathogens. However, these antimicrobial compounds are lipophilic and their poor water solubility and dispersibility makes it difficult to incorporate them in foods, impacting physical stability, sensory properties and antimicrobial effectiveness. This work presents simple and scalable processes to prepared capsules by spray drying emulsions with an oil phase of thymol in hexane emulsified by conjugates of whey protein isolate (WPI) and maltodextrin (MD). Hydration of spray dried capsules resulted in the formation of transparent and heat stable nano-dispersions containing thymol at concentrations well above its solubility limit. Further, effects of different MD chain lengths and WPI:MD mass ratios of 1:2, 1:1 and 2:1 on encapsulation and subsequent nano-dispersion properties were studied. Mass yields up to 82.8 % with an encapsulation efficiency of 55.8 % and a loading of 12.6 % volatile thymol were obtained after spray drying. Dispersions of spray dried powders at pH 3.0 and 7.0 were transparent after heating at 80°C for 15 min, with particles smaller than 100 nm. One formulation produced clear dispersions after heating at pH 5.0, which is near the isoelectric point of β-lactoglobulin, the major whey protein component, in contrast to gels for controls that were prepared from non-conjugated WPI and MD. Thus, conjugation of WPI with MD not only improved optical clarity but, also heat stability of thymol-containing nano-dispersions. The present study demonstrates a commercially viable technology to produce nanoscale systems to deliver lipophilic food components in foods, especially clear beverages.

Keywords: lipophilic antimicrobial, thymol, nanoscale delivery system, whey protein isolate, conjugate

2.1. Introduction

There is growing interest in novel food safety intervention strategies owing to the frequency and intensity of foodborne outbreaks caused by pathogens such as *Escherichia coli, Salmonella typhimurium, Listeria monocytogenes and Staphylococcus aureus*. The food industry has traditionally used aseptic handling, sanitization and environmental control as measures to address food quality and consumer health concerns. However, there is a need for new, more efficient and cost-effective approaches, such as the use of plant essential oils (EO) as antimicrobial agents to control the growth of pathogenic and spoilage microorganisms (Donsì and others 2011a; Donsì and others 2011b; Lambert and others 2001; Tserennadmid and others 2011). EO are strongly aromatic, volatile, phenolic compounds and can be extracted from parts of plants such as flowers, buds, leaves, seeds, twigs, barks, fruits and roots (Burt 2004). Traditionally, they have been used as flavoring agents in culinary applications and are drawing increasing interests due to their medicinal properties and preservative effects against a wide variety of microorganisms (Gutierrez and others 2008). EO of commonly used herb or spice ingredients such as thyme, oregano and clove, contain thymol, carvacrol, and eugenol as the major bioactive compound, respectively (Burt 2004). These are known to possess excellent antimicrobial properties against gram-negative and gram-positive pathogenic bacteria and many fungi (Kamble and Patil 2008).

However, the antimicrobial efficacy of EO is significantly reduced in foods, due to poor water solubility which results in uneven dispersion in food matrices (Gutierrez and others 2008). Additionally, due to their chemical structure and hydrophobic properties, they have a tendency to bind with lipids, proteins and other food constituents, making them less available for antimicrobial activity and in turn reduced efficacy (Burt 2004). This calls for use of EO at high concentrations in order to achieve desired efficacy as observed in growth media, which may affect sensory properties of foods such that they become unacceptable to consumers (Weiss and others 2009). Thus, a commonly used approach to overcome

these challenges is encapsulation of EO (Champagne and Fustier 2007) in protective materials such as gums (Beristain and others 2001), starches (Glenn and others 2010) and proteins (Charve and Reineccius 2009; Chen and others 2006). Milk protein and carbohydrate-based matrices have shown tremendous potential in encapsulating lipophilic volatiles (Chevalier and others 2001; Kato 2002). Conjugation of proteins and carbohydrates further improves the ability of proteins to adsorb strongly to the oil–water (or air–water) interface (offered by proteins) and the overall dispersibility in the aqueous phase (offered by carbohydrate) (Akhtar and Dickinson 2003). Therefore, Maillard-type protein-carbohydrate conjugates such as those of whey protein isolate (WPI) and maltodextrins (MDs) have been used to stabilize emulsions with oil phases of orange oil (Akhtar and Dickinson 2007), linoleic acid (Choi and others 2010) caraway EO (Bylaite and others 2001), oregano EO (Baranauskiene and others 2006), avocado oil (Bae and Lee 2008) and ginger oil (Toure and others 2007).

Recently, nanoscale encapsulation systems have attracted much interest to deliver bioactive compounds including lipophilic antimicrobials in food systems (Moraru and others 2009). Nanoscale systems may provide unique advantages such as increased surface area of EO droplets, improved EO-bacterial contact and transparent dispersions that scatter light less efficiently due to small particle size (Weiss and others 2009). There are several reports on nano-encapsulating lysozyme and nisin in liposomes (Were and others 2004), eugenol (Kriegel and others 2010), thymol (Ziani and others 2011) and terpene (Donsì and others 2011a) in nano-emulsions for improved antimicrobial activity. However, the process to make these systems may require the use of high energy methods, expensive ingredients or involve multiple steps. Additionally, some nanoscale systems for example, microemulsions (Gaysinsky and others 2007) require large amounts of surfactants and co-surfactants that are expensive and may be regulated, thus, limiting application in foods.

The current work is focused on the use of emulsion-evaporation to create food grade nanoscale capsules with encapsulated antimicrobials, as illustrated in Figure 2-1. The principle involves dissolving the lipophilic antimicrobial in a volatile organic solvent to form an oil phase, and emulsifying it into an aqueous phase containing dissolved WPI-MD conjugates, followed by spray drying to remove hexane. Spray drying is a simple, quick, and cost effective encapsulation method commonly used in the food industry to produce delivery systems for improved stability of volatile ingredients during processing and storage, and bioactivity of encapsulated compounds in food matrices. Besides, powdered products are convenient for storage, handling and transportation. WPI-MD conjugates were selected as emulsifiers based on the hypothesis that the amphiphilic proteins adsorb onto the lipophilic EO phase and the hydrophilic carbohydrate moiety can be solvated by the aqueous phase. This, in turn, can improve dispersibility and reduce particle aggregation due to steric hindrance.The chosen model lipophilic antimicrobial was Thymol, which has excellent antibacterial and antifungal activity. The limited water solubility of thymol, 1.0 g/l at 20°C (Burt 2004; Syracuse Research Corporation 2011), makes it difficult to be evenly dispersed in foods.

The objective of the present study was to create spray dried capsules of thymol in WPI-MD conjugates for dispersion in water and to study the effect of formulation parameters such as WPI:MD ratio and MD chain length on particle size, dispersibility, visual transparency and thermal stability of the novel thymol-containing dispersions.

2.2. Materials and Methods

2.2.1. Materials

Thymol (99 %) was purchased from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD of various chain lengths (MD40, MD100 and MD180, with an average dextrose equivalent of 4, 10 and 18, respectively) were products of Grain

Processing Corporation (Muscatine, IA). Other chemicals such as hexane and methanol were obtained from Fisher Scientific (Pittsburgh, PA).

2.2.2. Conjugate preparation

Conjugates were prepared by dry heating using a method described by Akhtar and Dickinson (2007), with some modifications including heating temperature, drying method, MD chain lengths and WPI:MD ratios. WPI and MD at a mass ratio 1:2, 1:1 or 2:1 were hydrated in deionized water at 3.7 % w/w WPI and varying MD amounts for 14 h and spray dried using a B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). Spray drying conditions included inlet temperature 150°C, 600 kPa compressed air pressure, air flow rate of 35 m^3/h and 6.67 ml/min feed rate. The spray dried powders were dry heated at 90°C for 2 h to form 'conjugates'. The conjugate powders were collected and stored in a freezer at -18°C.

2.2.3. Preparation of capsules by spray drying

For the first part of the study, emulsions were prepared by emulsifying an oil phase of thymol in hexane into an aqueous phase with these conjugates prepared at a WPI:MD180 mass ratio of 1:2, according to the formulations 1-16 in Table 2-1. Emulsions were prepared using a Virtis-Sentry Cyclone I.Q.2 microprocessor homogenizer equipped with a 12 inch shaft and an 1 inch disc blade operated at 15000 rpm for 3 min for 100ml. Emulsions in the first group (formulations 1-6) were prepared from an oil phase with 0.1-20 % w/v thymol in hexane emulsified at 10 % volume of the overall emulsion with a fixed amount of conjugates, corresponding to theoretical thymol loading levels of 1.1-69 %. The second group of emulsions (formulations 7-10) was prepared with a 1:9 ratio of oil:aqueous phase volume; but, the oil phase had 20-50 % thymol. The third set of emulsions (formulations 11-14) was prepared with 15-30 % volume fractions of the oil phase, with fixed concentrations of thymol and conjugates in each phase. In order to demonstrate the significance of conjugation of proteins with carbohydrates, controls to

formulations 3 and 7 were made using mixtures of WPI and MD, which were spray dried but, without the dry heat treatment as in the case of conjugates. For the second part of the study, the most promising formulation from the first part (formulation 7) was was selected as the basis to investigate the effect of conjugate structure on dispersion properties. WPI was used with MD40, MD100 and MD180 at mass ratios of 1:2, 1:1 and 2:1 (formulations 15-22 in Table 2-1) to create emulsions for spray drying.

2.2.4. Characterization of spray dried capsules

2.2.4.1. Mass Yield

Mass yield defined in Equation 1 was used to calculate percentages of the collected mass of spray dried products with reference to the non-solvent mass in the corresponding solution (feed) before spray drying.

$$Mass\ Yield\ \% = \frac{Mass\ of\ collected\ product}{Non-solvent\ mass\ of\ feed} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

2.2.4.2. Thymol loading in spray dried powders

Spray dried powders were dissolved at 8 mg/ml concentration in 40 % v/v aqueous methanol for quantification of thymol concentration using high performance liquid chromatography (HPLC) using a method of (Ghosheh and others 1999)) with modifications in Sample preparation. The 1200 series HPLC system from Agilent Technologies, Inc. (Santa Clara, CA) was equipped as follows: a 1200 series quaternary pump, a Diode Array Detector and a 1200 series vacuum degasser. A ZORBAX Eclipse Plus-C18 column (Agilent Technologies, Inc.) was used. HPLC grade water and methanol at 40:60 volume ratio was used as the mobile phase, in isocratic mode at a flow rate of 1 ml/min. 20 μL of Sample was injected. UV spectra were acquired between 190 and 370 nm and the chromatogram was extracted at

254 nm and analyzed using Chemstation Plus software (Agilent Technologies, Inc.). A calibration curve previously established using standard solutions with various thymol concentrations was used to determine thymol concentration based on Sample peak areas. Thymol loading (%w/w) was defined in Equation 2:

$$Loading\ \% = \frac{Mass\ of\ thymol}{Mass\ of\ collected\ product} \times 100 \dots\dots\dots (2)$$

2.2.4.3. Encapsulation efficiency

Encapsulation efficiency (EE) was defined as in Equation 3 to compare total thymol mass in a spray dried product and the corresponding thymol mass used in spray drying, while Equation 4 describes the total thymol concentration change before and after spray drying.

$$EE\ \% = \frac{Loading\ \% \times Mass\ of\ collected\ product}{Mass\ of\ thymol\ in\ feed} \times 100 \dots\dots\dots (3)$$

$$Thymol\ Concentration\ Change\ \% = \frac{Loading\ \%}{Theoretical\ loading\ \%} \times 100 \dots\dots\dots (4)$$

where theoretical loading % was the concentration of thymol in the feed excluding solvent:

$$Theoretical\ loading\ \% = \frac{Mass\ of\ thymol}{(Mass\ of\ thymol) + (mass\ of\ WPI - MD\ conjugate\ or\ mixture\ in\ feed)} \times 100 \dots\dots\dots (5)$$

2.2.4.4. Surface morphology

Spray dried capsules were mounted onto a two-way black adhesive tape mounted on a stainless steel stub. After sputter-coating with a gold layer of ~5 nm, structures of powders were imaged using a LEO 1525 surface scanning electron (SEM) microscope (LEO Electron Microscopy, Oberkochen, Germany).

2.2.5. Preparation and characterization of nano-dispersions

Spray dried capsules were hydrated at 5 % w/v powder concentration in deionized water at room temperature (20°C) for 14 h and adjusted to pH 3.0, 5.0, and 7.0 using 1 N NaOH or 1 N HCl and 0 and 50 mM NaCl for the following characterizations.

2.2.5.1. Turbidity and thermal stability

2 ml of above dispersions were tested in 4 ml glass vials each. Vials were placed in a water bath at 80°C for 15 min, followed by cooling in a room temperature water-bath immediately. To evaluate heat stability and visual clarity, photographs of dispersions were taken and the absorbance was measured at 421 and 600 nm before and after heating using a UV-vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA). The 421 nm wavelength corresponds to the highest absorbance by Maillard reaction products in the visible light spectrum (Yong and Foegeding 2008).

2.2.5.2. Particle size distribution of nano-dispersions

The above nano-dispersions were measured for size distributions before and after heating based on dynamic light scattering using Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc. Brea, CA). The volume-length mean particle diameter ($d_{4,3}$) was calculated using Equation 6 in which n_i is particle number corresponding to diameter d_i:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad \text{.....(6)}$$

2.2.6. Data and statistical analysis

Duplicate batches of spray dried Samples were prepared for each formulation inTable 2-1.. Each batch of capsules was tested in duplicate. Data from four replicate experiments were pooled to calculate the

statistical mean and standard error of the mean for each condition tested. All data was verified for normality, followed by mixed model analysis of variance (ANOVA, $p<0.05$) using SAS 9.2 (SAS Institute, NC).

2.3. Results and Discussion

2.3.1. Encapsulation Performance

The encapsulation performance of spray dried powders is presented in Table 2-2. All conjugate formulations and controls produced similar mass yields ($p>0.05$) with values ranging from 73.9 % to 82.8 %. Most conjugate formulations including formulations 1-6 and 11-14 showed higher EE and thymol loading than controls ($p<0.05$) containing non-conjugated WPI-MD mixtures. Sample 12 demonstrated the highest EE of 55.8 % and corresponding thymol concentration change of 70.1 % before and after spray drying. EE and thymol concentration change significantly reduced from formulations 7-10, which has increasing thymol and conjugate concentrations in their respective oil and aqueous phases ($p<0.05$). Adamiec and Kalemba (2006) reported that higher loading of oil may increase encapsulated flavor oil concentrations in spray dried powders; but, it may reduce the overall encapsulation efficiency, and this may depend on formulation parameters including type and concentration of wall materials used.

Both formulations 3 and 7 had 16.7 % theoretical thymol loading; but, they differed in the conjugate concentration in aqueous phase and thymol concentration in the oil phase. An interesting finding is that the concentration of thymol in Sample powders 3 and 7 was determined to be 1.5 and 10.6 % respectively, with EE of 7.1 and 51.3 % respectively (Table 2-2). With vapor pressure of 8.0 kPa at 150°C (Syracuse Research Corporation 2011), which was the inlet temperature for spray drying, it may be possible that thymol loss seemed higher in Sample 3, due to the evaporation of large amount of solvent during spray drying, when compared with Sample 7.

In contrast, controls C-3 and C-7 for Samples 3 and 7 respectively, produced lower EE of 11.8 and 25.1 % respectively, with lower thymol concentration change values of 15.0 and 33.1 % respectively. It is well established that conjugation of WPI with suitable carbohydrates such as dextran (Akhtar and Dickinson 2003) and maltodextrin (Akhtar and Dickinson 2007; Choi and others 2010), improves its surface-active properties. Based on the present findings, it can be concluded that optimization of emulsion preparation conditions and conjugation of WPI with MD can help minimize losses and enable reasonable encapsulation performance after spray drying.

2.3.2. Surface Morphology

The scanning electron micrographs show the structural characteristics of spray dried capsules, such as shape, size and surface defects. Most Samples demonstrated spherical capsules varying from 1-5 µm in size, containing shell structures (Figure 2-2). The formulation of shell structures may have resulted from quick evaporation of hexane from emulsion droplets during spray drying. Ruptured capsules were also observed, e.g., for Sample 8. Formulations 1-6 that varied in thymol concentrations, demonstrated numerous aggregated structures, while formulations 8-14, prepared with varying conjugate and oil phase volume concentrations, exhibited several disjointed, intact capsules with fewer surface defects. Defects such as holes and cracks were detected on the surface of capsules formed from formulation 14, which had the highest (30 % v/v) oil phase volume.

Capsules prepared with different WPI:MD ratios and MD chain length s are shown for Samples 15, 16, 18 and 21 in Figure 2-1. These Samples showed similar size and surface morphological characteristics as other treatments. Fundamentals interpreting microscapsule structure formations at the studied emulsion conditions however require future work.

2.3.3. Turbidity and thermal stability of dispersions

Pure thymol has poor solubility in deionized water, i.e. only up to 0.1 % w/v at 20°C (Syracuse Research Corporation 2011). When spray dried capsules were hydrated at 5 % w/v, an overall thymol concentration of up to 0.525 % w/v was obtained for some dispersions, which is much higher than its solubility limit. These dispersions were clear from pH 3.0-7.0, at 0 and 50 mM NaCl, before and heating. In comparison, when pure thymol at 0.525 % in deionized water, insoluble particulates were obtained after incubation at room temperature (21°C) for 24 h. Dispersions were photographed for their visual appearance and measured for absorbance to indirectly compare Sample turbidity. Results are presented below for impacts of solvent conditions (pH and ionic strength) and conjugate structure.

2.3.3.1. Effect of pH and ionic strength on visual clarity and heat stability

Photographs of dispersions are presented for Samples 3 and 7 only in Figure 2-2, and absorbance values of all treatments are tabulated in Table 2-3. At pH 3.0 and 7.0, all dispersions were clear, with an increase in visual clarity after heating at both salt concentrations. At pH 5.0, all dispersions except Samples 3 and 7 became turbid or formed gels after heating. The reduced thermal stability at pH 5.0 is expected because, near the isoelectric point of whey proteins, the minimized electrostatic repulsion due to reduced surface net charge favors protein aggregation. The presence of thymol strengthens hydrophobic attraction among particles, responsible for increased turbid appearance for Samples 4-6, 11-13 and 17 (Table 2-3) that had relatively higher thymol loading levels (Table 2-2).

It is well known that an increase in ionic strength reduces the effective distance of electrostatic repulsion and facilitates aggregation of whey proteins, with the extent dependent on concentration and type of ions present in solution (Tziboula and Donald Muir 1993). When compared to dispersions with 0 mM NaCl, dispersions with 50 mM NaCl showed slightly lower or higher absorbance values (Table 2-3). It is possible that this ionic strength was not high enough to significantly impact the aggregation of

particles in dispersions. As the NaCl concentration was increased to 100 mM, most dispersions except those formed by formulation 3 and 7, became more turbid (data not shown), indicating increased particle aggregation.

2.3.3.2. Effect of ratio of WPI:MD and MD chain length on visual clarity and heat stability

Functional properties of conjugates are known to be significantly affected by protein and carbohydrate structures (Dickinson 2008; Dunlap and Cote 2004; Shu and others 1996). In this work, capsules were prepared from conjugates containing three MD chain lengths and WPI:MD mass ratios (Samples 7, 15-22 in Table 2-1). After dispersing capsules, it was found that Samples made with a WPI:MD mass ratio of 1:2, regardless of MD chain length, produced dispersions with slightly higher clarity after heating, than those prepared with ratios of 1:1 or 2:1. At pH 5.0, aggregation of excess, non-glycosylated whey protein in formulations with WPI:MD mass ratio 2:1 may have occurred, contributing to higher turbidity among all dispersions. Where 1:1 WPI:MD ratio was used, MD concentration was not sufficient to bind with proteins and this may have resulted in available sites for aggregation, causing most dispersions to be clear at pH 3.0 and 7.0 but, turbid at pH 5.0 (Figure 2-3).

The effect of chain length of MD on dispersion properties was studied for MD40, MD100 and MD180. Most dispersions were clear at pH 3.0 and 7.0 but, turbid at pH 5.0 after heating at both NaCl concentrations, owing to the different MDs affecting hydration properties of conjugate-based dispersions. It has been suggested that size of the oligo- or polysaccharide may cause steric hindrance and impact the degree of conjugation during dry heat treatment and resulting functional properties (Ho and others 2000). However, there was no statistically significant effect of increasing MD chain length observed on absorbance of dispersions ($p>0.05$). Although the present study demonstrates the need for optimization of formulations to achieve visual clarity, improved dispersibility and heat stability in

dispersions, fundamental studies are needed to illustrate the impacts of conjugate structure on dispersion properties using well-defined emulsion preparation conditions.

2.3.4. Particle size distributions of nano-dispersions before and after heating

Dispersions were tested for particle size distributions before and after heating. Dispersions that were optically clear corresponded to $d_{4,3}$ smaller than 100 nm (all data not shown). Most formulations demonstrated $d_{4,3}$ less than 250 nm before heating, which reduced to less than 100 nm after heating at pH 3.0 and 7.0, at both salt concentrations. The reduced $d_{4,3}$ values after heating indicate improved hydration of spray dried powder at an elevated temperature, characteristic of disruption of inter-particle hydrogen bonding. At pH 5.0, the reduced $d_{4,3}$ after heating was observed for Samples 3 and 7 only, while other dispersions either became turbid or formed a gel. The $d_{4,3}$ of Sample 3 and 7 are listed in Table 2-4. . For Sample 7, $d_{4,3}$ of dispersions adjusted to pH 3.0, 5.0 and 7.0 without salt were 67, 100 and 58 nm , respectively, before heating and 64, 86, and 52 nm, respectively, after heating. The corresponding particle size distributions before and after heating are shown in Figure 2-2. The particle size information may suggest the formation of (thymol) core-(protein) shell structure illustrated in Figure 2-2 because $d_{4,3}$ values are much bigger than the hydrodynamic radius of whey protein components, which is 2.6-4.9 nm for β-lactoglobulin (Parker and others 2005), 2.0 nm for α-lactalbumin (Molek and Zydney 2007), and 3.7 nm for bovine serum albumin (Brownsey and others 2003), respectively, at neutral pH. Upon addition of 50 mM NaCl, diameters of Sample 7 dispersions at pH 3.0, 5.0, and 7.0 after heating were 66, 89, 60 nm (Table 2-4), respectively, and were not statistically different from the corresponding treatment with 0 mM NaCl.

2.3.5. Significance of conjugates to nano-dispersion properties

To illustrate the significance of conjugates on improvement of dispersion properties, controls for Samples 3 and 7 were prepared using non-conjugated mixtures of WPI and MD to emulsify the oil phase

with dissolved thymol (Samples C-3 and C-7 in Table 2-1). Capsules of Samples C-3 and C-7 were hydrated at 5 %w/v in deionized water, adjusted for pH and NaCl concentration. Visual appearance of dispersions before and after heating is presented in Figure2-5. Dispersions were slightly turbid at pH 3.0 and 7.0 which corresponded to higher absorbance values in Table 2-3 and larger $d_{4,3}$ Table 2-4 than those of Samples 3 and 7. At pH 5.0, $d_{4,3}$ of Samples C-3 and C-7 were even bigger (greater than 1 µm) before heating and both Samples formed opaque gels after heating (Figure 2-7). The enhanced dispersibility and thermal stability of dispersions prepared from conjugates supports the hypothesis in the introduction, enabled by the proposed core-shell structure (Figure 2-2) where the MD shell provides steric hindrance stabilizing particles. Future work may involve the use of advanced techniques such as atomic force microscopy to characterize particle structures in dispersions.

2.4. Conclusions

The present study demonstrated the success of encapsulating volatile thymol using spray drying and subsequent hydration of spray dried capsules to prepare transparent and heat stable dispersions at a thymol concentration well above its solubility limit. WPI-MD conjugates were advantageous than non-conjugated mixtures in improving dispersion, transparency and thermal stability. Variables in emulsion preparation impacted encapsulation performance of thymol and properties of nano-dispersions. Fundamental principles resulting in the observed differences however are not clear and require emulsion preparation conditions that are much more defined than in this work. Nevertheless, the presented scalable technology may be used to disperse various lipophilic components in clear liquid products, contributing to the growing functional beverage market.

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Appendix

Table 2-1. Formulations used to prepare emulsionsa for spray drying.

Sample ID	Type of MD	WPI:MD ratio	Thymol %w/v in hexane	Conjugate %w/v in water	Volume %v/v of oil phase	Theoretical thymol loading %w/w
1	180	1:2	0.1	1.0	10	1.1
2	180	1:2	1.25	1.0	10	12.2
3	180	1:2	1.8	1.0	10	16.7
4	180	1:2	5.0	1.0	10	35.7
5	180	1:2	10.0	1.0	10	52.6
6	180	1:2	20.0	1.0	10	69.0
7	180	1:2	20.0	11.1	10	16.7
8	180	1:2	30.0	16.7	10	16.7
9	180	1:2	40.0	22.2	10	16.7
10	180	1:2	50.0	27.8	10	16.7
11	180	1:2	20.0	11.1	15	24.1
12	180	1:2	20.0	11.1	20	31.1
13	180	1:2	20.0	11.1	25	37.5
14	180	1:2	20.0	11.1	30	43.6
15	40	1:2	20.0	11.1	10	16.7
16	100	1:2	20.0	11.1	10	16.7
17	40	1:1	20.0	7.4	10	23.1

Sample ID	Type of MD	WPI:MD ratio	Thymol %w/v in hexane	Conjugate %w/v in water	Volume %v/v of oil phase	Theoretical thymol loading (%w/w)
18	100	1:1	20.0	7.4	10	23.1
19	180	1:1	20.0	7.4	10	23.1
20	40	2:1	20.0	5.6	10	29.7
21	100	2:1	20.0	5.6	10	29.7
22	180	2:1	20.0	5.6	10	29.7
C-3	180	1:2	1.8	1.0[b]	10	16.7
C-7	180	1:2	20.0	11.1[b]	10	16.7

[a]Emulsions were prepared with an oil phase of thymol in hexane and an aqueous phase of whey protein isolate-maltodextrin (WPI-MD) conjugate (Samples 1-22) or mixture (Samples C-3 and C-7) in deionized water.

[b]A spray dried mixture of WPI and MD180 at a mass ratio of 1:2 without dry heating as in the preparation of conjugates was used to prepare emulsions to serve as a control to Samples 3 and 7

Table 2-2. Encapsulation performancea of spray drying emulsions prepared according to formulations in Table 2-1.

Sample ID	Mass yield (%)	Thymol loading (%w/w) in spray dried powder	Thymol concentration change (%)	Encapsulation efficiency (%)
1	73.9 ± 2.0	0.6 ± 0.0^{J}	54.9 ± 1.1^{DEFGHIJ}	40.6± 3.8^{CDEF}
2	76.6 ± 0.9	6.1 ± 0.5^{EFGHIJ}	50.3 ± 2.0^{CDEFGHI}	38.8 ± 4.7^{BCDE}
3	79.0 ± 0.9	1.5 ± 0.3^{J}	9.2 ± 0.8^{L}	7.1 ± 1.0^{J}
4	82.8 ± 1.8	10.5 ± 2.7^{ABCDEFG}	29.5 ± 3.8^{GHIJKL}	29.6 ± 5.8^{DEFGH}
5	78.8 ± 1.0	13.3 ± 0.1^{AB}	25.4 ± 1.0^{JKL}	20.0 ± 0.8^{FGHIJ}
6	79.7 ± 2.5	15.7 ± 3.5^{A}	22.8 ± 2.5^{KL}	17.3 ± 1.9^{FIJ}
7	81.2 ± 0.4	10.6 ± 0.6^{DEFGHI}	63.3 ± 1.9^{EFGHIJK}	51.32 ± 1.4^{AB}
8	78.6 ± 2.4	7.8 ± 2.1^{CDEFGH}	47.3 ± 6.4^{CDEFGHIJ}	36.9 ± 9.8^{BCDE}
9	81.0 ± 1.2	6.9 ± 0.0^{DEFGHI}	41.5 ± 0.1^{DEFGHIJK}	33.9 ± 2.0^{CDEF}
10	75.8 ± 2.3	4.4 ± 1.0^{GHIJ}	26.5 ± 3.0^{IJKL}	18.9 ± 1.9^{GHIJ}
11	78.1 ± 2.1	12.6 ± 0.2^{ABC}	52.3 ± 0.4^{BCDEFG}	40.7 ± 2.6^{BCD}
12	79.5 ± 2.0	11.7 ± 0.1^{ABCD}	70.1 ± 0.4^{ABC}	55.8 ± 0.7^{A}
13	78.5 ± 1.8	10.3 ± 0.1^{BCDEF}	61.9 ± 0.4^{ABCDE}	48.6 ± 1.7^{AB}
14	77.6 ± 4.6	9.4 ± 0.6^{BCDEFG}	56.4 ± 1.6^{ABCDEF}	44.2 ± 3.7^{ABC}
15	79.2 ± 0.7	6.22 ± 0.0^{EFGHIJ}	37.2 ± 0.0^{FGHIJK}	33.2 ± 0.3^{CDEFG}
16	80.6 ± 1.2	8.50 ± 0.1^{BCDEFGH}	50.9 ± 0.1^{CDEFGH}	42.3 ± 0.5^{ABCD}
17	78.2 ± 1.1	10.90 ± 0.2^{ABCDE}	65.3 ± 0.2^{ABCD}	42.4 ± 0.8^{ABCD}

Sample ID	Mass yield (%)	Thymol loading (%w/w) in spray dried powder	Thymol concentration change (%)	Encapsulation efficiency (%)
18	77.9 ± 1.3	7.1 ± 0.0^{DEFGHI}	42.2 ± 0.0^{DEFGHIJK}	28.2 ± 1.1^{DEFGH}
19	76.8 ± 1.4	4.5 ± 0.0^{EFGHIJ}	27.1 ± 0.0^{FGHIJKL}	18.4 ± 0.3^{FGHIJ}
20	75.5 ± 0.9	4.1 ± 0.0^{HIJ}	24.4 ± 0.0^{JKL}	11.9 ± 0.0^{IJ}
21	74.6 ± 0.6	3.3 ± 0.2^{HIJ}	19.7 ± 0.2^{KL}	9.6 ± 0.4^{J}
22	75.5 ± 1.5	4.5 ± 0.0^{GHIJ}	26.9 ± 0.0^{HIJKL}	13.6 ± 0.0^{IJ}
C-3	77.8 ± 1.3	2.5 ± 0.1^{IJ}	15.0 ± 0.3^{A}	11.8 ± 1.1^{IJ}
C-7	75.6 ± 1.6	3.5 ± 0.1^{AFGHIJ}	33.0 ± 1.5^{AB}	25.1 ± 2.5^{EFGH}

[a]Values are means ± standard errors of means from four measurements, two from each of two replicates. Different superscript letters in the same column represent statistical difference ($p<0.05$).

Table 2-3. Absorbance of thymol-containing dispersions before and after heating at 80°C for 15 min.

Sample ID	NaCl (mM)	Absorbance before heating						Absorbance after heating					
		421 nm			600 nm			421 nm			600 nm		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
3	0	0.408 ± 0.01	0.152 ± 0.01	0.285 ± 0.00	0.288 ± 0.00	0.080 ± 0.00	0.144 ± 0.00	0.207 ± 0.00	0.288 ± 0.07	0.416 ± 0.10	0.125 ± 0.00	0.115 ± 0.02	0.154 ± 0.03
	50	0.234 ± 0.00	1.271 ± 0.03	0.345 ± 0.05	0.100 ± 0.00	0.791 ± 0.03	0.160 ± 0.03	0.229 ± 0.00	3.005 ± 0.13	0.353 ± 0.05	0.090 ± 0.00	2.275 ± 0.14	0.133 ± 0.02
7	0	0.223 ± 0.00	0.438 ± 0.03	0.834 ± 0.34	0.230 ± 0.03	0.247 ± 0.02	0.453 ± 0.20	0.094 ± 0.00	0.806 ± 0.05	0.506 ± 0.03	0.093 ± 0.00	0.123 ± 0.03	0.242 ± 0.02
	50	0.240 ± 0.01	0.664 ± 0.01	0.228 ± 0.00	0.091 ± 0.00	0.490 ± 0.00	0.098 ± 0.00	0.327 ± 0.02	1.095 ± 0.10	0.306 ± 0.04	0.126 ± 0.02	0.872 ± 0.08	0.120 ± 0.01
15	0	1.652 ± 0.02	2.753 ± 0.02	1.694 ± 0.01	1.671 ± 0.47	2.386 ± 0.04	1.000 ± 0.02	0.568 ± 0.27	3.244 ± 0.02	0.341 ± 0.04	0.102 ± 0.01	3.076 ± 0.00	0.139 ± 0.01
	50	1.704 ± 0.00	1.937 ± 0.02	1.777 ± 0.00	0.903 ± 0.06	3.126 ± 0.04	0.589 ± 0.01	1.130 ± 0.01	1.320 ± 0.02	1.172 ± 0.01	0.517 ± 0.04	2.931 ± 0.10	0.320 ± 0.00
16	0	0.403 ± 0.11	1.822 ± 0.77	0.406 ± 0.16	0.283 ± 0.02	2.241 ± 0.71	0.534 ± 0.01	0.230 ± 0.05	1.345 ± 0.90	0.230 ± 0.08	0.143 ± 0.01	2.023 ± 0.74	0.288 ± 0.04
	50	0.399 ± 0.00	2.520 ± 0.00	0.410 ± 0.02	0.235 ± 0.02	3.183 ± 0.02	0.240 ± 0.00	0.215 ± 0.00	1.789 ± 0.01	0.219 ± 0.01	0.141 ± 0.01	3.040 ± 0.01	0.130 ± 0.00
18	0	1.824 ± 0.01	2.527 ± 0.02	1.952 ± 0.02	1.534 ± 0.00	2.176 ± 0.05	1.655 ± 0.02	0.487 ± 0.00	3.347 ± 0.02	0.551 ± 0.04	0.232 ± 0.00	3.067 ± 0.01	0.284 ± 0.03
	50	1.814 ± 0.01	2.450 ± 0.01	1.839 ± 0.03	1.501 ± 0.00	2.101 ± 0.01	1.521 ± 0.04	0.531 ± 0.07	3.279 ± 0.04	0.429 ± 0.02	0.252 ± 0.05	3.142 ± 0.01	0.205 ± 0.00
21	0	0.449 ± 0.19	1.032 ± 0.32	0.399 ± 0.18	0.764 ± 0.22	0.712 ± 0.23	1.292 ± 0.64	0.275 ± 0.11	Gel	0.245 ± 0.10	0.327 ± 0.11	Gel	0.993 ± 0.55
	50	0.635 ± 0.01	1.065 ± 0.03	0.726 ± 0.01	0.282 ± 0.01	0.569 ± 0.00	0.339 ± 0.02	0.383 ± 0.00	Gel	0.422 ± 0.01	0.150 ± 0.00	Gel	0.159 ± 0.00

Sample ID	NaCl (mM)	Absorbance before heating						Absorbance after heating					
		421 nm			600 nm			421 nm			600 nm		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
C-3	0	1.949 ± 0.03	2.045 ± 0.06	2.202 ± 0.06	1.568 ± 0.08	1.595 ± 0.01	2.012 ± 0.02	0.469 ± 0.00	Gel	0.609 ± 0.01	0.240 ± 0.01	Gel	0.322 ± 0.01
	50	2.081 ± 0.05	2.017 ± 0.02	2.292 ± 0.02	1.821 ± 0.02	1.656 ± 0.03	2.024 ± 0.01	0.617 ± 0.03	Gel	0.642 ± 0.01	0.316 ± 0.01	Gel	0.267 ± 0.02
C-7	0	2.690 ± 0.02	2.596 ± 0.04	2.411 ± 0.00	2.565 ± 0.07	2.359 ± 0.01	2.126 ± 0.05	0.270 ± 0.01	Gel	1.174 ± 0.00	0.139 ± 0.00	Gel	0.538 ± 0.01
	50	2.483 ± 0.04	2.599 ± 0.00	2.529 ± 0.02	2.241 ± 0.03	2.412 ± 0.01	2.168 ± 0.00	0.235 ± 0.01	Gel	0.972 ± 0.03	0.131 ± 0.00	Gel	0.332 ± 0.01

[a]Values are means ± standard errors of means from four measurements, two from each of two replicates.

Table 2-4. $d_{4,3}$ of thymol-containing dispersions adjusted to pH 3.0, 5.0 and 7.0 before and after heating at 80°C for 15 min.

Sample ID	NaCl (mM)	$d_{4,3}$ before heating (nm)			$d_{4,3}$ after heating (nm)		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
3	0	194 ± 4^{HI}	298 ± 9^{IJ}	104 ± 7^{FGHIJ}	54 ± 1^{J}	148 ± 8^{IJ}	35 ± 2^{J}
	50	245 ± 8^{HI}	317 ± 5^{IJ}	138 ± 3^{FGHIJ}	52 ± 2^{J}	165 ± 3^{IJ}	44 ± 1^{J}
7	0	67 ± 1^{IJ}	100 ± 1^{IJ}	58 ± 1^{IJ}	64 ± 1^{IJ}	86 ± 1^{IJ}	52 ± 1^{IJ}
	50	70 ± 2^{IJ}	113 ± 6^{IJ}	64 ± 2^{IJ}	66 ± 2^{IJ}	89 ± 1^{IJ}	60 ± 2^{IJ}
15	0	203 ± 2^{HIJ}	420 ± 2^{EFGHIJ}	124 ± 1^{IJ}	99 ± 1^{IJ}	Turbid^{A}	82 ± 2^{IJ}
	50	227 ± 1^{HIJ}	367 ± 2^{EFGHIJ}	136 ± 2^{IJ}	64 ± 1^{IJ}	Turbid^{A}	80 ± 1^{IJ}
16	0	130 ± 2^{IJ}	393 ± 1^{EFGHIJ}	103 ± 1^{IJ}	80 ± 0^{IJ}	Turbid^{A}	96 ± 4^{IJ}
	50	155 ± 1^{IJ}	451 ± 2^{EFGHIJ}	91 ± 1^{IJ}	76 ± 1^{IJ}	Turbid^{A}	89 ± 1^{IJ}
18	0	198 ± 1^{GHIJ}	308 ± 2^{FGHIJ}	207 ± 1^{HIJ}	107 ± 1^{IJ}	Turbid^{A}	93 ± 1^{IJ}
	50	212 ± 1^{GHIJ}	357 ± 0^{FGHIJ}	196 ± 2^{HIJ}	93 ± 1^{IJ}	Turbid^{A}	82 ± 2^{IJ}
21	0	134 ± 1^{IJ}	488 ± 2^{DEFGHIJ}	126 ± 3^{IJ}	102 ± 2^{IJ}	Turbid^{A}	87 ± 1^{IJ}
	50	217 ± 2^{IJ}	557 ± 1^{DEFGHIJ}	179 ± 2^{IJ}	70 ± 1^{IJ}	Turbid^{A}	71 ± 1^{IJ}
C-3	0	353 ± 5^{FGHIJ}	1444 ± 21^{B}	596 ± 13^{DEFGHI}	1000 ± 3^{CD}	Gel^{A}	715 ± 17^{CDEFG}
	50	298 ± 6^{FGHIJ}	3631 ± 46^{B}	521 ± 3^{DEFGHI}	988 ± 6^{CD}	Gel^{A}	751 ± 10^{CDEFG}
C-7	0	325 ± 4^{DEFGH}	1267 ± 19^{C}	328 ± 8^{EFGHIJ}	935 ± 14^{CDE}	Gel^{A}	765 ± 5^{CDEF}
	50	343 ± 15^{DEFGH}	1335 ± 50^{C}	461 ± 17^{EFGHIJ}	855 ± 4^{CDE}	Gel^{A}	748 ± 9^{CDEF}

[a]Values are means ± standard errors of means from four measurements, two from each of two replicates. Different superscript letters represent statistical difference ($p<0.05$).

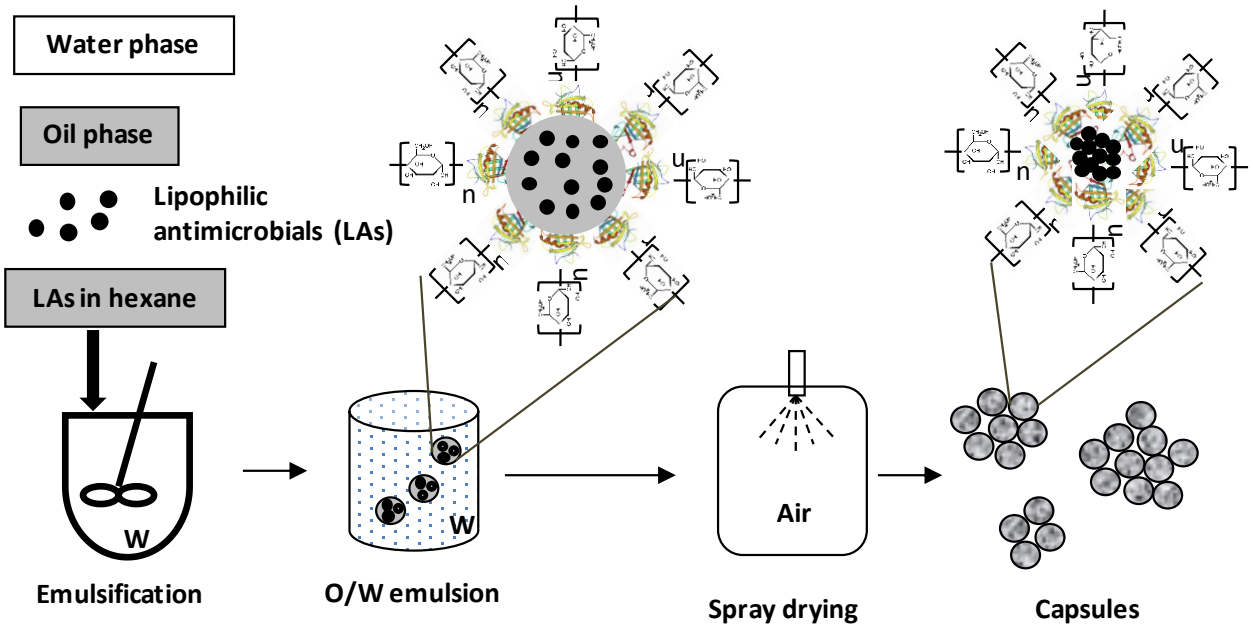

Figure 2-1. Emulsion-evaporation process for encapsulation of thymol in whey protein isolate-maltodextrin conjugates using spray drying.

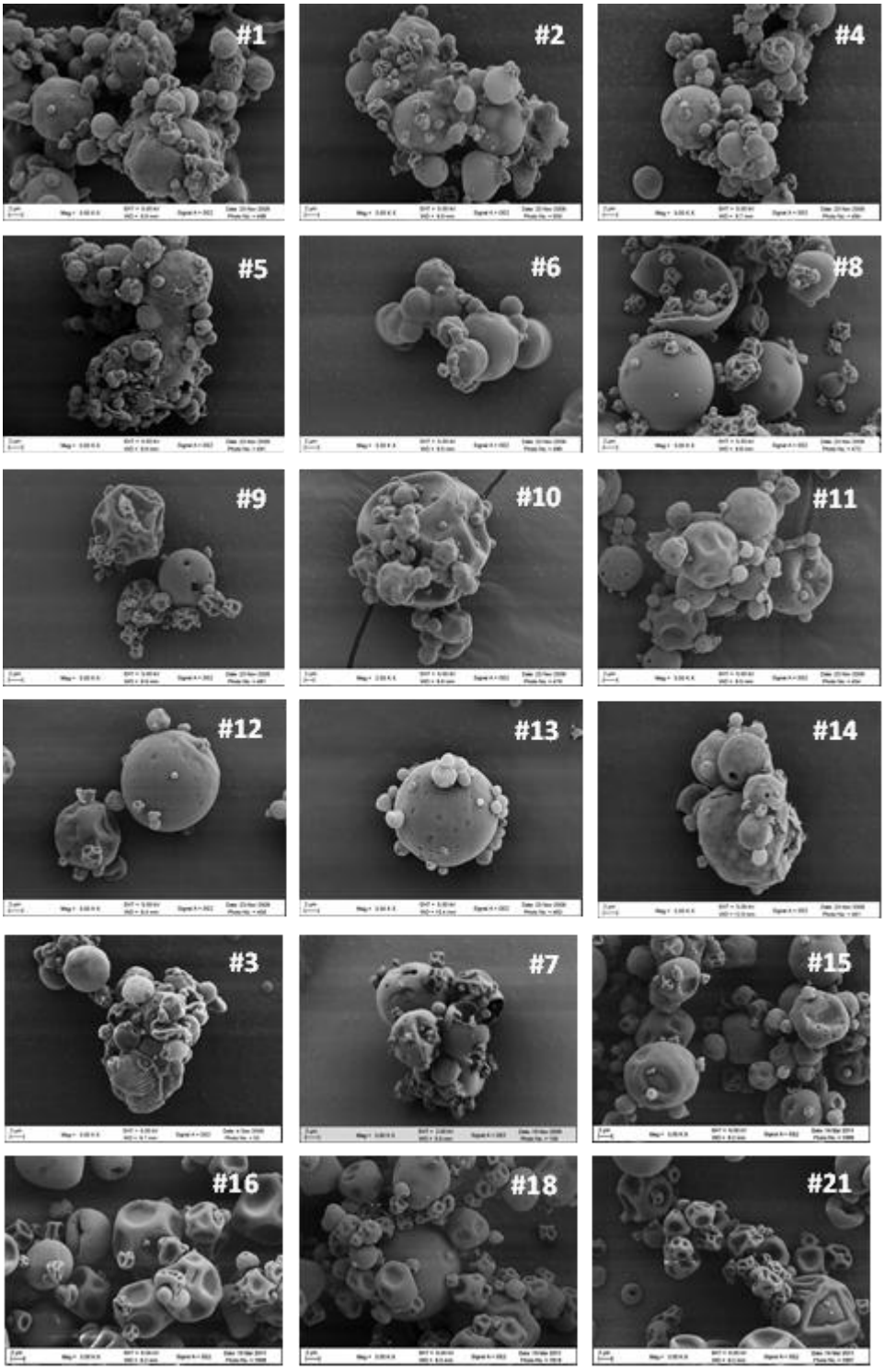

Figure 2-2. Scanning electron micrographs of spray dried powders prepared from formulations in Table 2-1.

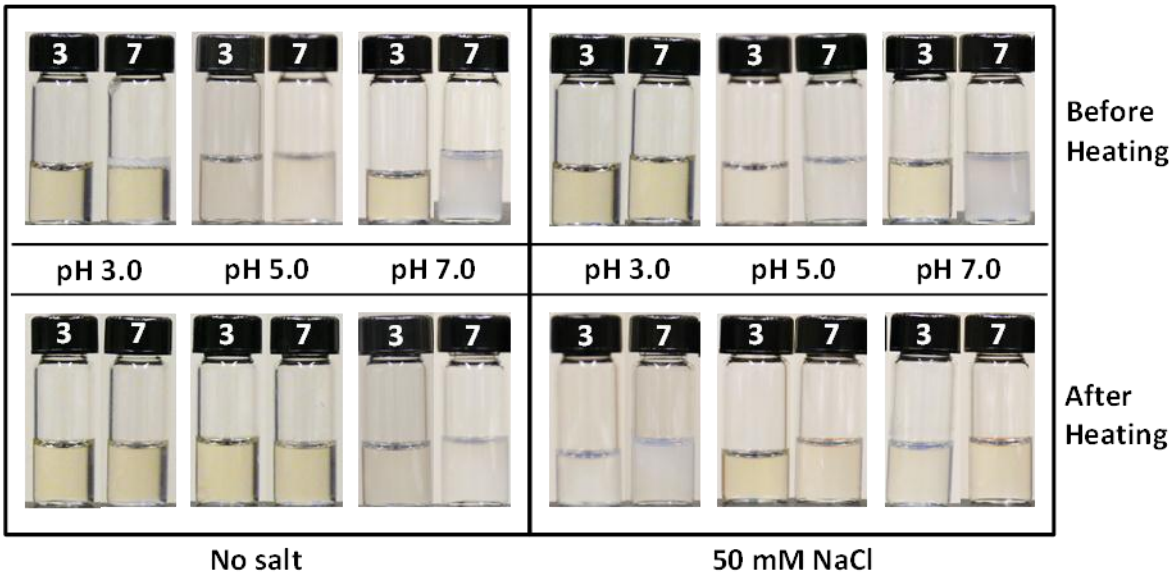

Figure 2-3. Photographs of thymol-containing dispersions before and after heating at 80°C for 15 min. Dispersions were hydrated with 5 %w/v spray dried capsules prepared according to formulations 3 and 7, labeled on vials, in Table 2-1 and adjusted to pH 3.0, 5.0 and 7.0, with 0 and 50 mM NaCl, before heating.

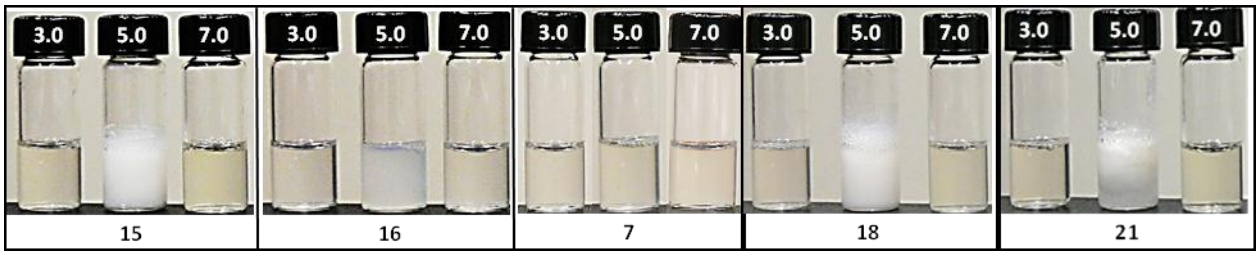

Figure 2-4. Photographs of thymol-containing dispersions after heating at 80°C for 15 min. Dispersions were hydrated with 5 %w/v spray dried capsules prepared according to formulations 15, 16, 7 made with conjugates at 1:2 WPI:MD40, MD100 or MD180 mass ratio respectively, and formulations 18 and 21 made with conjugates at WPI:MD40 at mass ratios 1:2 and 2:1 respectively, in Table 2-1 and adjusted to pH 3.0, 5.0 and 7.0, before heating.

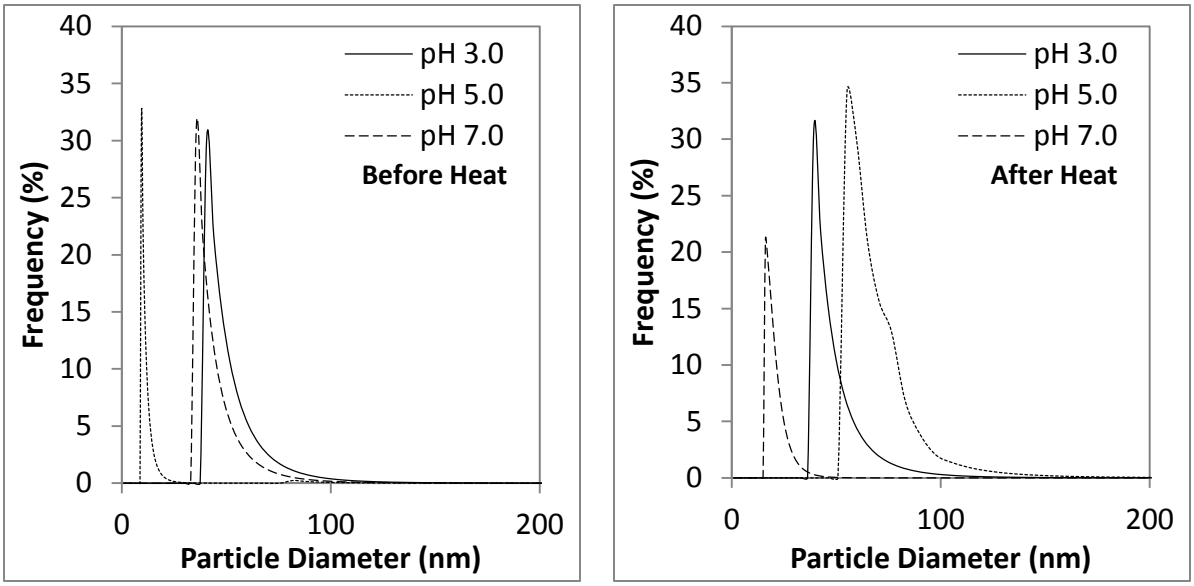

Figure 2-5. Comparison of particle size distribution changes of nano-dispersions before (A) and after (B) heating at 80°C for 15 min. Nano-dispersions contained 5 %w/v capsules prepared by spray-drying emulsions based on formulation 7 in Table 2-1. Dispersions were adjusted to pH 3.0, 5.0 and 7.0 before heating.

Figure 2-6. Photograph of free thymol suspended at 0.525 %w/v at 20°C after 24 h.

3. Nano-dispersing thymol for enhanced dispersibility and antimicrobial effectiveness: Part 2. Antimicrobial activity in growth media

Abstract

Food safety is a continuous challenge within the food industry, due to numerous outbreaks caused by foodborne pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* (*Lm*) and consumer demands for 'natural' foods. Plant essential oils are potent antimicrobials against a broad spectrum of bacteria. However, their lipophilic nature makes it challenging to apply them to foods because of the adverse impacts on quality and antimicrobial efficacy. In the present study, thymol was dispersed in nanocapsules prepared with conjugates of whey protein isolate (WPI) and maltodextrin (MD) at 1:2 WPI:MD180 mass ratio. The antimicrobial efficacy of nano-dispersed and free thymol was tested against *E. coli* O157:H7 ATCC 43889 and 43894, *Salmonella typhimurium* strain 2576, *Lm* strains Scott A and 101 and *Staphylococcus aureus* strains 27708 and SA113 in tryptic soy broth adjusted to pH 6.8 at 35°C for *E. coli* and 32°C for *Lm*. Results indicate that the minimum inhibitory concentration (MIC) for nano-dispersed and free thymol against all strains at pH 6.8 was 0.5 g/l, with the exception of *E. coli* ATCC 43889 and *Lm* strain Scott A which were inhibited by 0.3 g/l free oil. Nano-dispersed and free thymol were further tested for bacterial inhibition of *E. coli* and *Lm* at pH 5.5 and 3.5 in tryptic soy broth at 35, 32, 25 and 4°C. Results indicate that *Lm* was completely inhibited by 0.5 g/l nano-dispersed thymol at pH 5.5 after 3 h, while *E. coli* showed 1.0-3.0 log CFU ml^{-1} reduction after 48 h. At pH 3.5, *Lm* controls did not grow; but, *E. coli* survived. Both strains were inhibited by 0.5 g/l nano-dispersed thymol after 3 h. No significant effect of temperature was found on thymol activity. The current technology produced clear nano-dispersions of thymol, with promising antimicrobial activity against a broad spectrum of foodborne pathogens.

Keywords: lipophilic antimicrobial, thymol, nano-dispersion, pH, temperature, foodborne pathogens

3.1. Introduction

The food industry continually faces challenges concerning microbiological contamination of food products. One of the major consequences include reduction in quality of food products caused by spoilage microorganisms such as common yeasts and molds, thus reducing consumer acceptance and causing heavy economic losses to the industry and consumers. However, the more serious concern is that regarding consumer health. Foods may be contaminated with pathogenic bacteria such as *Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella typhimurium* and *Listeria monocytogenes* (*Lm*), post-pasteurization, during handling, storage or just prior to consumption of foods. It is know that some *E. coli* strains are capable of surviving and growing in low pH and some *Lm* strains at refrigerated temperatures, eventually causing infections and gastrointestinal illness (Solomakos and others 2008). The number of foodborne illness outbreaks in the last few decades worldwide is the driving force behind innovation in strategies to control microbial growth.

Traditionally, several approaches have been adopted in food preservation such as aseptic packaging, physical sanitization and use of chemical additives in foods (Weiss and others 2009; Donsì and others 2011). There are several reports on the use of bacteriocins, salts, organics acids to retard microbial growth or kill microorganisms in foods (Guarda and others 2011). However, these additives are faced with inactivation due to enzymes, loss of antimicrobial activity due to interactions with food constituents, or inefficiency due to microbial resistance (Ghalfi and others 2007). Additionally, there is increasing consumer demand for minimally processed, safe and shelf-stable foods with fewer synthetic additives, thus leading to efforts in developing and using natural antimicrobial ingredients (Tippayatum and Chonhenchob 2007).

Plant essential oils (EO) from thyme (thymol), oregano (carvacrol) and clove (thymol) are among the best candidates for natural food antimicrobials. Thymol is a nontoxic, GRAS ingredient (Arfa and others

2007), a major EO component of an aromatic plant 'thyme' (*Thymus vulgaris*), traditionally used as flavoring in culinary applications (Baranauskiene and others 2006). It is well established that thymol possesses antimicrobial activity against a broad spectrum of gram negative and positive bacteria (Burt 2004; Burt and Reinders 2003; Tippayatum and Chonhenchob 2007; Solomakos and others 2008). There are several reports on the use of thymol against *Lm, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, E. coli, Salmonella enterica, Salmonella typhimurium* (Pei and others 2009; Tohidpour and others 2010), *Pseudomonas aeruginosa* and *Campylobacter jejuni* (Friedman and others 2004) and some fungi (Burt 2004).

However, there are some challenges involved in the application of thymol to foods, the primary one being its intense herbal aroma, restricting the levels at which it can be incorporated into foods without adversely affecting sensory properties. Thymol, being lipophilic, has low solubility in water, thus making it difficult to disperse uniformly in foods. The phase-separated thymol may be visible, impacting product appearance, especially that of transparent beverages. Relatively high vapor pressure of thymol is a challenge in processing, transportation and storage, especially at higher temperatures. Like any other lipophilic bioactive, thymol may bind with food constituents such as proteins, thus reducing the overall effective concentration of the antimicrobial, which calls for the need to use high doses in order to deliver concentrations sufficient to successfully inhibit microbial growth, compromising visual, aroma, and taste qualities of foods (Weiss and others 2009; Liolios and others 2009; Guarda and others 2011).

Encapsulation is a common approach to overcome challenges associated with the delivery of such sensitive compounds. Recently, incorporation of bioactives in nano-particles has attracted much interest because the subcellular-sized nano-particles may enhance cellular absorption via both active and passive mechanisms (Donsì and others 2011), which may increase activity of antimicrobials as compared to delivery in microcapsules. Nano-dispersed plant essential oils have increased solubility and surface area

in water-rich phase or liquid-solid interfaces in foods, areas where microorganisms may be preferably located (Weiss and others 2009). Enhanced antimicrobial activity has been observed for carvacrol encapsulated into liposomal delivery systems (Liolios and others 2009) and eugenol dispersed by surfactant micelles (Gaysinsky and others 2005a; Gaysinsky and others 2005b). With limited work on developing nanoscale food materials, reports in literature on encapsulating thymol are even scarce.

Chapter 2 presents a simple, scalable, low energy approach to prepare nano-dispersions that were transparent and heat stable at a thymol concentration higher than its solubility limit. The emulsion-evaporation approach involved preparation of emulsions with an oil phase of thymol in hexane and an aqueous phase with dissolved conjugates prepared with whey protein isolate (WPI) and maltodextrin (MD) at different WPI:MD mass ratios and MD chain lengths, followed by spray drying. Transparent, heat stable nano-dispersions were observed after hydration of spray dried capsules. The objective of the present study was to evaluate the antimicrobial efficacy of nano-dispersed thymol against *E. coli* O157:H7, *Lm, S. aureus* and *S. typhimurium* in growth medium (Tryptic Soy Broth; TSB) at different pH and temperature conditions.

3.2. Materials and Methods

3.2.1. Materials

Thymol (99 %) was purchased from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD180 with an average dextrose equivalent of 18 was obtained from Grain Processing Corporation (Muscatine, IA). TSB, peptone, and agar (chemical grade) were products of Becton, Dickinson and Company (Sparks, MD). Other chemicals such as hexane and methanol were purchased from Fisher Scientific (Pittsburgh, PA).

3.2.2. Nano-dispersion preparation

The optimized conditions identified in Chapter 2 were used for preparing nano-dispersions. Conjugates were prepared by spray drying solutions containing WPI and MD180 at a mass ratio of 1:2, followed by dry heating the spray dried powders at 90°C for 2 h, via Maillard reaction. Thymol was encapsulated using emulsion-evaporation, for which emulsions were prepared by emulsifying an oil phase (with 20 %w/v thymol in hexane) at 10 %v/v volume into an aqueous phase with 11.1 % conjugates, followed by spray drying (Sample 7 in Chapter 2). All spray drying experiments were performed at an inlet temperature of 150°C, feed rate of 6.67 ml/min, air flow rate of 35 m^3/h, 600 kPa compressed air pressure, and a recorded outlet temperature of 80-90°C, using a model B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). The spray dried powders were collected and stored in a freezer at -18°C. Spray dried capsules were hydrated either at various concentrations of thymol in deionized water for 14 h at room temperature. Antimicrobial efficacy of dispersions was tested as described below. Dispersions were measured for size distribution based on dynamic light scattering using Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc. Brea, CA), and volume-length mean particle diameter ($d_{4,3}$) was calculated using Equation 1, where n_i is the number of particles with a particle diameter of d_i:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad \text{.....(1)}$$

3.2.3. Culture preparation

E. coli O157:H7 ATCC 43889 and ATCC 43894, *Lm* strains Scott A and 101, *S. aureus* strain 2576, *S. typhimurium* strains 27708 and SA113 were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville. All cultures were grown in TSB and

stored at -20°C in glycerol as stocks. Working cultures were obtained by inoculating 50 ml TSB with 100 µl stock cultures and incubating for 24 h at 35 or 32°C.

3.2.4. Antimicrobial susceptibility test in Tryptic Soy Broth

Microbial inhibition was studied using time-kill assays. Bacterial cultures were harvested at late logarithmic phase (overnight) and used at approximately 5.0-6.0 log CFU ml^{-1}. A total volume of 25 ml consisting of 12.5 ml of TSB, 10 ml of thymol-containing nano-dispersion or deionized water (used as control), and 2.5 ml of inoculum was used. The final overall thymol concentrations in the TSB mixture were 0.3, 0.5, 0.75 and 1.0 g/l. The mixture was adjusted to pH 6.8, 5.5 and 3.5 using 1 N NaCl and 1 N HCl respectively, and incubated at 35, 32, 25 or 4°C. At regular intervals (0, 3, 6, 12 and 24 h), a bacterial suspension Sample (1.0 or 0.1 ml) was serially diluted in 0.1 % peptone, plated in duplicate (1.0 or 0.1 ml) using tryptic soy agar, incubated for 24 h at optimum growth temperatures, and then enumerated using pour plating for viable count as CFU ml^{-1}. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of thymol inhibiting visible growth of the test microorganisms above the original inoculums approximately 4.0-5.0 log CFU ml^{-1} (inhibition) after 48 h, while the minimum bactericidal concentration (MBC) was defined as the lowest concentration tested where bacterial death (inactivation) was observed after 48 h (Burt 2004). The growth medium with and without bacterial culture was used as positive and negative controls in all treatments.

3.2.5. Data and statistical analysis

Data from four replicate experiments were pooled to calculate the statistical mean and standard error of the mean for each treatment. Data was verified for normality, followed by mixed model analysis of variance (ANOVA, $p<0.05$) using SAS 9.2 (SAS Institute, NC).

3.3. Results and Discussion

3.3.1. Nano-dispersion properties

Conjugates play an important role in the stabilization of essential oil emulsions. The hydrophobic residues in protein molecules anchor the oil droplets during emulsion formation, while the carbohydrate positions its hydrophilic moieties into the aqueous phase after emulsion preparation, thus, forming a steric stabilizing layer on the oil droplet surface and preventing coalescence (Donsì and others 2011). Nano-dispersions prepared from WPI-MD conjugates showed improved physical properties including optical clarity, heat stability and oil particle diameter when compared to controls prepared from non-conjugated mixture of WPI and MD, detailed in Chapter 2. Nano-dispersions were transparent at pH 3.0, 5.0, and 7.0, with 99.5 % particles measuring less than 100 nm (Figure 3-1), corresponding to $d_{4,3}$ of 67, 100 and 58 nm respectively (data presented in Chapter 2).

3.3.2. Antimicrobial activity of thymol against gram-positive and gram-negative pathogens at pH 6.8

It is often challenging to incorporate plant essential oils and their components in foods, because the effective antimicrobial concentrations may exceed levels in food that are acceptable to the consumer (Burt 2004). Thus, it is important to determine the minimum inhibitory and bactericidal concentrations of these antimicrobial compounds and optimize their mode of application, minimize losses and thus establish a balance between the efficacy of the antimicrobial and desirable organoleptic properties of food products (Rivas and others 2010). Table 3-1. 1 presents the MIC and MBC values for thymol contained in the nano-dispersions and as free oil, evaluated for growth inhibition and inactivation activity against different strains of *E. coli* O157:H7, *S. aureus, Lm and S. typhimurium* in TSB. Overall, nano-dispersed thymol demonstrated MIC of 0.5 g/l and MBC of 1.0 g/l which was effective against all gram-positive and gram-negative foodborne pathogens at pH 6.8 (Table 3-1. 1). With a starting inoculum

population of 4.0-5.0 log CFU ml^{-1}, both *E. coli* O157:H7 strains showed 2.0-3.0 log CFU ml^{-1} reduction after 48 h when exposed to 0.5 g/l nano-dispersed thymol at pH 6.8 (Figure 3-2). Similar results for this concentration were observed for *Lm* strains Scott A and 101 (Figure 3-3). (Burt 2004) and Rivas and others (2010) reviewed several studies and summarized that an average thymol concentration of ca. 0.45 g/l is needed for antimicrobial inhibition for *E. coli, S. typhimurium* and *S. aureus*.

While 0.3 g/l nano-dispersed thymol was too low for complete bacterial kill, no growth was observed after 3 h by nano-dispersions and free oil at 1.0 g/l thymol for all strains when dispersions were used (Table 3-1. 2 and Figure 3-3). Free oil demonstrated slightly lower MIC and MBC values as *E. coli* and *Lm* were inhibited at 0.3 g/l and completely killed at 0.5 g/l. (Rasooli 2007) and (Guarda and others 2011) found that 0.25 g/l thymol was effective against *Lm* and *L. innocua.*

Contrary to previously described trends, 0.5 g/l thymol was not a bactericidal concentration for *S. aureus* strains 27708 and SA113 (Figure 3-4) and *S. typhimurium* strain 2576 (Figure 3-5). Nano-dispersed and free thymol showed similar log reduction values after 48 h, with *Staphylococcus* being less susceptible than *Salmonella*. MIC and MBC values of free and nano-dispersed thymol for *Staphylococcus* and *Salmonella* strains and *Lm* strain 101 were 0.5 and 0.75 g/l respectively at pH 6.8 (Table 3-1). The present findings are in line with reports of (Friedman and others 2004; Tippayatum and Chonhenchob 2007; Guarda and others 2011), in that they found that thymol was active against *Lm, E. coli*, *C. jejuni*, *S. enterica* and *S. t*yphimurium at similar concentrations, with indication that *E. coli* showed the highest sensitivity.

Some studies suggest that gram-negative bacteria are more resistant than the gram-positive ones when different essential oils were tested, due to the protective outer membrane containing lipopolysaccharides (LPS) restricting the diffusion of hydrophobic compounds (Burt 2004). The present

findings are in line with studies that reported a variation in sensitivity and susceptibility of different bacterial pathogens, including *E. coli* O157:H7 to thymol (Falcone and others 2007; Rivas and others 2010). It may be possible that the chemical structure of thymol enable it to disintegrate the outer membrane of gram-negative bacteria, releasing the lipopolysaccharides, and thus increasing the permeability of the cytoplasmic membrane of the cell and resulting in similar effects as observed in gram-positive bacteria (Guarda and others 2011).

3.3.3. Effect of temperature on antimicrobial activity of thymol at pH 5.5 and 3.5

Temperature and pH are among many factors that affect the microbial growth and survival. Some strains of *E. coli* O157:H7 are known to be acid tolerant, while *Lm* has been identified to grow slowly at lower temperatures (Rivas and others 2010). Thus, the antimicrobial efficacy of nano-dispersed thymol in TSB adjusted to pH 5.5 (Figure 3-6) and 3.5 (Figure 3-7) was evaluated against *E. coli* O157:H7 strains ATCC 43889 and 43894 and *Lm* strains Scott A and 101 at respective optimum growth (35 and 32°C), ambient (25°C) and refrigerated (4°C) temperatures. *Lm* controls did not grow at pH 3.5 and thus these results are not discussed here. Overall, the antimicrobial resistance of bacteria followed the order of *E. coli* O157:H7 ATCC 43894 > *E. coli* O157:H7 ATCC 43889 and *Lm* strain 101 > *Lm* strain Scott A. 1.0 g/l nano-dispersed thymol completely inhibited all bacteria after 3 h for all pH and temperature treatments, while 0.3 g/l nano-dispersed thymol was ineffective at inhibiting all strains, leading to increase in populations of *E. coli* O157:H7 and *Lm* by 1- and 3-log CFU ml^{-1} respectively, after 48 h (Figure 3-6 and Figure 3-7).

At 0.5 g/l thymol, at pH 5.5, populations of all strains (Figure 3-6) showed bacteriostatic behavior at 25, 32/35°C and were reduced by ca. 3 log CFU ml^{-1} at 4°C after 48 h. Some studies reported bactericidal activity of encapsulated thymol at ≥ 0.25 g/l at 4 and 10°C, and at 2 g/l at 15 and 37°C against *E. coli* O157:H7 (Rivas and others 2010) and similar bacteriostatic values for *Lm* at 4 and 25°C (Burt and

Reinders 2003). At pH 3.5, *E. coli* O157:H7 was inhibited after 6 h at all temperatures; while *Lm* was inhibited after 3 h at 25, 32 and 35°C and after 6 h at 4°C, by 0.5 g/l nano-dispersed thymol (Figure 3-7). The present findings are in line with other reports that suggested increased susceptibility of *S. aureus, Lm* and *E. coli* O157:H7 to rosemary and thyme essential oils at lower pH (Juven and others 1994; Burt and Reinders 2003; Raybaudi-Massilia and others 2006). This may be due to increased contact with bacterial cell structure, facilitating better binding of the thymol molecules to cellular proteins, in turn bacterial permeability and inhibition by antimicrobial (Rivas and others 2010). Thymol has limited solubility of less than 1.0 g/l at 20°C (ChemIDPlus, 2011), which further reduces with a decrease in temperature. Although the present findings did not show a significant temperature effect, the use of nano-dispersions looks promising as new, natural antimicrobial ingredients in foods, without affecting appearance, especially in clear beverages, at low temperatures.

3.4. Conclusions

The current research confirms the efficacy of nano-dispersed thymol against *E. coli* O157:H7, *Lm, Staphylococcus aureus and Salmonella typhimurium.* The hereby presented novel nanoscale antimicrobial delivery system is effective at 4-35°C from pH 3.5-6.8 in TSB. Further research can be explored to apply this system to complex food matrices such as apple cider and milk to confirm its potential and viability in beverage applications.

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Appendix

Table 3-1. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of thymol (g/l) required for inhibition and inactivation of foodborne pathogenic bacteria in tryptic soy broth at pH 6.8.

Bacteria Species	Escherichia coli O157:H7				Listeria monocytogenes				Staphylococcus aureus		Salmonella typhimurium			
Strain	ATCC 43889		ATCC 43894		Scott A		101		SA113		27708		2576	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Nano-dispersed[a] Thymol (g/l)	0.50	0.75	0.50	0.75	0.50	0.75	0.50	0.75	0.50	1.00	0.50	1.00	0.50	1.00
Free Thymol (g/l)	0.30	0.50	0.30	0.50	0.30	0.50	0.50	0.75	0.50	0.75	0.50	0.75	0.50	0.75

[a]Nano-dispersions were made using conjugates at 1:2 WPI:MD180 mass ratio.

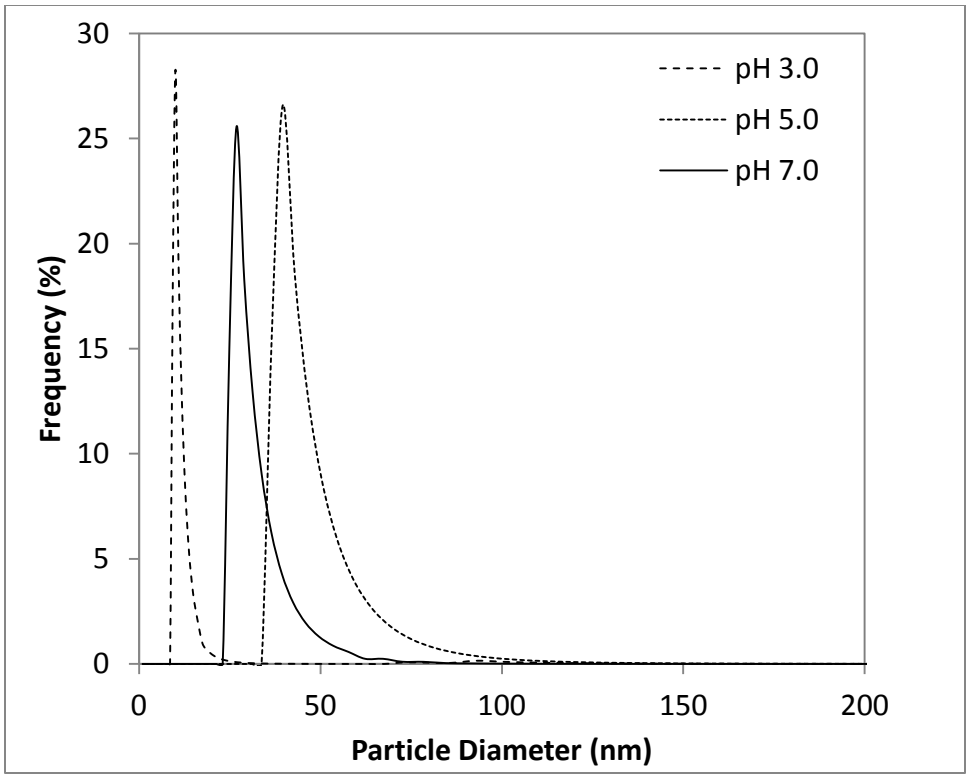

Figure 3-1. Particle size distribution of thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, at pH 3.0, 5.0 and 7.0. Values represent means from four measurements, two measurements from each of two replicates.

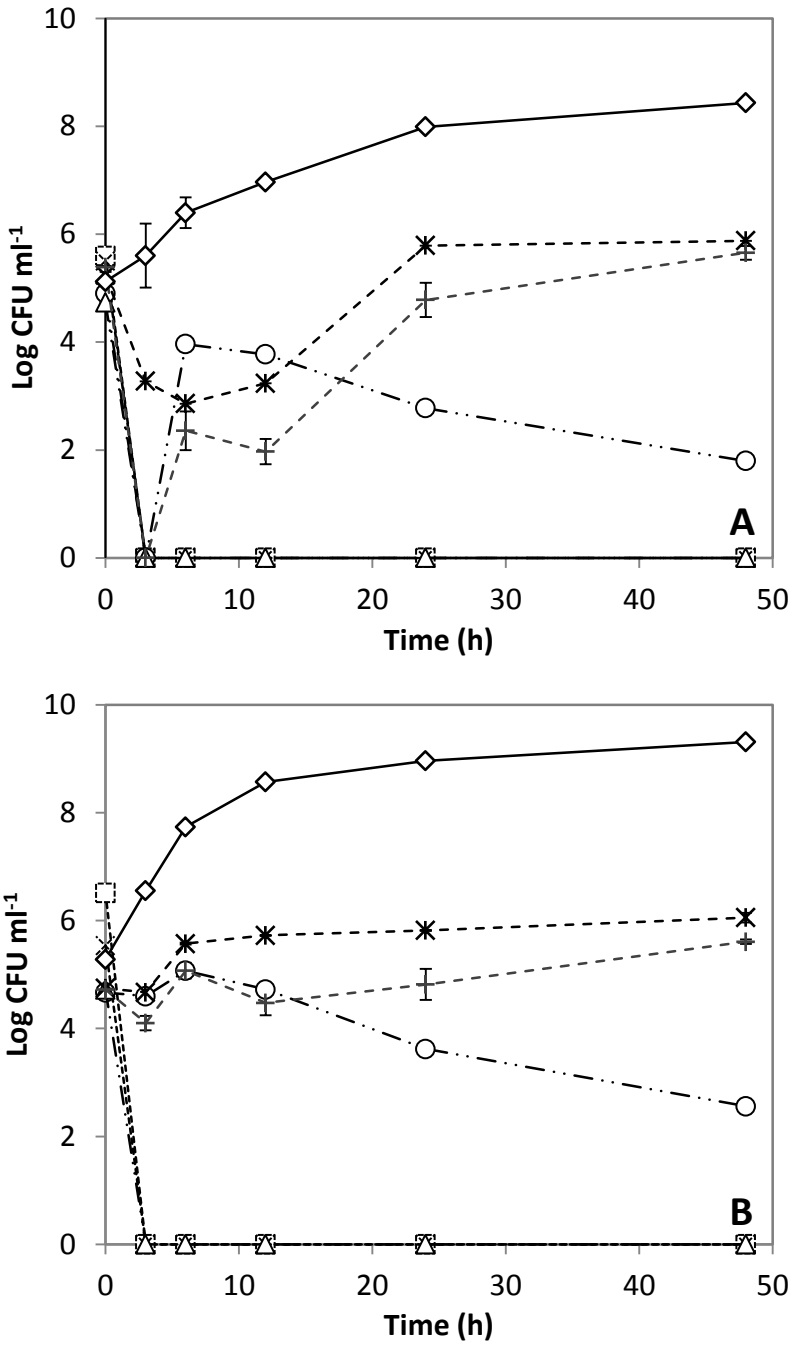

Figure 3-2. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in tryptic soy broth adjusted to pH 6.8, against *Escherichia coli* O157:H7 ATCC 43889 (A) and 43894 (B) at 35°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed thymol: (□) 1 g/l, (○) 0.5 g/l, (✳) 0.3 g/l; Free thymol: (×) 1 g/l, (+) 0.5 g/l, (△) 0.3 g/l and (◇) control of bacterial culture with no thymol.

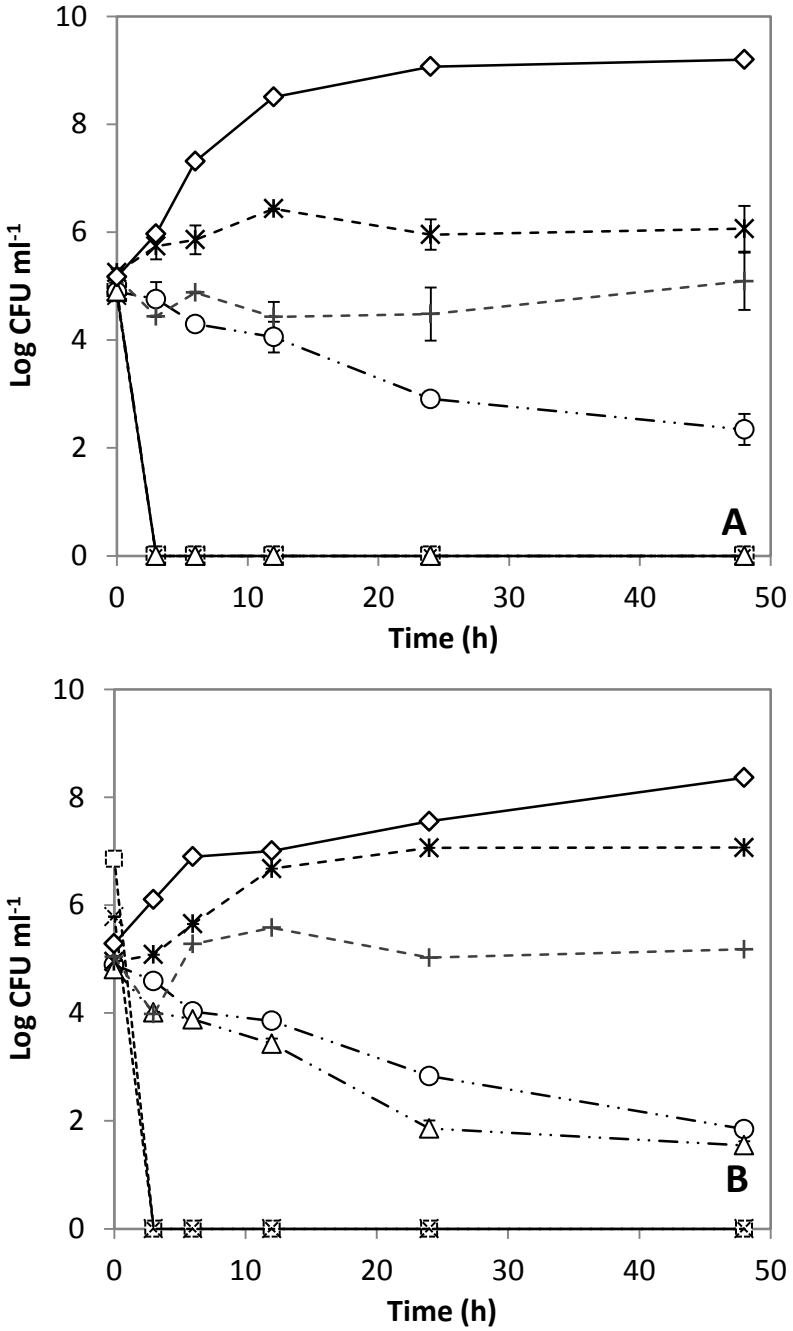

Figure 3-3. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in tryptic soy broth adjusted to pH 6.8, against *Listeria monocytogenes* strain Scott A (A) and 101 (B) at 32°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 3-2.

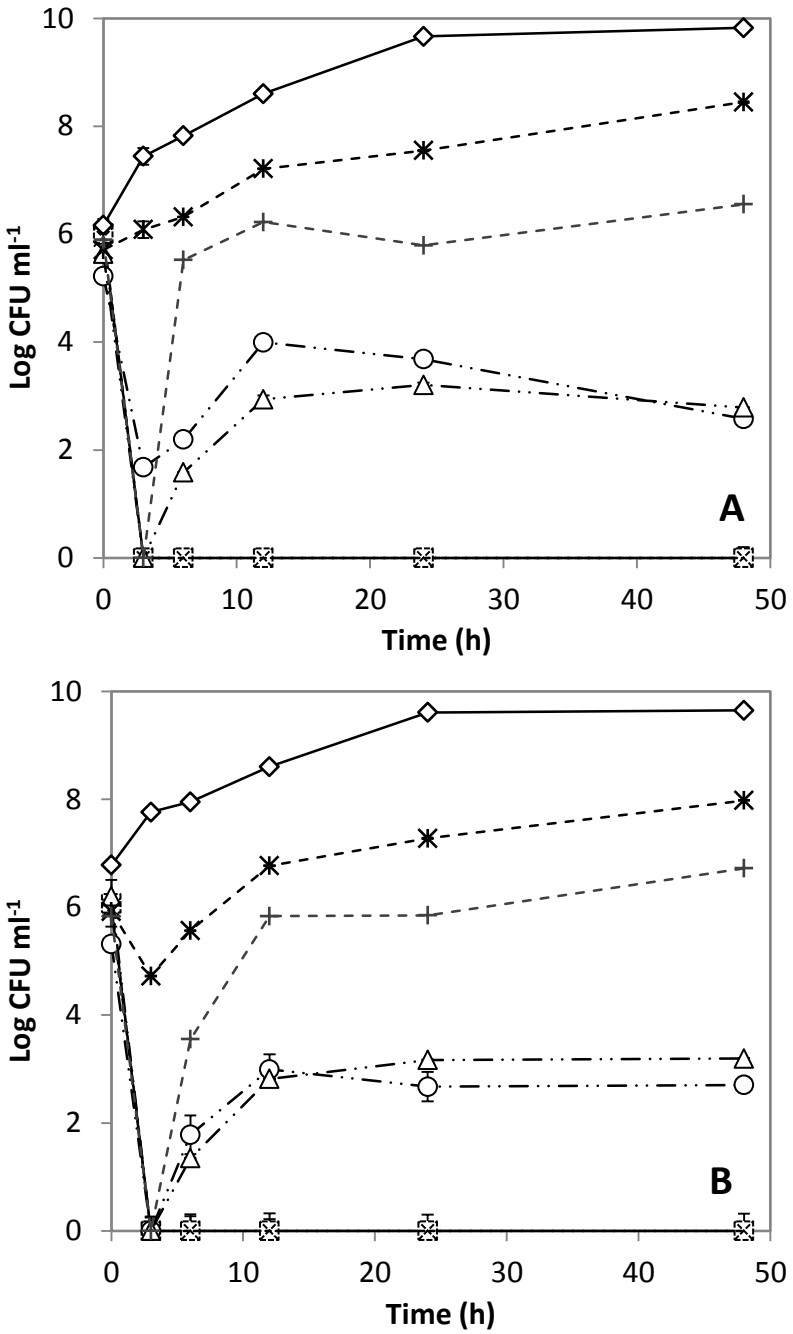

Figure 3-4. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in tryptic soy broth adjusted to pH 6.8, against *Staphylococcus aureus* strains SA113 (A) and 27708 (B) at 35°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 3-2.

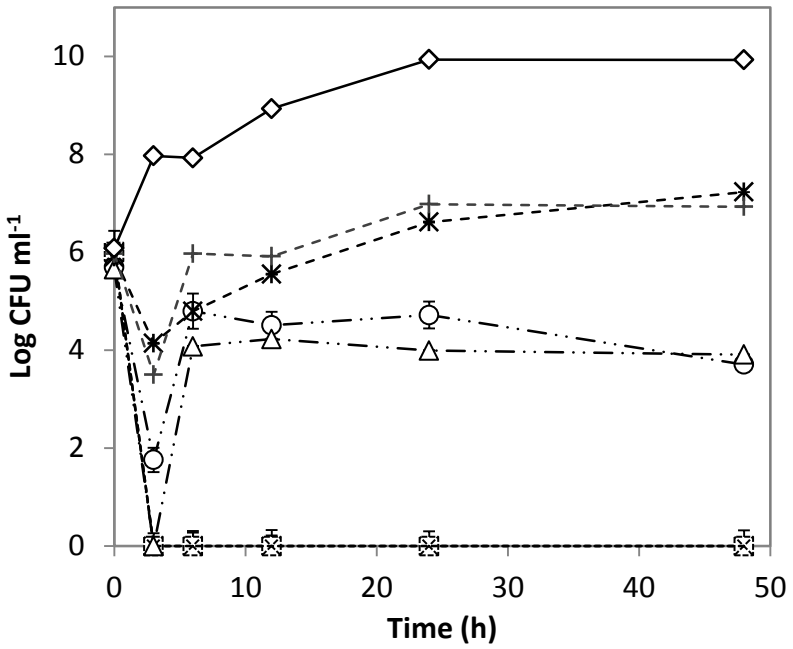

Figure 3-5. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in tryptic soy broth adjusted to pH 6.8, against *Salmonella typhimurium* strain 2576 at 35°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 3-2.

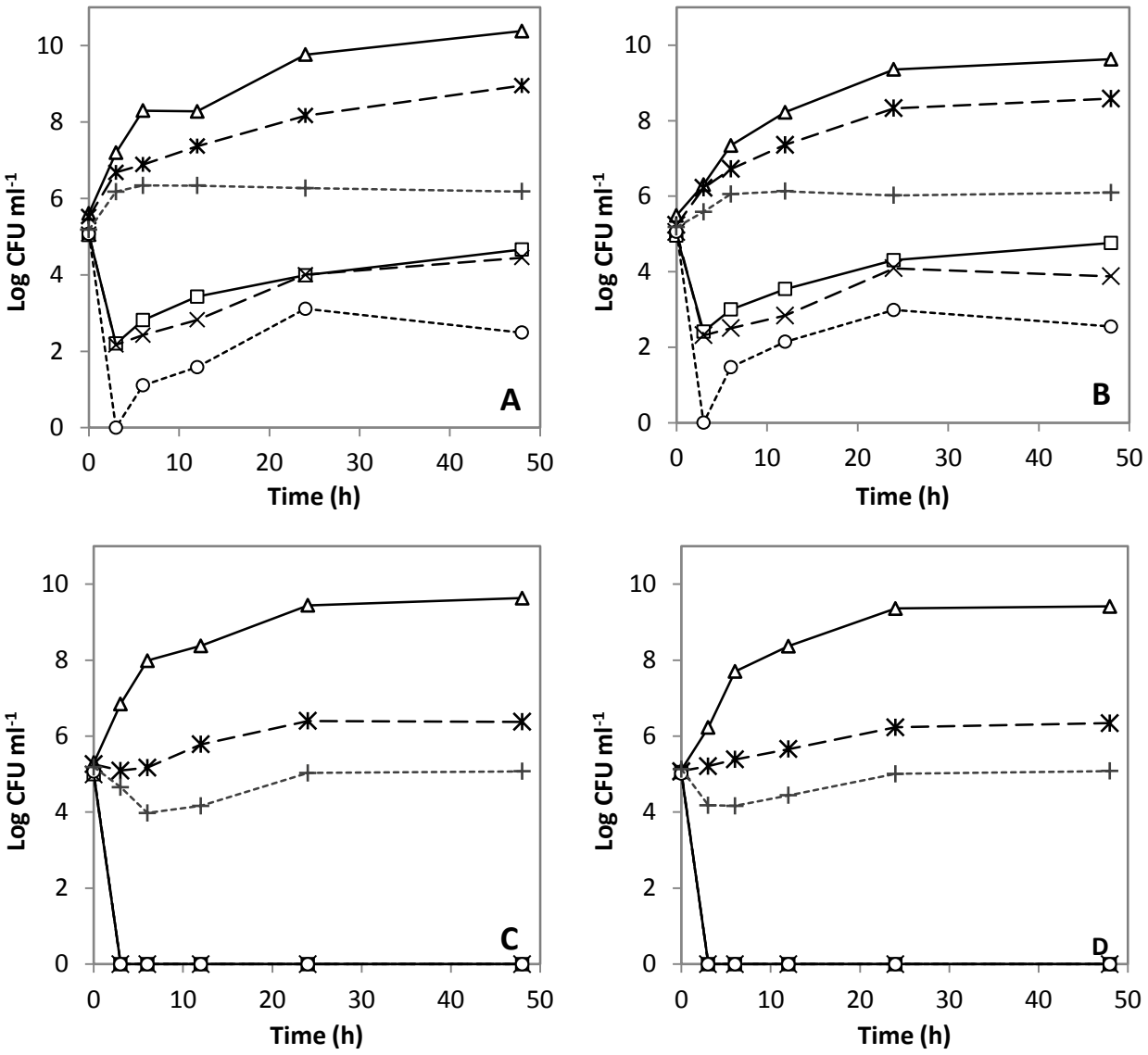

Figure 3-6. Antimicrobial activity of thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.5 g/l in tryptic soy broth adjusted to pH 5.5, against *Escherichia coli* O157:H7 ATCC 43889 (A) and ATCC 43894 (B), and *Listeria monocytogenes* strains Scott A (C) and 101 (D). Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed thymol: (□) 35 or 32°C, (×) 25°C and (○) 4°C; Control of bacterial culture with no thymol (△) 35°C for *E. coli* or 32°C for *Lm*, (✳) 25°C and (+) 4°C.

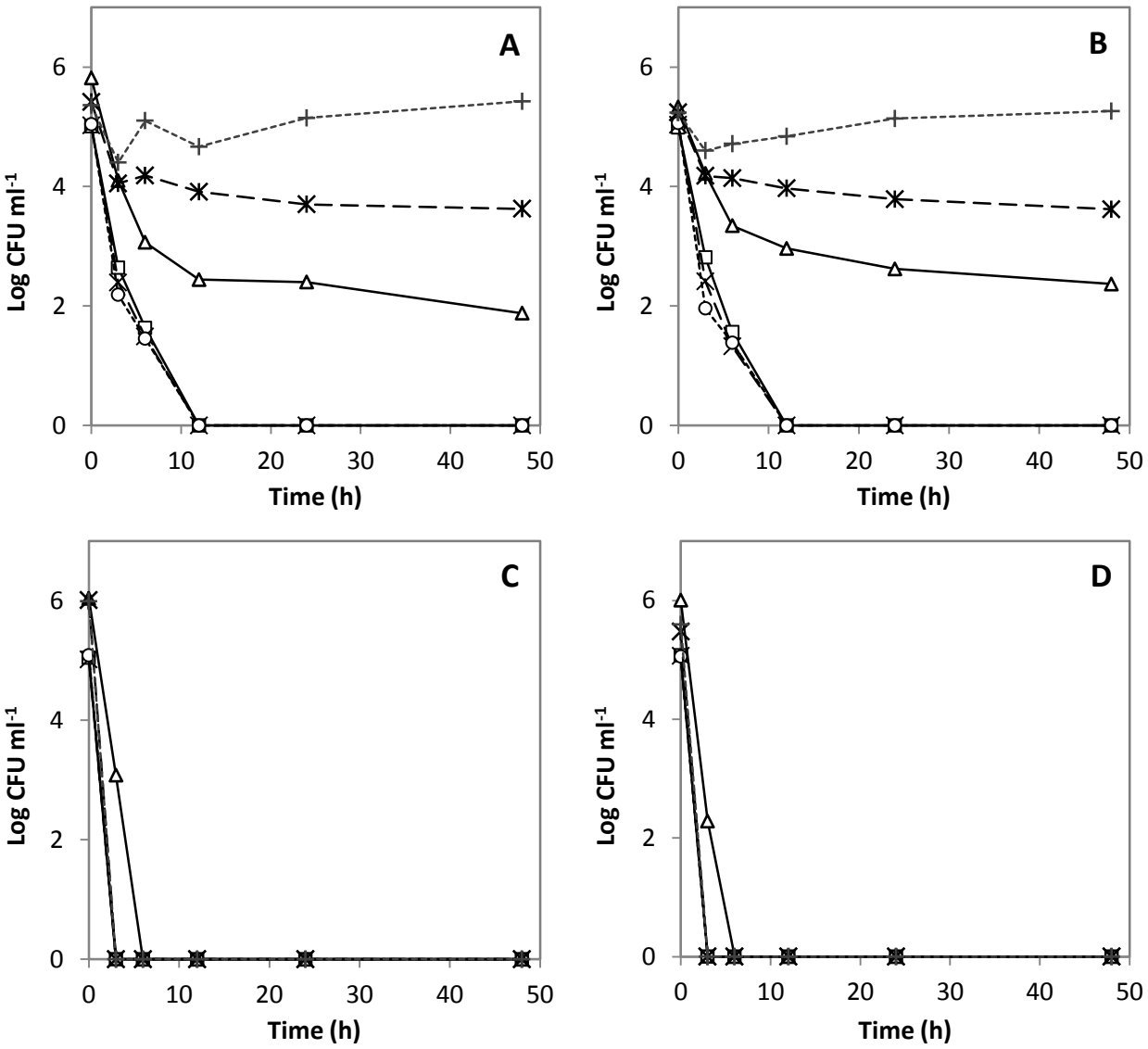

Figure 3-7. Antimicrobial activity of thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.5 g/l in tryptic soy broth adjusted to pH 3.5, against *Escherichia coli* O157:H7 ATCC 43889 (A) and ATCC 43894 (B), and *Listeria monocytogenes* strains Scott A (C) and 101 (D). Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 3-6.

4. Nano-dispersing thymol for enhanced dispersibility and antimicrobial effectiveness. Part 3. Antimicrobial activity in model food systems

Abstract

Foodborne outbreaks caused by pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes (Lm)* are growing concerns in the food industry. Moreover, consumer demand for minimally processed foods with few chemical additives has sparked an interest in the use of natural plant essential oils as antimicrobial agents. However, essential oils are marginally soluble in water, making it challenging to evenly disperse them to foods and causing undesirable changes, and tend to bind with food lipid and protein components, resulting in lowered antimicrobial efficacy. In the previous study, transparent dispersions of thymol encapsulated with conjugates of whey protein isolate (WPI) and maltodextrin (MD) were created. In the current study, nano-dispersed and free thymol were compared for their antimicrobial efficacy against *E. coli* O157:H7 ATCC 43889 and 43894, and *Lm* strains Scott A and 101 in apple cider and 2 % reduced fat milk. Apple cider was adjusted to pH 5.5 and 3.5 and antimicrobial tests were performed at 0.3, 0.5, 0.75 and 1.0 g/l thymol concentrations at 35, 32, 25 and 4°C. Overall, 0.5 and 1.0 g/l thymol in nano-dispersion and free form were inhibitory and bactericidal, respectively, against bacterial strains in all treatment conditions. At pH 5.5, 0.5 g/l nano-dispersed thymol was bacteriostatic against *Lm* and *E. coli* up to 48 h. At pH 3.5, *Lm* controls did not survive beyond 12 h; but, *E. coli* survived and was inhibited by 0.5 g/l nano-dispersed thymol after 12 and 48 h in apple cider. *E. coli* strains were significantly sensitive to 4°C and pH 3.5 ($p<0.05$). When bacteria were tested in 2 % reduced fat milk at 35 or 32°C, nano-dispersed and free oil demonstrated inhibition at 4.5 g/l. Thus, the current technology seems to be promising and novel, enabling thymol-containing nano-dispersions that are not only clear but, also effective against pathogens in food applications, especially clear beverages.

Keywords: lipophilic antimicrobial, thymol, nano-dispersion, pH*,* temperature*, Escherichia coli* O157:H7*, Listeria monocytogenes,* apple cider, milk

4.1. Introduction

Preservation of food quality and consumer health are two of the primary concerns today, considering the frequent occurrence and severity of foodborne outbreaks caused by *Escherichia coli* O157:H7 and *Listeria monocytogenes (Lm).* These are most commonly addressed by pasteurization of foods, use of chemical sanitizing agents and clean handling practices. However, consumers are increasingly demanding minimally processed foods with 'natural' flavors and few synthetic additives (Baskaran and others 2010). Unpasteurized apple cider and raw milk are examples of such products. Typically considered a 'low risk' food due to acidic pH of 4.0 (Friedman and others 2004; Miller and Kaspar 1994), apple cider has seen multiple outbreaks from contamination by *E. coli* O157:H7 and related *E. coli* strains from 1991-1998 (Ukuku and others 2009). Deaths resulted from hemorrhagic colitis and hemolytic uremic syndrome caused by the pathogen. There is concern regarding its ability to cause severe illness with low infectious dose (Lin and others 1996). Investigations have revealed that some *E. coli* O157:H7 strains are acid tolerant or resistant (Leyer and others 1995; Benjamin and Datta 1995; Jordan and others 1999; Hsin-Yi and Chou 2001)and able to survive refrigeration temperatures for one to two weeks (Roering and others 1999). Although *Lm* has not been directly linked to apple cider outbreaks, a case of *Listeriosis* was reported in 2008, and the pathogen was isolated from unpasteurized apple juice, due to possible cross contamination of apple cider from milk, indicating a concern for future outbreaks (Bock 2008). It is also suggested that the widespread occurrence, relative heat resistance and ability to grow under refrigerated/frozen conditions makes *Lm* an appropriate target organism for investigation of innovative preservative strategies (Roering and others 1999).

The industry is exploring new ways to develop effective natural antimicrobials to extend product shelf life. Plant essential oils (EO) are being evaluated as alternative, natural antimicrobial ingredients. EO are

classified as generally regarded as safe (GRAS) and are responsible for the aroma and flavor of many spices and herbs (Arfa and others 2007). Several reports confirm the antibacterial and antifungal properties of EO with the most effective ones coming from thyme (thymol), oregano (carvacrol) and clove (eugenol) (Baranauskiene and others 2006). Thymol, the major antimicrobial component from an aromatic plant 'thyme' (*Thymus vulgaris*), is a hydrophobic, phenolic compound able to bind with bacterial proteins, which results in cell membrane disintegration and permeability, thus making it a potent, broad-spectrum antimicrobial (Falcone and others 2007). However, its hydrophobicity makes its application in foods challenging because of the difficulty of even dispersion in food matrices. Binding of thymol with hydrophobic constituents (lipids and proteins) of complex food systems, such as milk, reduces its antimicrobial effectiveness. Phase separation of thymol due to kinetic instability of oil droplets impacts visual quality and antimicrobial availability. Being a volatile essential oil, it also has an intense spicy/medicinal aroma particularly upon heating, thus adversely affecting sensory properties of foods upon incorporation in free form or at higher concentrations (Burt 2004; Weiss and others 2009; Liolios and others 2009; Guarda and others 2011)

Challenges in dealing with such compounds are usually overcome by dissolving them in a solvent or solvent mixture with decreased polarity, or encapsulating them in emulsions for better dispersibility. Droplets of the oil of interest are kinetically stabilized in a continuous aqueous phase by emulsifiers. Milk proteins are among the best natural surfactants for food applications (Ly 2008). Interfacial properties of milk proteins, particularly whey proteins, can be improved by conjunction with more hydrophilic oligosaccharides and polysaccharides (Kato 2002). Compared to emulsions stabilized by proteins, the oligo- or polysaccharide moiety of conjugates on droplet surfaces provides extra stability for the emulsion. As such, conjugates of whey protein isoate (WPI) and maltodextrin (MD) have been

applied to prepare emulsions of tomato volatiles (Christiansen and others 2011) and conjugated linoleic acid (Choi and others 2010) to minimize degradation and improve dispersibility in foods.

Encapsulation of lipophilic antimicrobials in nanoscale systems has been attempted to obtain small oil droplets with low light scattering ability and increased surface area for bacterial contact (Weiss and others 2009). Proof of concept has been given by liposomal delivery systems containing carvacrol (Liolios and others 2009) and nanoscale surfactant micelles containing eugenol (Gaysinsky and others 2005) when tested in growth media and milk.

In the present study, there is interest in the use of nano-encapsulated thymol for beverage applications. The objective was to evaluate the efficacy of thymol-containing WPI-MD conjugate based dispersions in inhibition of *E. coli* O157:H7 and *Lm* in model food systems such as apple cider and 2 % reduced fat milk at different pH and temperature conditions.

4.2. Materials and methods

4.2.1. Materials

Thymol (99 %) was obtained from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD180, with an average dextrose equivalent of 18, was a product of Grain Processing Corporation (Muscatine, IA). TSB, peptone, and agar (chemical grade) were purchased from Becton, Dickinson and Company (Sparks, MD). Other chemicals such as hexane and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Apple cider was a product of M.H. Zeigler and Sons, LLC (Lansdale, PA). Ultra high temperature (UHT) pasteurized organic milk with 2 % fat was purchased from Private Selection, a brand of the Kroger company (Cincinnati, OH).

4.2.2. Nano-dispersion preparation

An emulsion-evaporation process was used to encapsulate thymol. An oil phase (with 20 %w/v thymol in hexane) was emulsified at 10 %v/v volume into an aqueous phase with 11.1 % conjugates, followed by spray drying. Conjugates were previously prepared by spray drying solutions containing WPI and MD180 at a mass ratio of 1:2, followed by dry heating the spray dried powders at 90°C for 2 h for conjugation (Maillard reaction). All spray drying experiments were performed using a model B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland) at inlet temperature of 150°C, feed rate of 6.67 ml/min, 600 kPa compressed air pressure, 35 m^3/h air flow rate, and a recorded outlet temperature of 80-90°C. The spray dried capsules were collected and stored in a freezer at -18°C. Spray dried capsules were hydrated either at 5 %w/w or to a specific concentration of thymol (%w/v) in deionized water for 14 h at room temperature and dispersions were used in antimicrobial tests respectively, described below. Dispersions were measured for size distribution based on dynamic light scattering using Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc. Brea, CA), and volume-length mean particle diameter ($d_{4,3}$), where *n* is particle number and *d* is particle diameter, was calculated using Equation 1:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad \text{.....(1)}$$

4.2.3. Culture preparation

Stock cultures of *E. coli* O157:H7 ATCC 43889 and ATCC 43894, *Lm* strains Scott A and 101 were obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville. All

cultures were grown in TSB and stored at -20°C in glycerol as stocks and 100 µl were inoculated in 50 ml TSB and incubated for 24 h at 35 or 32°C to obtain working cultures.

4.2.4. Antimicrobial susceptibility test in model food systems

Inhibition of *E. coli* and *Lm* was studied in apple cider and milk using time-kill assays. Both apple cider and milk Samples were used within 2-3 h but, always within 24 h after opening the container. The bacterial cultures were harvested at late logarithmic phase (overnight) and diluted to approximately 5.0-6.0 log CFU ml^{-1} for use. A total volume of 25 ml consisting of 12.5 ml of apple cider or milk, 10 ml of thymol-containing nano-dispersions or deionized water (as a control), and 2.5 ml of inoculum was used. Nano-dispersions were used such that the mixture contained an overall final thymol concentration of 0.3, 0.5, 0.75 or 1.0 g/l thymol for apple cider and 4.5 g/l for milk. The mixture was adjusted to pH 6.8 for milk treatments and to pH 5.5 and 3.5 for apple cider treatments using 1 N HCl or 1 N NaOH, followed by incubation at 35, 32, 25 and 4°C. At regular intervals (0, 3, 6, 12 and 24 h), a bacterial suspension Sample (1.0 or 0.1 ml) was serially diluted in 0.1 % peptone, plated in duplicate (1.0 or 0.1 ml) using tryptic soy agar, incubated for 24 h at optimum growth temperatures, and then enumerated using pour plating for viable count as CFU ml^{-1}. The growth medium with and without bacterial culture was used as positive and negative controls in all treatments.

4.2.5. Statistical analysis

Values presented were data from four replicate experiments pooled to calculate the statistical mean and standard error of the mean for each treatment. The mixed model analysis of variance (ANOVA, $p<0.05$) test was used to analyze data, using SAS 9.2 (SAS Institute, NC).

4.3. Results and Discussion

4.3.1. Nano-dispersion properties

The nano-dispersions containing thymol and conjugates of whey proteins with MDs showed improved physical properties, detailed in Chapter 2. The dispersions were transparent at pH 3.0, 5.0, and 7.0, corresponding to $d_{4,3}$ of 67, 100 and 58 nm respectively, and absorbance values at 600 nm all below 0.5 (Table 4-1). It is well established that conjugation of proteins and carbohydrates can improve emulsifying properties. This occurs because the protein molecules provide hydrophobic residues which anchor the oil droplets during emulsion formation, while the hydrophilic moieties of the carbohydrate positions themselves into the aqueous layer after emulsion preparation thus forming a steric stabilizing layer at the oil and water interface, and preventing oil droplets from coalescing (Donsì and others 2011; Akhtar and Dickinson 2007).

4.3.2. Effect of pH on antimicrobial activity of thymol in apple cider

The antimicrobial efficacy of 0.3-1.0 g/l thymol in apple cider adjusted to pH 5.5 at optimum incubation temperature for *E. coli* ATCC 43889 and ATCC 43894, *Lm* strains Scott A and 101 is presented in Figure 4-1 and Figure 4-2. 1.0 g/l thymol resulted in complete bacterial inhibition at pH 5.5. When 0.5 g/l thymol was used, bacterial populations reduced by 1.0 and 2.0 log CFU ml^{-1} from an initial population of 4.0-5.0 log CFU ml^{-1} at 4 and 25°C, respectively. 0.5 g/l thymol was bacteriostatic at 35°C for *E. coli* and 32°C for *Lm.* Free thymol was generally more effective than nano-dispersed thymol. However, at 4°C, insoluble particles were observed based on visual inspection when free thymol was suspended in water above 0.5 g/l (data not shown). This visual defect, although microbiologically more potent, may impact consumer acceptance, especially for transparent beverages. In contrast, transparent nano-dispersions were

observed at all tested thymol concentrations. Thus, from here on, results will be discussed for nano-dispersed thymol only.

It was observed that bacteria were able to recover only at thymol concentrations below 0.5 g/l at pH 5.5, which indicates that thymol had bactericidal activity rather than bacteriostatic against all tested pathogenic strains at optimum growth temperature (Figure 4-3). In a similar study, Knight and McKellar (2007) demonstrated bactericidal action of >0.25 g/l clove oil, the major active ingredient of which is a phenolic compound eugenol, with a chemical structure similar to thymol. The difference may be because different strains are known to react differently to similar oils, mechanisms of which are yet under-investigated. An initial decrease in counts of the more sensitive strains *E. coli* O157:H7 ATCC 43889 and *Lm* strain Scott A to their lowest detectable levels was observed, possibly due to sudden stress caused by exposure to antimicrobial. Pathogens then recovered and started growing at pH 5.5 after 3 h. This type of rebound phenomenon was reported previously in a similar study (Ghalfi and others 2007) for oregano oil, which contains an isomer of thymol, carvacrol as its main EO. The similar mechanism may have occurred in the case of thymol, where pathogens adapt to sublethal antimicrobial doses, spontaneously develop resistance (Davidson and Harrison 2002), or are protected by food constituents or are able to recover from injury (Gill and Holley 2006).

While 0.3 g/l thymol was ineffective at inhibiting pathogen growth in apple cider, this was the bacteriostatic concentration at pH 5.5 in TSB, reported in Chapter 3. This means that more thymol was needed for the same antimicrobial effect when applied in apple cider. Studies have previously demonstrated that essential oils generally lose some antibacterial properties when used in foods, due to interactions of their phenolic compounds with food components such as lipids, proteins and salts (Cosentino and others 1999; Gill and Holley 2006; Burt 2004). It is now suggested that the

concentrations to be used in actual food systems for preservation can be as high as 100-fold of those recorded in *in vitro* studies (Solomakos and others 2008; Gutierrez and others 2009; Gutierrez and others 2008), which may adversely affect sensory properties and consumer acceptance of foods. However, the nano-encapsulated thymol system is able to deliver higher concentration of antimicrobial without affecting visual clarity of apple cider; but, the impacts on organoleptic properties are to be studied.

Figure 4-4 presents the antimicrobial activity of 0.5 g/l nano-dispersed thymol against *E. coli* O157:H7 ATCC 43889 and 43894, and *Lm* strains Scott A and 101 respectively in apple cider adjusted to pH 3.5 at different temperatures, with temperature impact discussed in the next section. pH played a significant role ($p<0.001$) on the antimicrobial activity of thymol. Both *E. coli* strains demonstrated survival at pH 3.5 and thus acid tolerance, with ATCC 43894 being more resistant than ATCC 43889. It is interesting to note that bacterial populations did not show the decline and recovery immediately following inoculation at pH 3.5 as in the case of pH 5.5. It may be possible that the characteristics responsible for acid resistance in *E. coli* 0157:H7 must be present prior to introduction into the acidic environment and not produced as a sudden response to the drastic change in pH (Miller and Kaspar 1994; Friedman and others 2004). The present findings are in agreement with TSB studies (Friedman and others 2004; Miller and Kaspar 1994) that reported that *E. coli* ATCC 43889 and ATCC 43895 were both acid tolerant strains and the tolerance may have been induced when the growth medium became slightly acidic (from pH 7.4 to 6.0) upon prior overnight incubation of cultures.

However, the low pH of apple cider simultaneously played an advantageous role in the antimicrobial activity, with increased susceptibility of bacterial cells to thymol due to alterations in fatty acid composition, dissolution of lipopolysaccharide (LPS) fractions of bacterial cellular structures. The

resulting sublethal injury to *E. coli* O157:H7, LPS membrane disintegrity, cell permeability and disruption of cellular activity may explain the effectiveness of thymol against the otherwise resistant gram-negative *E. coli* (Knight and McKellar 2007). Similar results were observed by Baskaran and others (2010) for trans-cinnamaldehyde in apple cider with *E. coli*. Although *Lm* controls did not grow at pH 3.5, the bacteria survived and were detectable up to 3.0 log CFU ml^{-1} at 6 h and reduced to undetectable levels at 12 h after pH adjustment. This indicates that it is possible for consumers to get sick within 12 h of contamination of apple cider and similar products with *Lm,* especially higher initial load and more so with more acid tolerant *Lm* strains.

4.3.3. Effect of temperature on antimicrobial activity of thymol in apple cider

Antimicrobial activity of 0.5 g/l nano-dispersed thymol against *E. coli* O157:H7 ATCC 43889, *E. coli* O157:H7 ATCC 43894, *Lm* strains Scott A and 101 in apple cider adjusted to pH 5.5 (Figure 4-3) and 3.5 (Figure 4-4) was studied at incubation temperatures 35/32, 25 and 4°C. Overall, temperature did not have a significant effect on susceptibility of *E. coli* or *Lm* to thymol at pH 3.5 and 5.5. Bactericidal concentration remained at 1.0 g/l, while bacteriostatic concentration was 0.5 g/l for both bacteria at pH 5.5. Bacteria in controls survived better at higher temperatures at pH 5.5. On the contrary, when pH was lowered to 3.5, *E. coli* O157:H7 strains in controls survived better at lower temperatures (4°C), while *Lm* in controls survived only up to 12 h in apple cider. It has been reported that temperature did not affect the acid tolerance of *E. coli* ATCC 43895; but, above 25°C, the sensitivity was slightly increased at an alkaline pH above 8.0, whereas the effect was not obvious at pH 2.0-4.0 (Miller and Kaspar 1994). Bactericidal activity of essential oils against *E. coli* has been shown to increase with incubation temperature when tested at 4, 21, and 37°C in apple juice (Friedman and others 2004). However, the testing times were 5, 60, and 120 min, which may have been too short to capture recovery or stress

response of pathogens upon change in environment conditions. A noteworthy finding in their study was that *E. coli* strains were viable at refrigerated temperatures for up to 2 h and that carvacrol, cinnamaldehyde and thymol retained their antimicrobial properties. Continued inactivation of pathogens by these natural antimicrobials in apple cider during refrigerated storage is promising in developing intervention strategies.

4.3.4. Antimicrobial efficacy of nano-dispersed thymol in milk

Figure 4-5 shows similar antimicrobial activity of 4.5 g/l free and nano-dispersed thymol against *E. coli* O157:H7 ATCC 43889 and *Lm* strain Scott A. As discussed previously, the effective antimicrobial activity may be contributed to its phenolic chemical structure and hydrophobic nature that favors its interaction with bacterial cell components, thus facilitating inhibition of growth of gram-negative and gram-positive bacteria. The need for higher concentrations of antimicrobials in foods than in growth media can be explained by possible loss of oil due to interactions with milk fat globules and proteins (Gutierrez and others 2009; Gutierrez and others 2008). It has been previously reported that essential oils up to 100-fold concentrations than *in vitro* studies may be needed to achieve the same efficacy in actual food systems (Solomakos and others 2008). Higher levels of plant EO may adversely affect sensory properties and lower consumer acceptance of foods. Unfortunately, sensory tests were not conducted in this work. Nevertheless, the nano-dispersed systems provide a unique advantage of being able to deliver poorly water-soluble thymol at concentrations well beyond its solubility limit ca. 1.0 g/l at 20°C; (Syracuse Research Corporation 2011), up to 6.5 g/l, in the form of dispersions, thus expanding its applications in the advancement of value added foods.

4.4. Conclusions

The efficacy of thymol against *E. coli* O157:H7 and of *Lm* was affected by pH and food matrix. Nano-dispersed and free thymol showed similar effectiveness in both model foods. *E. coli* was significantly sensitive to treatment combination of 4°C and pH 3.5, while *Lm* did not survive at such a low pH. Thymol concentration needed to inhibit both pathogens at pH 5.5 showed a 9-fold increase from 0.5 g/l in TSB and apple cider at pH 5.5 to 4.5 g/l in 2 % reduced fat milk at pH 6.8. Additional studies are needed to test the efficacy of lower concentrations of thymol in milk and sensory thresholds for use of higher concentrations of thyme or similar plant essential oils.

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Appendix

Table 4-1. Absorbance and mean particle diameters[a] of thymol-containing nano-dispersions.

pH	Absorbance at 600 nm	Mean particle diameter $d_{4,3}$ (nm)
3.0	0.230 ± 0.03	67 ± 1
5.0	0.453 ± 0.20	100 ± 1
7.0	0.247 ± 0.02	58 ± 1

[a]Values are means ± standard errors of mean from four measurements, two measurements from each of two replicates.

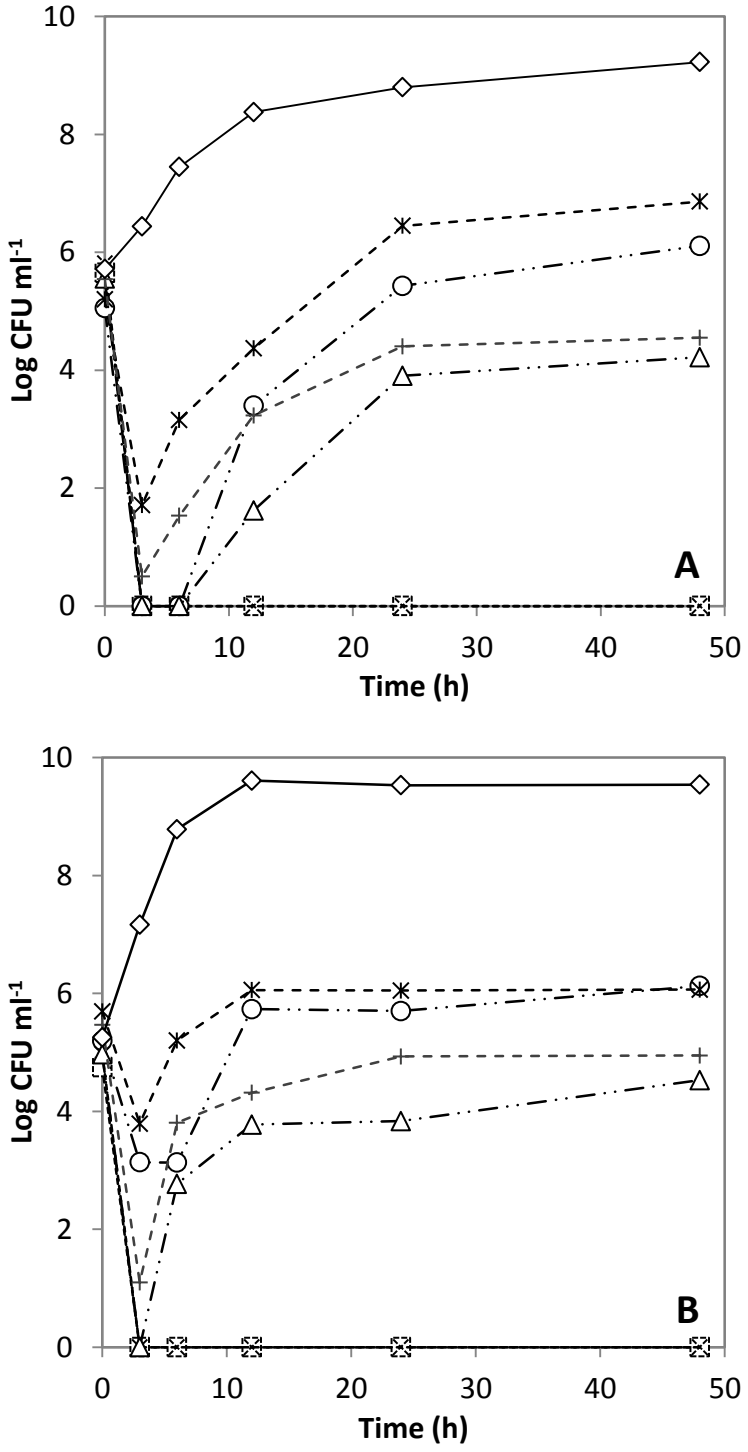

Figure 4-1. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in apple cider adjusted to pH 5.5, against *Escherichia coli* O157:H7 ATCC 43889 (A) and 43894 (B) at 35°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed thymol: (□) 1 g/l, (○) 0.5 g/l, (✳) 0.3 g/l; Free thymol: (×) 1 g/l, (+) 0.5 g/l, (△) 0.3 g/l and Control of bacterial culture with no thymol (◇).

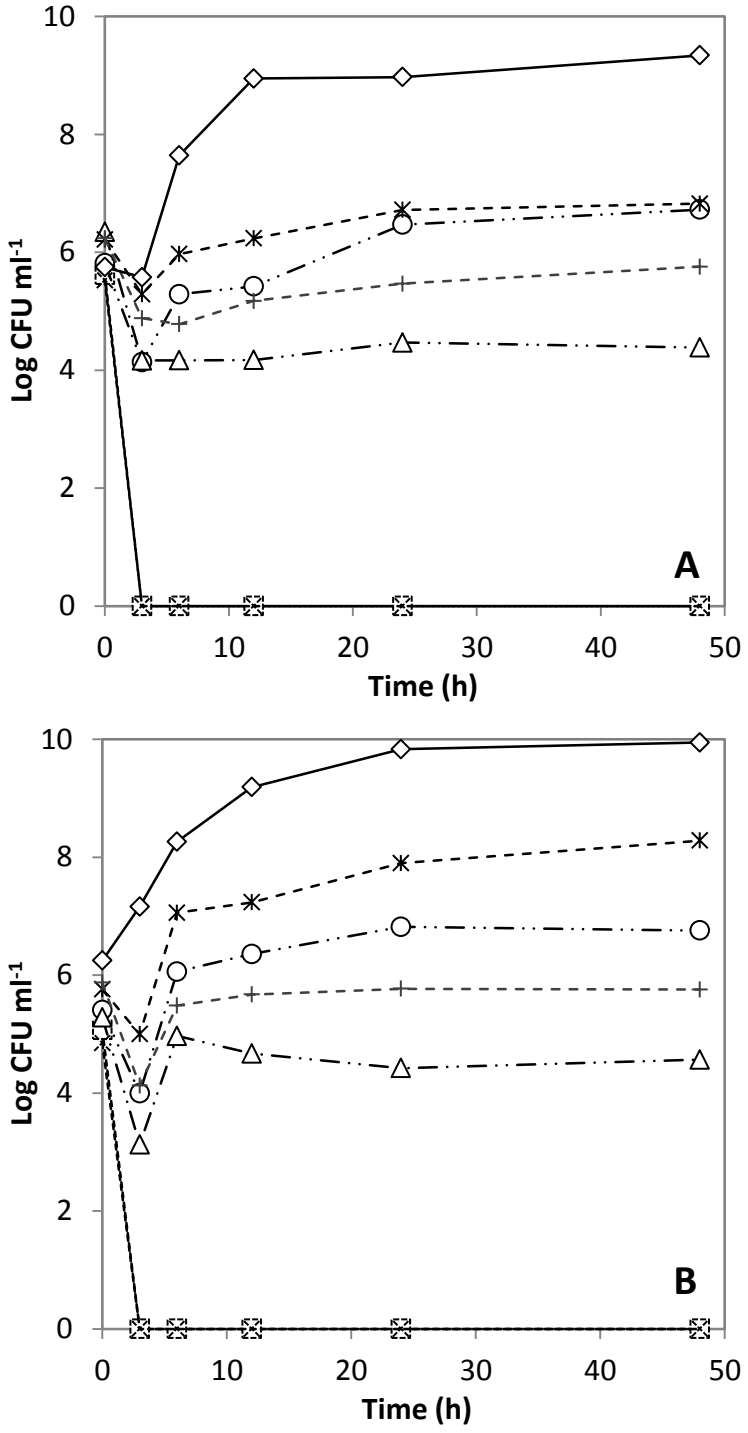

Figure 4-2. Antimicrobial activity of free thymol and thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.3, 0.5 and 1.0 g/l in apple cider adjusted to pH 5.5, against *Listeria monocytogenes* strains Scott A (A) and 101 (B) at 32°C. Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 4-1.

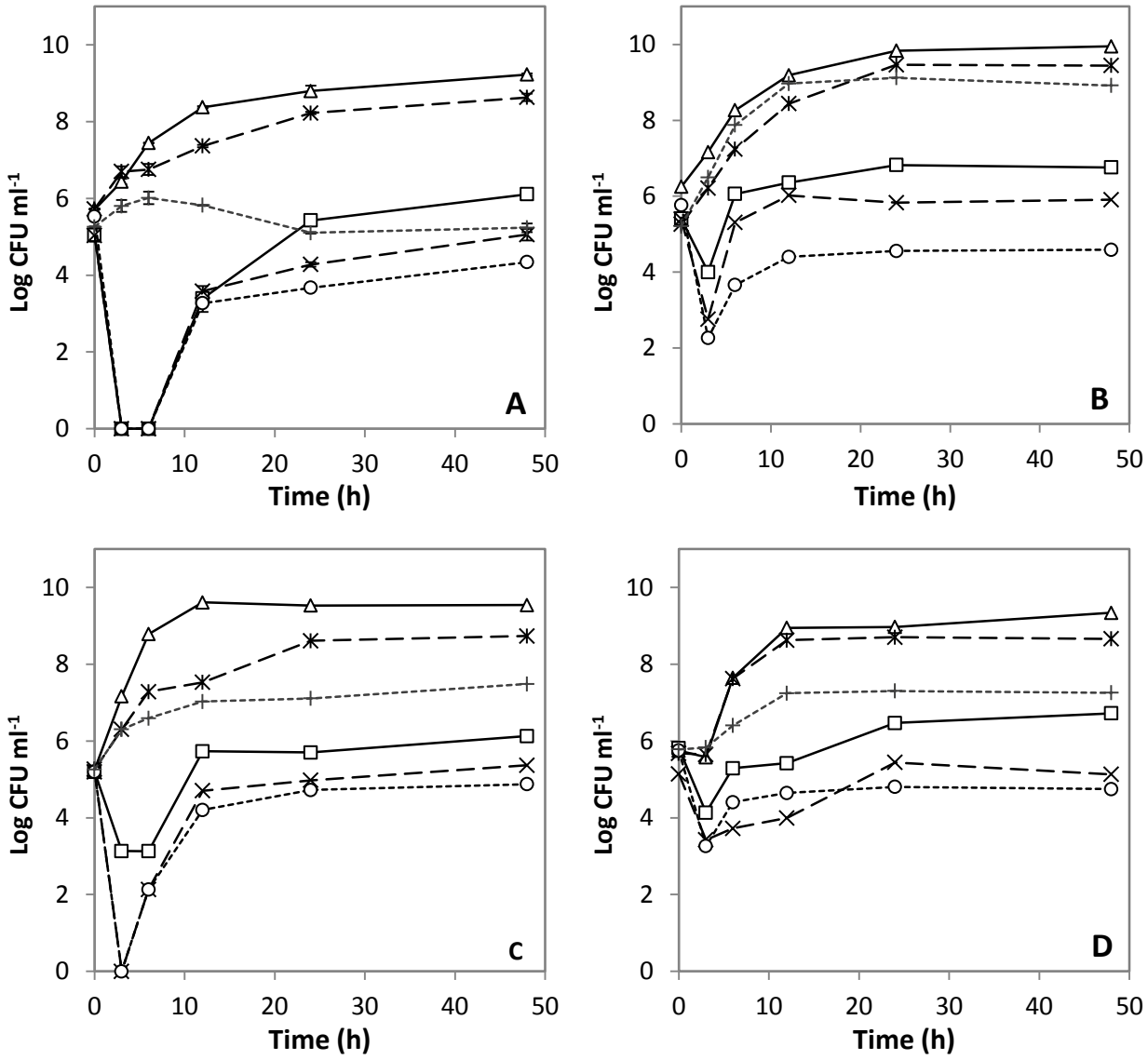

Figure 4-3. Antimicrobial activity of thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.5 g/l in apple cider adjusted to pH 5.5, against *Escherichia coli* O157:H7 ATCC 43889 (A) and ATCC 43894 (B), and *Listeria monocytogenes* strains Scott A (C) and 101 (D). Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed thymol: (□) 35 or 32°C, (×) 25°C and (○) 4°C; Control of bacterial culture with no thymol (△) 35°C for *E. coli* or 32°C for *Lm*, (✳) 25°C and (+) 4°C.

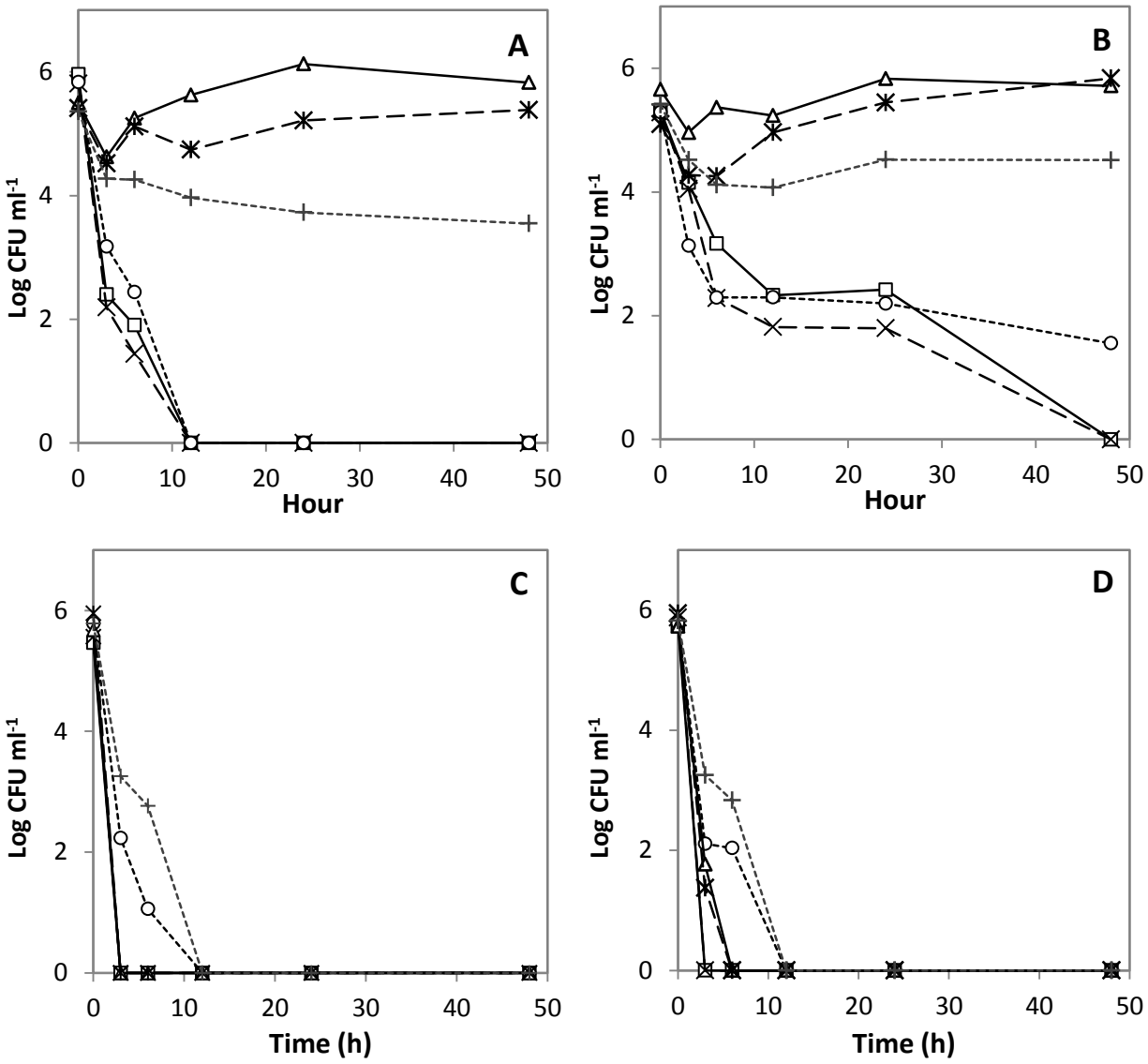

Figure 4-4. Antimicrobial activity of thymol-containing nano-dispersion made using 1:2 WPI:MD180 conjugate, used at an overall thymol concentration of 0.5 g/l in apple cider adjusted to pH 3.5, against *Escherichia coli* O157:H7 ATCC 43889 (A) and ATCC 43894 (B), and *Listeria monocytogenes* strains Scott A (C) and 101 (D). Error bars represent standard error of mean from four measurements, two from each of two replicates. Symbols defined in Figure 4-3.

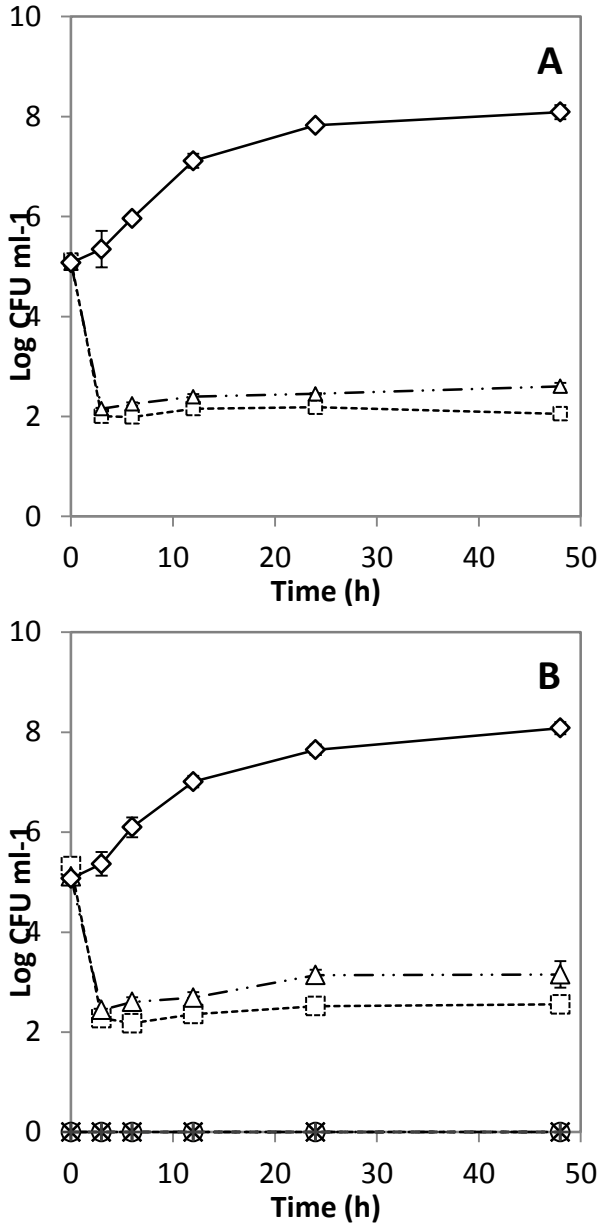

Figure 4-5. Antimicrobial activity of free thymol (△) and thymol-containing nano-dispersion (□) made using 1:2 WPI:MD180 conjugate, used at 4.5 g/l in 2 % reduced fat milk, against *Escherichia coli* O157:H7 ATCC 43889 at 35°C (A) and *Listeria monocytogenes* strain Scott A at 32°C (B), respectively. Error bars represent standard error of mean from four measurements, two from each of two replicates. Control of bacterial culture with no thymol (◇) at pH 6.8 and 35°C for *E. coli* and 32°C for *Lm*.

5. Preparation and characterization of nano-dispersions of eugenol for visual clarity and heat stability

Abstract

Plant essential oils such as eugenol have strong antimicrobial properties; but, their lipophilic nature limits their application in foods due to poor dispersibility in water, adverse effects on visual appearance and loss of antimicrobial efficacy. Maillard-type conjugates of whey proteins and carbohydrates such as maltodextrins are excellent emulsifying agents and have been commonly used to encapsulate volatile oils and flavor compounds. The goal of this study was to develop a low cost technology to encapsulate eugenol in spray dried capsules of conjugates of whey protein isolate (WPI) and maltodextrins (MD) made using different WPI:MD mass ratios and MD chain lengths. Mass yield up to 82.68 % with a maximum encapsulation efficiency of 47.88 % volatile eugenol were observed among spray drying treatments. Upon hydration of spray dried capsules and adjustment pH to 3.0 and 7.0, most dispersions were transparent after heating at 80°C for 15 min, corresponding to diameters smaller than 100 nm. One promising formulation also showed clear dispersions after heating at pH 5.0, which is near the isoelectric point of whey proteins in contrast to gel formation which occurred for a control prepared with non-conjugated WPI and MD mixture. The present study provides simple and economical technology to produce nanoscale systems for delivering lipophilic bioactives in foods, without adversely affecting their visual appearance.

Keywords: lipophilic antimicrobial, eugenol, nanoscale delivery system, whey protein isolate, conjugates

5.1. Introduction

Encapsulation is the most commonly employed approach in the food industry to protect sensitive compounds from degradation, mask unpleasant tastes of certain compounds, reduce losses due to evaporation, prevent binding or interaction between with other food matrix components, or facilitate controlled release under desired conditions (de Vos and others 2010). Essential oils (EO) are a group of lipophilic compounds that are commonly encapsulated due to their volatile nature and intense aroma (Toure and others 2007). EO are ethereal compounds obtained from herbs and spices (Burt 2004) and are traditionally used as flavoring ingredients in culinary applications. Recently, EO are receiving attention for additional functional properties such as antioxidant, antibacterial, and antifungal activity (De Martino L 2009). Particularly, thymol, carvacrol and eugenol, which are EO of thyme, oregano and clove, respectively, act as strong 'natural' preservative agents against bacterial foodborne pathogens such as *Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus,* and many fungi (Chao and others 2000; Devi and others 2010; Bajpai and others 2011; Oussalah and others 2007). However, binding of EO and food constituents and inhomogeneity in distribution in food matrices due to their lipophilic nature reduce their antimicrobial efficiency (Pei and others 2009; Gutierrez and others 2008; Ly 2008). Thus, the food industry is constantly faced with the challenges of applying effective concentrations of EO to achieve maximum antimicrobial efficacy, without compromising on sensory quality and consumer acceptability. Encapsulation of EO in food materials like proteins and carbohydrates may solve these challenges (Zeller and others 1998; Gharsallaoui and others 2007; Charve and Reineccius 2009; Sliwinski and others 2003).

Further, encapsulation of bioactive ingredients in nanoscale systems provides unique functional properties that are impossible for conventional microencapsulation systems (Augustin and Hemar 2009).

Nanoparticles usually are referred to those with a diameter of less than 100 nm that enable stable dispersions and possibly transparent appearance due to reduced scattering of light (Farhang 2009). Particularly for antimicrobial compounds, nanoscale systems have been studied for encapsulating lysozyme and nisin in liposomes (Were and others 2003), thymol in nanoemulsions (Ziani and others 2011) and eugenol in microemulsions (Kriegel and others 2008; Gaysinsky and others 2005). However, these technologies may have drawbacks such as the adoption of high energy methods and the use of large concentrations of surfactants and possibly co-surfactants which may not be GRAS and thus raise toxicity concerns.

The goal of this work is to develop an emulsion-evaporation-based cost-effective approach to encapsulate antimicrobials in nanoscale structures of food grade materials enabling transparent and heat stable dispersions. Emulsion-evaporation is based on formation of an emulsion consisting of an oil phase with a volatile solvent that can be evaporated off to form shrinking droplets, eventually enabling nano-meter sized particles (Chu and others 2007). To make the approach more practical for industrial use, the present study adopted spray drying to produce powdered products from emulsions containing an oil phase of hexane with dissolved eugenol. Spray-drying is the most commonly used as a one-step, low-energy and economical encapsulation method (Adamiec and Kalemba 2006). Although spray dried capsules typically have a diameter of several micrometers, it is hypothesized, in this study, that depletion of hexane creates capsules with a mostly empty shell and subsequent hydration of capsules enables much shrunk structures. Whey proteins are extensively studied as emulsifiers. However, aggregation of whey protein-stabilized particles may be a problem during thermal treatments, particularly for systems with an increased ionic strength and acidity near isoelectric points of whey proteins. In the present study, conjugates of whey protein isolate (WPI)-maltodextrin (MD) were

produced by dry heating (via the Maillard reaction), with the rationale that the MD moiety may provide an additional mechanism stabilizing particles (Akhtar and Dickinson 2007). There are several reports on the use of various WPI-MD based materials for encapsulating lipophilic ingredients such as caraway EO (Bylaite and others 2001), oregano EO (Baranauskiene and others 2006), avocado oil (Bae and Lee 2008) and ginger oil (Toure and others 2007). The studied model antimicrobial agent, eugenol, has low solubility of only up to 0.25 %w/v in water at 20°C (Research Corporation 2011) and is volatile, with a vapor pressure of 4.2 kPa at 150°C, a typical spray drying temperature (Research Corporation 2011). The solubility of eugenol presents a challenge for preparing transparent dispersions above its solubility limit, while the volatility may influence encapsulation performance in emulsion-evaporation technologies. Additionally, it is slightly more water-soluble than thymol and serves as a better candidate for antimicrobial applications for foods containing higher amounts of lipid and lipid-soluble components, such as milk and meat.

5.2. Materials and methods

5.2.1. Materials

WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD of various chain lengths (MD40, MD100 and MD180, with an average dextrose equivalent of 4, 10 and 18, respectively) were supplied by Grain Processing Corporation (Muscatine, IA). Eugenol (99 %) was obtained from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). Hexane, methanol and other chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

5.2.2. Preparation of WPI-MD conjugates

WPI was dissolved at 3.7 %w/w in deionized water and MD was dissolved at WPI:MD mass ratios of 1:2, 1:1 or 2:1. After hydration up to 14 h, Samples were spray dried at 150°C inlet temperature, 6.67 ml/min

feed rate, 600 kPa compressed air pressure and 35 m^3/h air flow rate, using B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). The outlet temperature was recorded to be 80-90°C. Spray dried powders were heated at 90°C for 2 h, collected and stored in a -18°C freezer. This method was adapted from that of Akhtar and Dickinson (2007), by substituting freeze drying with spray drying to prepare powders by drying heating.

5.2.3. Encapsulation of eugenol by spray drying emulsions

Emulsions were prepared using compositions given in Table 5-1, using a Virtis-Sentry Cyclone I.Q.2 microprocessor homogenizer equipped with a 12 inch shaft and an 1 inch disc blade operated at 15000 rpm for 3 min for 100ml. The oil phase contained 20 %w/v eugenol in hexane and was used at 10 % volume of the overall emulsion. The aqueous phase was dissolved with conjugates at an overall 3.7 % protein concentration. A mixture of WPI and MD40 at a WPI:MD mass ratios of 1:2 was prepared by spray drying but, without dry heat treatment at 90°C for 2 h as in the case of preparing conjugates. This mixture was used to prepare emulsions (Sample C-1 in Table 5-1) to serve as controls for conjugate treatment (Sample 1 in Table 5-1). Emulsions were spray dried right after preparation, using the same spray dryer and conditions stated in section 5.2.2. The powdered emulsion was collected and stored at -18°C until further analysis.

5.2.4. Encapsulation performance of spray dried capsules

5.2.4.1. Mass Yield

Mass yield refers to the percentages of the collected mass with reference to the non-solvent mass in the corresponding emulsion (feed) before spray drying:

$$Mass\ yield\ \% = \frac{Mass\ of\ collected\ product}{Non-solvent\ mass\ of\ feed} \times 100 \ldots\ldots\ldots\ldots (1)$$

5.2.4.2. Eugenol loading

Eugenol loading in spray dried powders was quantified using high performance liquid chromatography (HPLC). Capsules were dissolved at 8 mg/ml in 40 %v/v aqueous methanol. The Sample injection volume was 20 μL. The mobile phase consisted of 60 %v/v aqueous methanol, applied with the isocratic mode at a flow rate of 1 ml/min. A 1200 series quaternary pump, a 1200 series Diode Array Detector, a 1200 series vacuum degasser, and a ZORBAX Eclipse Plus-C18 column were used (all from Agilent Technologies, Inc. Santa Clara, CA). The UV spectra were acquired between 190-370 nm and the chromatogram was extracted at 254 nm (Geng and others 2007). Data was acquired and analyzed using Chemstation Plus software (Agilent Technologies, Inc. Santa Clara, CA). A calibration curve was previously established using standard solutions with various concentrations of eugenol. Sample peak area was used to determine eugenol concentration in the Sample for calculation of loading % using Equation 2:

$$Loading\ \% = \frac{Mass\ of\ eugenol}{Mass\ of\ collected\ product} \times 100 \dots\dots\dots (2)$$

5.2.4.3. Encapsulation efficiency

Spray drying experiments were evaluated for their encapsulation efficiency (EE), defined as the percentage of total eugenol mass in a spray dried product with reference to the corresponding eugenol mass used in spray drying (Equation 3).

$$EE\ \% = \frac{Loading\ \% \times Mass\ of\ collected\ product}{Mass\ of\ eugenol\ in\ feed} \times 100 \dots\dots\dots (3)$$

The concentration of eugenol in the feed excluding solvent was calculated as the theoretical eugenol loading % in the spray drying feed, given in Equation 4:

$$Theoretical\ loading\ \%$$

$$= \frac{Mass\ of\ eugenol}{(Mass\ of\ eugenol) + (mass\ of\ WPI - MD\ conjugate\ or\ mixture\ in\ feed\ )} \times 100 \ldots\ldots\ldots (4)$$

Finally, the overall eugenol concentration change before and after spray drying was calculated using Equation 5.

$$Eugenol\ concentration\ change\ \% = \frac{Loading\ \%}{Theoretical\ loading\ \%} \times 100 \ldots\ldots\ldots (5)$$

5.2.4.4. Microstructure of spray dried powders

Surface morphology of Sample powders, after sputter-coating the spray dried capsules with a gold layer of ~5 nm, was imaged using a surface scanning electron microscope (LEO 1525 SEM, LEO Electron Microscopy, Oberkochen, Germany) at 3000X magnification. The powder Sample was fixed onto a two-way black adhesive tape mounted on a stainless steel stub.

5.2.5. Preparation and characterization of nano-dispersions

Dispersions were prepared by hydrating spray dried capsules at 5 %w/v concentration in deionized water for 14 h at room temperature (21°C). After adjusting to pH 3.0, 5.0, and 7.0 using 1 N NaOH or 1 N HCl and 0 and 50 mM NaCl, dispersions were characterized for heat stability and size distributions as follows.

5.2.5.1. Visual clarity and heat stability of nano-dispersions

The above dispersions were heated in a water bath at 80°C for 15 min, followed by cooling to room temperature. The dispersions were photographed and measured for changes in absorbance at 421 and 600 nm (Yong and Foegeding 2008) before and after heating using a UV-vis spectrophotometer (model

Biomate 5, Thermo Electron Corp., Woburn, MA). The 421 nm wavelength corresponds to the highest absorbance by Maillard reaction products (Yong and Foegeding 2008).

5.2.5.2. Particle size distribution of nano-dispersions

Particle size distributions of the dispersions were recorded before and after heating based on dynamic light scattering using Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc. Brea, CA). The obtained data was used to calculate the volume-length mean particle diameter ($d_{4,3}$) based on Equation 6:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad \text{..... (6)}$$

where n_i is the number of particles with a diameter of d_i.

5.2.6. Data and statistical analysis

Two spray dried replicates were prepared separately for each formulation in Table 5-1. Each spray dried Sample was tested in duplicate. Statistical means and standard errors of the means were calculated from four measurements. Data was analyzed by mixed model analysis of variance (ANOVA, $p<0.05$) using SAS version 9.2 (SAS Institute, NC).

5.3. Results and Discussion

5.3.1. Encapsulation performance

The mass yield, eugenol loading and EE of spray dried capsules are presented in Table 5-2. Formulations 1, 2 and 7 achieved highest eugenol loading level of 7.9 %, EE of 35.7 % and mass yield of 82.7 % after spray drying (Table 5-2), respectively. Eugenol is a highly volatile essential oil, with a vapor pressure of

4.2 kPa at 150°C (ChemIDPlus, 2011). An overall low loading (less than 8 % eugenol) in the formulations (Table 5-2) may have resulted from losses caused from the evaporation of eugenol due to high inlet temperature of 150°C and 600 kPa compressed air pressure used during spray drying. Current findings indicate that conjugate structure did not have a significant effect on encapsulation performance of formulations after spray drying. However, controlled emulsion preparation conditions and a more comprehensive experimental design than that in the present study are needed to better understand the effect of WPI:MD ratio and MD chain length on encapsulation performance of Samples.

When compared to the control prepared with non-conjugated mixture of WPI and MD (Sample C-1 in Table 5-1), the encapsulation performance of Sample 1 was significantly better ($p<0.05$). The loading % of Sample 1 was 7.3 %, higher than 6.6 % of the Sample C-1. The EE of Sample 1 (35.8 %) was also much higher than that of Sample C-1 (14.3 %) (Table 5-2). Mechanisms enabling the improved encapsulation performance of conjugate Samples are to be studied.

5.3.2. Surface morphology of spray dried capsules

Table 5-2 presents scanning electron micrographs of spray dried microcapsules. All Samples demonstrated spherical, micrometer sized capsules with shell structures. Formulations 1-6 made with MD40 and MD100, exhibited small size (1-2 µm) and intact structures, with smooth surface (Table 5-2). Formulations 7, 8 and 9 were made with MD 180 and showed relatively higher number of cracks and broken shells. A noteworthy finding was that these ruptured microcapsules were relatively larger (2-5 µm) and demonstrated an internal structure with pockets that accommodated smaller, spherical structures.

No obvious effect of MD chain length was observed on the morphological characteristics of Samples made from MD40, MD100 or MD180 at the same mass ratio. It is known that the microstructure of encapsulated structures can be affected by formulation ingredients (emulsion composition) and spray drying conditions, which in turn can affect functional properties of the encapsulated oil (Adamiec and Kalemba 2006), detailed optimization is needed to further investigate the effect of conjugate structure on encapsulation and release of the oil from Samples.

5.3.3. Turbidity and thermal stability of dispersions

Photographs of nano-dispersions (Figure 5-2, Figure 5-2 and Figure 5-1) were used to compare visual clarity, before and after heating, and the absorbance of nano-dispersions (Table 5-3) was used as an indirect, qualitative comparison of Sample turbidity. Mackay (2000) estimated that only 0.16 % w/w pure eugenol is soluble in deionized water at room temperature, beyond which it separates out into a distinct oil phase. However, many dispersions with a eugenol content much higher than the solubility limit were transparent before and after heating, in the present study. The following discussion is presented for impacts of solvent chemistry and conjugate structure on dispersion properties.

5.3.3.1. Effect of pH and ionic strength on turbidity and thermal stability of dispersions

Overall, dispersions were clearer at pH 3.0 and 7.0 than at pH 5.0. Some Samples at pH 3.0 and 7.0 became clearer after heating, indicating enhanced dispersion of capsules, while some became more turbid or even formed gels, particularly at pH 5.0. At pH 3.0 and 7.0, dispersions of Sample 1 were clear, while dispersions of other Samples were slightly turbid after heating (Figure 5-2, Figure 5-2 and Figure 5-2). At pH 5.0, which is near the isoelectric point of β-lactoglobulin, the major whey protein component, all Sample dispersions except Sample 1 were turbid or formed gels at both salt concentrations after heating (Figure 5-2, Figure 5-2 and Figure 5-2). At this pH, the net charge of β-

lactoglobulin is close to zero and the electrostatic repulsion is not strong enough to prevent protein aggregation (Kulmyrzaev and Schubert 2004). It is not clear why only Sample 1, prepared using conjugates with MD40 at a 1:2 WPI:MD40 ratio, enabled better dispersibility and heat stability than other treatments.

Dispersions with 50 mM NaCl had similar absorbance values to those with 0 mM NaCl, after heating. It is known that the effective distance of electrostatic repulsion is reduced at an increased ionic strength and thus the chance of particle aggregation is increased (Schmitt and others 2007). No significant differences for treatments with 0 and 50 mM NaCl may have resulted from steric hindrance provided by the MD moiety of the conjugates.

5.3.3.2. Effect of conjugate structure on turbidity and thermal stability of dispersions

When dispersions corresponding to different conjugates were tested, Samples prepared with a WPI:MD mass ratio of 1:2, regardless of MD chain length, demonstrated slightly lower absorbance values after heating than those at WPI:MD mass ratios of 1:1 and 2:1 ratios (Table 5-3). These results are similar for thymol-containing dispersions discussed in Chapter 2. It may be possible that the presence of a higher concentration of MD leads to a higher degree of conjugation during dry heating, which in turn improved dispersibility and heat stability of capsules. The degree of conjugation however requires future quantification.

Studies report that the effectiveness of protein-carbohydrate conjugates as emulsifying agents are significantly affected by protein type and conformation, carbohydrate size, and their interactions (Akhtar and Dickinson 2007; Doublier and others 2000). Clear dispersions were produced by Sample 1, which contained MD40 at 1:2 WPI:MD mass ratio. In contrast, Sample 7 containing thymol, previously

discussed in Chapter 2, was made using MD180 at the same mass ratio. The differences in results may be attributed to the difference in conjugate composition and essential oils used in both formulations., Eugenol being slightly more water-soluble than thymol, higher losses may have occurred during spray drying, resulting in lower oil loading in Sample 1 when compared to Sample 7 (discussed in Chapter 2), which may affect dispersion properties. Thus, there is a need to further investigate the significance of MD chain length in comparison to physical chemistries of oils on the properties of dispersions, in addition to employing more controlled emulficiation procedures to form capsules.

5.3.4. Particle size distributions of dispersions

Sample dispersions were measured for particle size using light scattering. Most dispersions were visually clear at pH 3.0 and 7.0, with 0 and 50 mM NaCl, after heating, corresponding to$d_{4,3}$ less than 200 nm (all data not shown). Since dispersions of Sample 1 were transparent even after heating at pH 5.0, particle size distributions of these dispersions are presented in Figure 5-5, with $d_{4,3}$ summarized in Figure 5-2. $d_{4,3}$ ranging from 115 to 242 nm before heating and 36 to 91 nm after heating at 80°C for 15 min, were observed at pH 3.0-7.0, with and without 50mM NaCl (Table 5-4). Generally, $d_{4,3}$ of dispersions with 50 mM NaCl were higher than those with 0 mM NaCl, indicating the increased aggregation due to the screening of electrostatic repulsion by electrolytes (Ye 2010). The changes in $d_{4,3}$ may not have been significant enough to impact absorbance values in Table 5-4. Particle size distributions of most dispersions of Sample 1 showed two peaks (Figure 5-5), especially after heating. Peaks after heating shifted to the smaller diameter range, corresponding to improved clarity discussed above.

5.3.5. Significance of conjugation on dispersion properties

The importance of conjugation to dispersion properties was demonstrated for Sample C-1 (Table 5-1), which was prepared as a control to Sample 1 by using a mixture of non-conjugated WPI and MD.

Dispersions of Sample C-1 were more turbid than those of Sample 1, especially at pH 5.0, before heating (Figure 5-5 and Figure 5-2). Same observations were recorded after heating, with gels formed for dispersions of Sample C-1 at pH 5.0 . Contrasting to the reduced particle size for Sample 1 after heating, the control Sample C-1 showed shifting of peaks toward bigger diameters (Figure 5-6 and Table 5-4). The comparison indicates the importance of conjugating MD with WPI for improved dispersibility and thermal stability of resultant nano-dispersions.

5.4. Conclusions

The present study demonstrated the success of encapsulating volatile eugenol using spray drying and subsequent hydration of spray dried capsules to prepare transparent and heat stable dispersions at a eugenol concentration well above its solubility limit. WPI-MD conjugates were advantageous than non-conjugated mixtures in improving dispersion transparency and thermal stability. Variables in emulsion preparation impacted encapsulation performance of eugenol and properties of nano-dispersions. Further investigation is needed to understand fundamental principles resulting in the observed differences by adopting more comprehensive experimental designs and more precisely controlled emulsion preparation conditions. Nevertheless, the presented low cost and simple technology may be used to disperse various lipophilic components in clear liquid products, contributing to the growing functional beverage market.

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Appendix

Table 5-1. Formulations used to prepare emulsions[a] for spray drying.

Sample ID	Type of MD	WPI:MD ratio	Eugenol %w/v in hexane	Conjugate %w/v in water	Volume %v/v of oil phase	Theoretical eugenol loading %w/w
1	40	1:2	20.0	11.1	10	16.7
2	40	1:1	20.0	7.4	10	23.1
3	40	2:1	20.0	5.6	10	29.7
4	100	1:2	20.0	11.1	10	16.7
5	100	1:1	20.0	7.4	10	23.1
6	100	2:1	20.0	5.6	10	29.7
7	180	1:2	20.0	11.1	10	16.7
8	180	1:1	20.0	7.4	10	23.1
9	180	2:1	20.0	5.6	10	29.7
C-1	40	1:2	20.0	11.1[b]	10	16.7

[a]Emulsions were prepared with an oil phase of eugenol in hexane and an aqueous phase of whey protein isolate-maltodextrin (WPI-MD) conjugate (Samples 1-9) or mixture (Sample C-1) in deionized water.

[b]A spray dried mixture of WPI and MD40 at a mass ratio of 1:2, without dry heating as in the preparation of conjugates, was used to prepare emulsions to serve as a control to Sample 1.

Table 5-2. Encapsulation performance[a] of spray drying emulsions prepared according to formulations in Table 5-1.

Sample ID	Mass Yield %	Eugenol %w/w in spray dried powder	Eugenol concentration change %	Encapsulation efficiency %
1	81.8 ± 0.8^{AB}	7.3 ± 0.1^{AB}	43.7 ± 0.31^{A}	35.7 ± 0.6^{A}
2	77.4 ± 0.9^{BCD}	7.8 ± 0.0^{A}	34.6 ± 0.1^{C}	26.8 ± 0.3^{B}
3	73.2 ± 1.6^{D}	7.2 ± 0.0^{AB}	24.3 ± 0.1^{F}	17.8 ± 0.3^{E}
4	79.6 ± 1.5^{ABC}	5.0 ± 0.0^{D}	30.2 ± 0.0^{E}	24.1 ± 0.5^{D}
5	77.8 ± 0.3^{BCD}	7.5 ± 0.0^{AB}	32.6 ± 0.0^{D}	25.4 ± 0.2^{CD}
6	76.9 ± 0.7^{CD}	6.5 ± 0.1^{BC}	21.8 ± 0.0^{G}	16.8 ± 0.2^{E}
7	82.7 ± 0.8^{A}	7.2 ± 0.0^{AB}	42.9 ± 0.1^{B}	35.4 ± 0.3^{A}
8	77.7 ± 1.1^{BCD}	7.9 ± 0.0^{A}	34.4 ± 0.1^{C}	26.7 ± 0.3^{BC}
9	74.7 ± 0.8^{D}	6.6 ± 0.0^{B}	22.1 ± 0.1^{G}	16.5 ± 0.1^{E}
C-1	67.8 ± 1.3^{E}	3.2 ± 0.0^{CD}	21.1 ± 0.1^{H}	14.3 ± 0.3^{F}

[a]Values are means ± standard errors of means from four measurements, two from each of two replicates. Different superscript letters in the same column represent statistical difference ($p<0.05$).

Table 5-3. Absorbance of eugenol-containing dispersions before and after heating at 80°C for 15 min.

Sample ID	NaCl (mM)	Absorbance Before Heating						Absorbance After Heating					
		421 nm			600 nm			421 nm			600 nm		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
1	0	2.61 ± 0.08	3.01 ± 0.02	2.61 ± 0.05	2.24 ± 0.23	2.96 ± 0.01	2.09 ± 0.18	1.01 ± 0.11	3.09 ± 0.09	0.91 ± 0.15	0.64 ± 0.29	3.04 ± 0.13	0.44 ± 0.37
	50	2.57 ± 0.08	3.07 ± 0.07	2.53 ± 0.09	2.22 ± 0.21	2.95 ± 0.01	1.90 ± 0.06	0.95 ± 0.11	3.10 ± 0.08	0.97 ± 0.25	0.60 ± 0.24	3.05 ± 0.15	0.37 ± 0.21
2	0	2.51 ± 0.4	2.65 ± 0.04	2.74 ± 0.05	2.22 ± 0.05	2.38 ± 0.01	2.38 ± 0.11	1.95 ± 0.7	3.31 ± 0.06	1.21 ± 0.07	1.60 ± 0.05	3.04 ± 0.01	0.68 ± 0.01
	50	2.43 ± 0.01	2.64 ± 0.03	2.75 ± 0.06	2.17 ± 0.04	2.39 ± 0.00	2.39 ± 0.11	1.98 ± 0.04	3.50 ± 0.05	1.66 ± 0.06	1.60 ± 0.03	3.09 ± 0.02	1.11 ± 0.00
3	0	2.35 ± 0.06	2.86 ± 0.01	2.41 ± 0.09	1.86 ± 0.00	2.49 ± 0.01	1.90 ± 0.00	0.49 ± 0.11	3.40 ± 0.03	0.49 ± 0.14	0.17 ± 0.02	3.13 ± 0.03	0.18 ± 0.03
	50	2.33 ± 0.09	2.94 ± 0.04	2.40 ± 0.11	1.83 ± 0.02	2.46 ± 0.01	1.88 ± 0.00	0.84 ± 0.15	3.45 ± 0.02	0.76 ± 0.16	0.32 ± 0.06	3.16 ± 0.01	0.25 ± 0.00
4	0	0.47 ± 0.05	1.64 ± 0.27	0.73 ± 0.02	0.83 ± 0.12	2.91 ± 0.24	0.72 ± 0.06	0.26 ± 0.17	1.24 ± 0.20	0.38 ± 0.09	0.54 ± 0.02	2.54 ± 0.18	0.40 ± 0.02
	50	0.37 ± 0.06	1.33 ± 0.29	0.63 ± 0.13	0.71 ± 0.05	3.12 ± 0.00	0.69 ± 0.03	0.23 ± -.06	0.89 ± 0.01	0.36 ± 0.03	0.40 ± 0.03	2.97 ± 0.20	0.33 ± 0.07
5	0	2.20 ± 0.03	2.37 ± 0.02	2.57 ± 0.01	1.94 ± 0.11	2.08 ± 0.00	2.18 ± 0.00	1.47 ± 0.03	3.61 ± 0.02	1.70 ± 0.00	1.00 ± 0.10	3.10 ± 0.09	1.13 ± 0.01
	50	2.21 ± 0.04	2.24 ± 0.06	2.55 ± 0.00	1.96 ± 0.07	1.94 ± 0.00	2.18 ± 0.07	1.27 ± 0.04	3.33 ± 0.06	1.24 ± 0.00	0.78 ± 0.07	3.10 ± 0.05	0.65 ± 0.08
6	0	0.34 ± 0.04	1.49 ± 0.01	0.38 ± 0.02	0.43 ± 0.07	2.59 ± 0.36	0.30 ± 0.01	0.16 ± 0.14	0.67 ± 0.36	0.15 ± 0.03	0.21 ± 0.02	2.51 ± 0.00	0.10 ± 0.01
	50	0.24 ± 0.02	0.84 ± 0.11	0.28 ± 0.03	0.41 ± 0.02	2.68 ± 0.33	1.30 ± 0.57	0.13 ± 0.10	0.37 ± 0.29	0.12 ± 0.55	0.14 ± 0.00	2.52 ± 0.04	1.09 ± 0.00

Sample ID	NaCl (mM)	Absorbance Before Heating						Absorbance After Heating					
		421 nm			600 nm			421 nm			600 nm		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
7	0	1.69 ± 0.37	1.52 ± 0.30	1.80 ± 0.39	1.62 ± 0.33	1.85 ± 0.52	1.56 ± 0.30	1.39 ± 0.36	1.79 ± 0.38	1.43 ± 0.33	1.29 ± 0.35	2.09 ± 0.52	1.17 ± 0.37
	50	1.63 ± 0.34	2.00 ± 0.30	1.78 ± 0.39	1.66 ± 0.33	2.31 ± 0.15	2.05 ± 0.29	1.29 ± 0.35	2.19 ± 0.29	1.41 ± 0.18	1.29 ± 0.33	2.52 ± 0.41	1.42 ± 0.37
8	0	2.38 ± 0.00	2.50 ± 0.13	2.52 ± 0.01	2.13 ± 0.11	2.23 ± 0.01	2.20 ± 0.00	1.71 ± 0.11	3.37 ± 0.14	1.66 ± 0.01	1.28 ± 0.09	3.12 ± 0.05	1.10 ± 0.00
	50	2.16 ± 0.10	2.52 ± 0.04	2.49 ± 0.00	1.89 ± 0.05	2.24 ± 0.00	2.17 ± 0.03	1.40 ± 0.00	3.46 ± 0.03	1.60 ± 0.00	0.88 ± 0.05	3.09 ± 0.03	1.01 ± 0.03
9	0	1.62 ± 0.35	2.30 ± 0.03	1.73 ± 0.33	1.49 ± 0.27	3.14 ± 0.02	1.56 ± 0.28	1.20 ± 0.34	2.25 ± 0.37	1.15 ± 0.32	1.01 ± 0.30	3.12 ± 0.29	0.95 ± 0.28
	50	1.53 ± 0.34	2.46 ± 0.05	1.67 ± 0.32	1.54 ± 0.26	2.81 ± 0.10	1.96 ± 0.23	1.07 ± 0.31	2.53 ± 0.25	1.13 ± 0.12	0.99 ± 0.28	2.95 ± 0.33	1.10 ± 0.27
C-1	0	3.26 ± 0.00	3.32 ± 0.00	3.27 ± 0.00	3.26 ± 0.00	Gel	3.16 ± 0.00	2.74 ± 0.00	2.91 ± 0.00	2.75 ± 0.00	2.51 ± 0.02	Gel	1.87 ± 0.00
	50	3.20 ± 0.02	3.27 ± 0.00	3.33 ± 0.07	3.06 ± 0.00	Gel	2.99 ± 0.00	2.75 ± 0.00	2.89 ± 0.00	2.77 ± 0.00	2.30 ± 0.02	Gel	1.69 ± 0.01

[a]Values are means ± standard errors of means from four measurements, two from each of two replicates.

Table 5-4. $d_{4,3}$ values[a] of dispersions with capsules prepared by spray drying emulsions according to formulations 1 and C-1 in Table 5-1.

Sample ID	NaCl (mM)	$d_{4,3}$ (nm) before heating			$d_{4,3}$ (nm) after heating		
		pH 3.0	pH 5.0	pH 7.0	pH 3.0	pH 5.0	pH 7.0
1	0	116 ± 0^{D}	217 ± 1^{D}	127 ± 7^{D}	40 ± 1^{E}	86 ± 1^{DE}	50 ± 3^{E}
	50	134 ± 14^{D}	242 ± 1^{CD}	160 ± 0^{D}	36 ± 0^{E}	91 ± 4^{DE}	64 ± 2^{E}
C-1	0	274 ± 1^{C}	5488 ± 20^{B}	546 ± 4^{BC}	256 ± 1^{BC}	Gel^{A}	813 ± 1^{BC}
	50	321 ± 1^{C}	5327 ± 10^{B}	740 ± 13^{BC}	335 ± 3^{BC}	Gel^{A}	301 ± 12^{C}

[a]Values are means ± standard errors of mean from four measurements, two measurements from each of two replicates. Different superscript letters represent statistical difference ($p<0.05$).

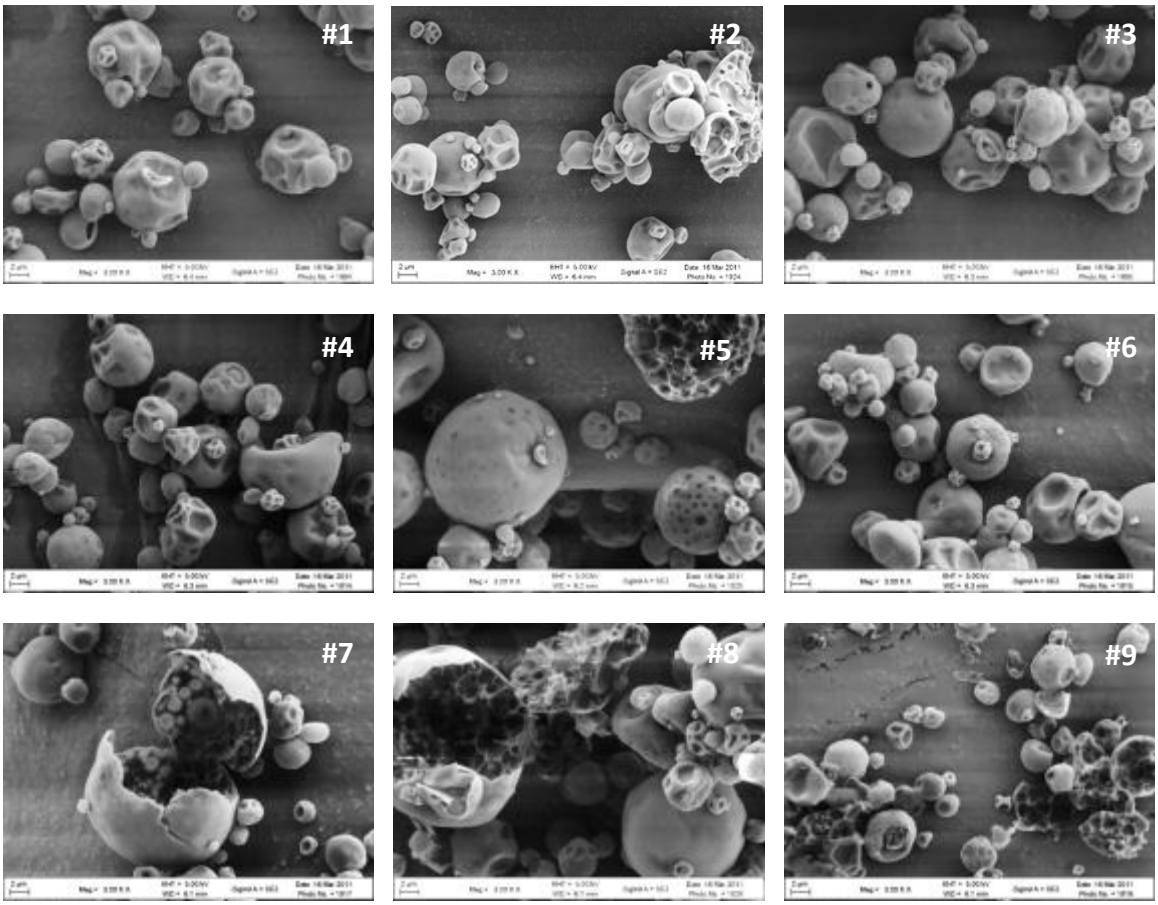

Figure 5-1. Scanning electron micrographs of capsules prepared by spray drying emulsions based on formulations in Table 5-1.

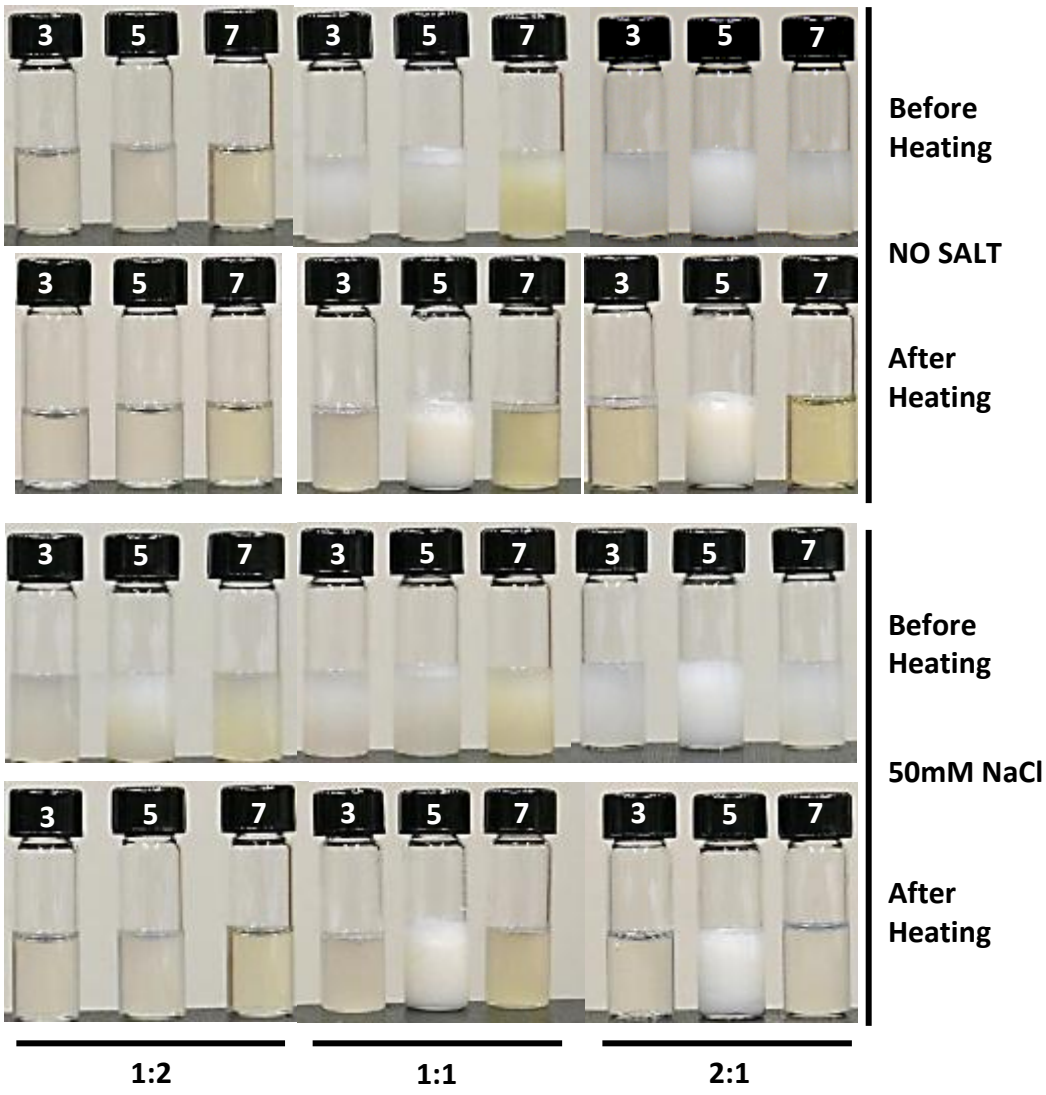

Figure 5-2. Photographs of 5 %w/v eugenol-containing dispersions, before and after heating at 80°C for 15 min, adjusted to pH 3.0, 5.0 and 7.0, as labeled on each vial, and 0 and 50 mM NaCl. Capsules were prepared by spray drying emulsions emulsified with WPI-MD40 conjugates at mass ratios of 1:2, 1:1 and 2:1 (Samples 1-3 in Table 5-1).

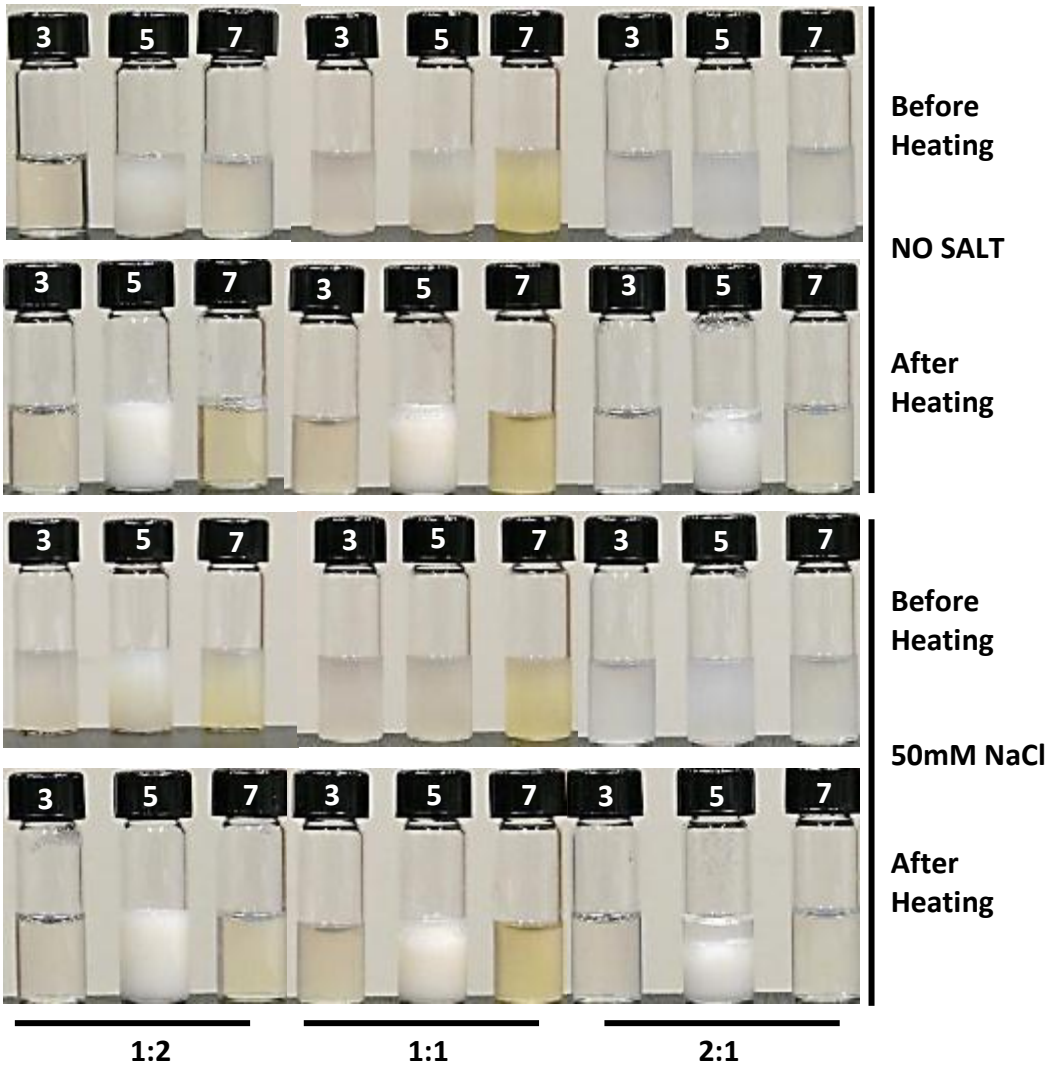

Figure 5-3. Photographs of dispersions made using 5 %w/v spray dried capsules, before and after heating at 80°C for 15 min, and adjusted to pH 3.0, 5.0 and 7.0, as labeled on each vial, and 0 and 50 mM NaCl. Capsules were prepared by spray drying emulsions emulsified with WPI and MD100 conjugates at mass ratios of 1:2, 1:1 and 2:1 (Samples 4-6 in Table 5-1).

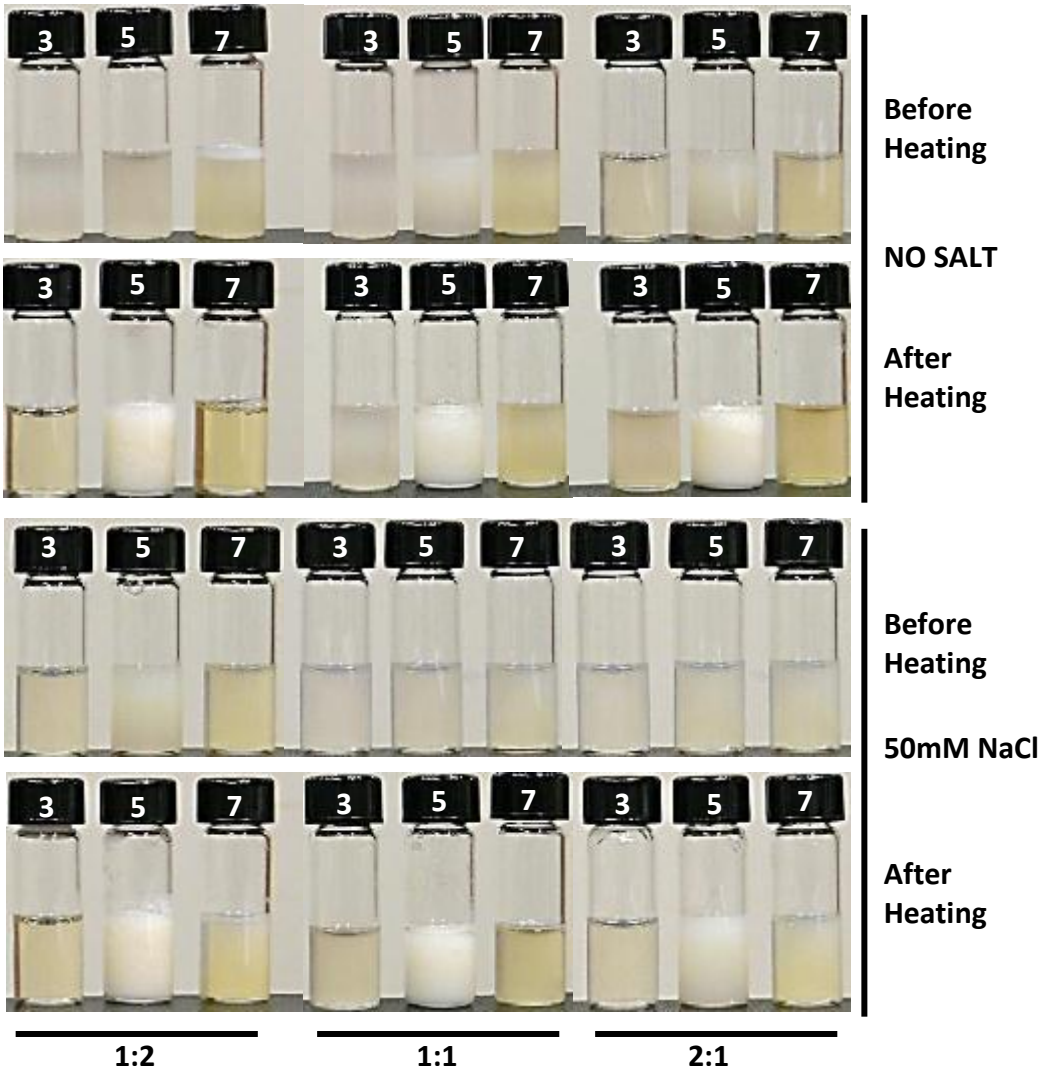

Figure 5-4. Photographs of dispersions with 5 %w/v spray dried capsules before and after heating at 80°C for 15 min. Dispersions were adjusted to pH 3.0, 5.0 and 7.0, as labeled on each vial, and 0 and 50 mM NaCl. Capsules were prepared by spray drying emulsions emulsified with WPI and MD180 conjugates at mass ratios of 1:2, 1:1 and 2:1 (Samples 7-9 in Table 5-1).

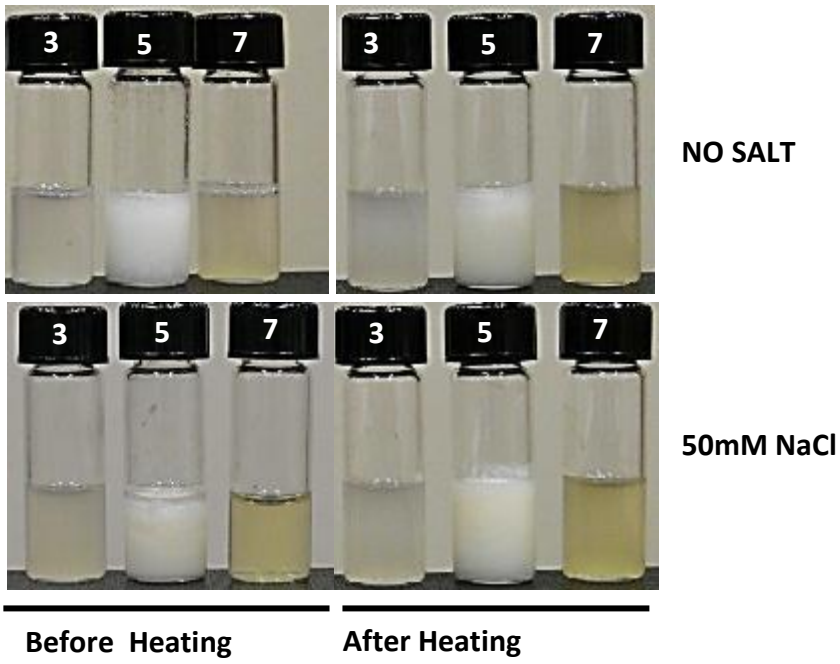

Figure 5-5. Photographs of the control for formulation 1 (Sample C-1 in Table 5-1), hydrated at 5 %w/v and adjusted to pH 3.0, 5.0 and 7.0, with 0 and 50 mM NaCl, before and after heating at 80°C for 15 min.

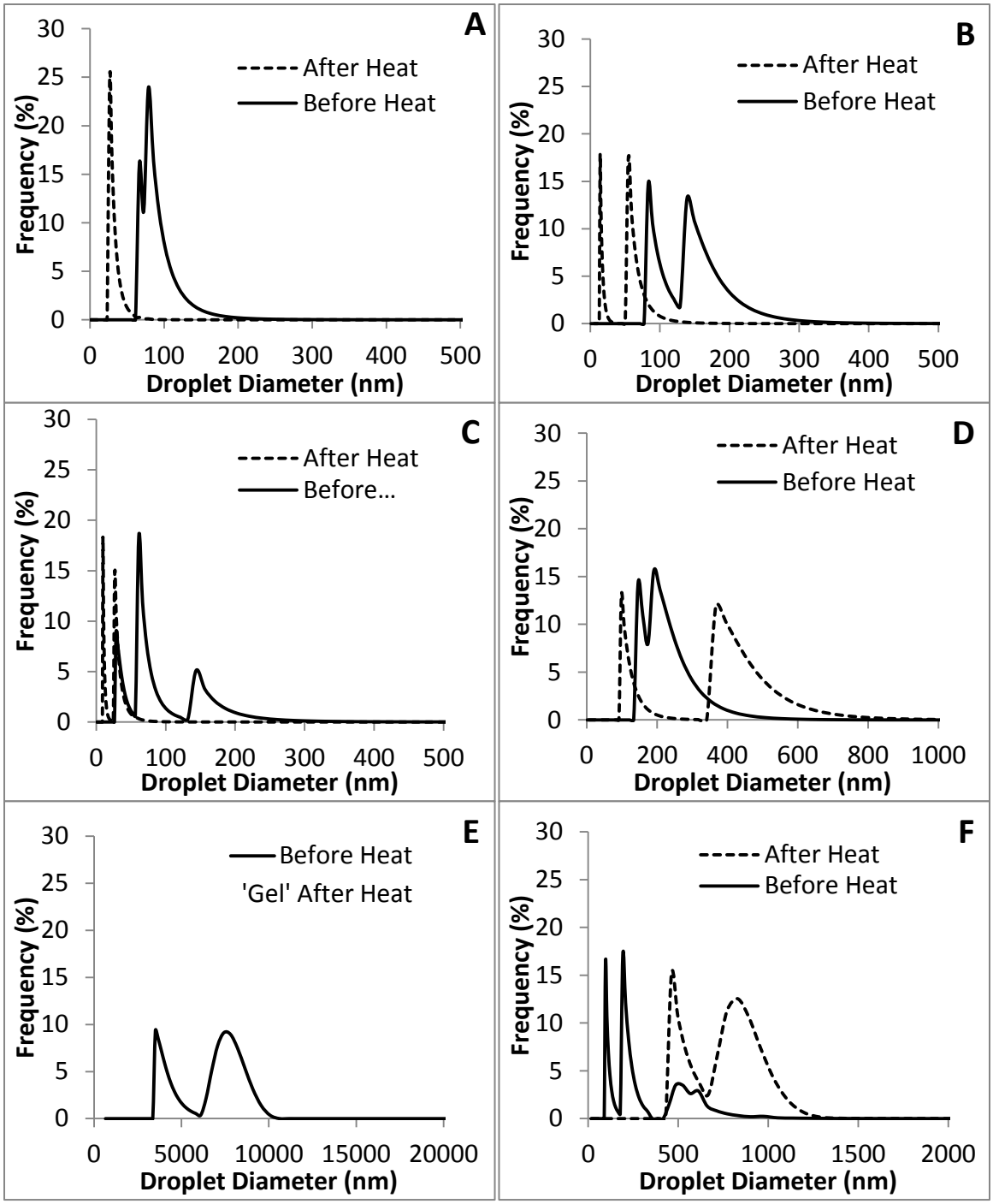

Figure 5-6. Comparison of particle size distribution changes of dispersions before and after heating at 80 °C for 15 min. Dispersions contained 5 %w/v capsules prepared by spray-drying emulsions based on formulations of Sample 1 (A-C) and its control Sample C-1 (D-F) in Table 5-1. Dispersions were adjusted to pH 3.0 (A and D), 5.0 (B and E) and 7.0 (C and F) before heating.

6. Antimicrobial activity of nano-dispersed eugenol against *Escherichia coli* O157:H7 and *Listeria monocytogenes* in tryptic soy broth and in milk

Abstract

The food industry is constantly faced with the need to control pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes (Lm),* while preserving food quality desired by consumers. Plant essential oils are known for their excellent antimicrobial efficacy; but, their poor solubility in water makes it difficult to disperse evenly in food matrices, impacting food quality and antimicrobial efficacy. In the present study, eugenol was dispersed in nanocapsules prepared with conjugates of whey protein isolate (WPI) and maltodextrin (MD) at 1:2, 1:1 and 2:1 WPI:MD mass ratios. Antimicrobial efficacies against *E. coli* O157:H7 ATCC 43889 and 43894, and *Lm* strains Scott A and 101 were compared for nano-dispersed and free eugenol in tryptic soy broth (TSB) and 2 % reduced fat milk and 35 and 32°C. Results indicate that the MIC values for nano-dispersed eugenol were 1.25 g/l in TSB and 4.5 g/l in milk against *E. coli* and were 1.75 g/l in TSB and 5.5 g/l in milk against *Lm*. Nano-dispersed eugenol was more effective than free oil in milk ($p<0.05$) but, similar in TSB. No statistical difference was found in bacterial inhibition for capsules prepared with different ratios of WPI and MD and MD types. Nano-dispersing eugenol not only enabled transparent dispersions but, improved antimicrobial efficacy in foods, making the technology promising for food applications, especially beverages, to inhibit a broad spectrum of foodborne pathogens.

Keywords: lipophilic antimicrobial, eugenol, nano-dispersion, *Escherichia coli* O157:H7, *Listeria monocytogenes,* milk, tryptic soy broth

6.1. Introduction

Escherichia coli O157:H7 and *Listeria monocytogenes (Lm)* are two of the most common foodborne pathogens. The U.S. Department of Agriculture estimates an annual expenditure of approximately $2.3 billion on health care related to acute infections caused by *Lm* and $0.7 billion for *E. coli* O157 and related strains (Gaysinsky and others 2007; Gill and Holley 2006). *E. coli* O157:H7 has been known to cause outbreaks involving hemorrhagic colitis and fatal hemolytic uremic syndrome and other illnesses, most commonly associated with the consumption of undercooked or contaminated ground beef (Amalaradjou and others 2010), unpasteurized apple cider (Baskaran and others 2010) and contaminated fresh produce such as spinach (Viazis and others 2011) and lettuce (Boyer and others 2011). In May 2011, a newly emerged mutant strain *E. coli* O104:H4 that contaminated sprouts caused a severe outbreak in Germany, and smaller outbreaks in France, Spain and UK (CDC 2011). Listeriosis, caused by *Lm* may result in abortion or stillbirths among pregnant women, meningitis, pneumonia or sepsis in newborns and infants, septicemia, encephalitis or meningitis in elderly and adults with compromised immunity (Sandasi and others 2008; Todd and Notermans 2011). Most Listeriosis outbreaks have been associated with raw or unpasteurized milk and dairy products (Greenwood and others 1991).

Thus, the food industry is constantly looking new, efficient and cost-effective food safety intervention strategies to control the growth of pathogenic and spoilage microorganisms, preserve the microbiological and sensory quality, extend shelf life and ensure safety of consumer foods (Lambert and others 2001). Some ways, by which manufacturers control microbial growth, include pasteurization, aseptic packaging or addition of preservatives. In addition, temperature control strategies are employed for most foods to prevent contamination post-processing. For example, refrigeration has been used as the principal method of preservation for foods including dairy products and fruit juices (Tornambé and

others 2008). In addition to thermal processing, the use of natural antimicrobial compounds to reduce growth of or possibly even eliminate foodborne pathogens is being preferred as a more effective approach in food preservation and microbial control (Tserennadmid and others 2011).

Essential oils from aromatic plants, generally herbs or spices, are one such group of naturally occurring antimicrobials. Traditionally used as flavoring agents, they are now gaining popularity due to their preservative effects such as antifungal, antibacterial and antioxidant properties (Burt 2004). Thymol, carvacrol and eugenol are some of the most potent compounds in essential oils extracted from commonly used culinary ingredients thyme, oregano and clove, respectively (Ananda and others 2009). Their antimicrobial activity is attributed to the presence of phenolic groups in their chemical structures (Michiels and others 2007). They are effective against a broad spectrum of gram-negative and gram-positive pathogenic bacteria such as *E. coli, Lm , S. aureus, S. typhimurium* and many fungi (Bajpai and others 2011; De Martino L 2009; Oussalah and others 2007). However, these essential oils are poorly soluble in water, difficult to incorporate into foods and have tendency to bind with food constituents due to their hydrophobic nature and become less available for antimicrobial action (Friedman and others 2004; Gaysinsky and others 2007; Juven and others 1994). This means that in order to achieve the same level of efficacy as observed in model growth media systems, they need to be incorporated in foods at concentrations higher than those acceptable to consumers, due to their strong herbal aroma.

In order to overcome these challenges, many essential oils are emulsified and stabilized by proteins and other surface active ingredients, to enhance solubility and dispersibility in aqueous media, reduce losses due to binding with other food constituents, and increase antimicrobial efficacy by promoting contact with bacterial cell components. Several studies have demonstrated the potential of microencapsulating essential oils in matrices of milk proteins in conjunction with gums, starch and dextran, resulting in improved protective film forming effects, controlled release and effective antimicrobial functionality

(Baranauskiene and others 2006; Beristain and others 2001; Bylaite and others 2001). The stability of these emulsion-based systems is highly dependent on many factors: properties of the stabilizing protein particles and bacteria, such as hydrophobicity and surface charge, which affect not only stability of the system but, also bacterial interaction, contact and inhibition when antimicrobial oils are used (Weiss and others 2009). Furthermore, depending on the interfacial properties, bacteria interact with other food components such as proteins and lipids, and these interactions may affect the stability of emulsions, localization of bacteria in the different regions of the food matrix and antimicrobial efficacy (Pérez-Conesa and others 2011). Thus, there is growing interest in designing structured emulsions with controlled interfacial and droplet characteristics, for improved stability and effective antimicrobial activity of the essential oil, such as nano-emulsions for thymol, microemulsions for eugenol and liposomes for other antimicrobial compounds such as nisin and lysozyme (Donsì and others 2011a; Donsì and others 2011b; Gaysinsky and others 2007; Pérez-Conesa and others 2011; Weiss and others 2009). However, preparation of nano-emulsions may require high energy, while microemulsion systems require expensive and large amounts of surfactants and co-surfactants, which may not be food grade.

The objective of the present study was to create eugenol-containing nano-dispersions made from whey protein isolate (WPI)-maltodextrin (MD) conjugates, using spray drying and to investigate their antimicrobial efficacy against *E. coli* O157:H7 and *Lm ,* in growth medium (Tryptic Soy Broth; TSB) and a model food system (2 % reduced fat milk).

6.2. Materials and Methods

6.2.1. Materials

Eugenol (99 %) was purchased from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD of various chain lengths (MD40, MD100 and MD180) corresponding to an average dextrose equivalent of 4, 10 and 18, were obtained from Grain

Processing Corporation (Muscatine, IA). TSB, peptone, and agar (chemical grade) were products of Becton, Dickinson and Company (Sparks, MD). Other chemicals such as hexane and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Ultra-high temperature (UHT)-pasteurized organic milk with 2 % fat was obtained from Private Selection, a brand of the Kroger Company (Cincinnati, OH).

6.2.2. Preparation of nano-dispersions

To encapsulate eugenol by emulsion-evaporation, emulsions were prepared by emulsifying an oil phase (with 20 %w/v eugenol in hexane) at 10 %v/v volume into an aqueous phase with conjugates corresponding to a net protein concentration of 3.7 %w/v. Emulsions were then spray dried. Conjugates were previously prepared by spray drying solutions containing WPI and MD at a mass ratio of 1:2, 1:1 and 2:1, followed by dry heating the spray dried powder at 90°C for 2 h for conjugation (Maillard reaction). All spray drying experiments were performed using a model B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland) at an inlet temperature of 150°C, 35 m^3/h air flow rate, 600 kPa compressed air pressure, feed rate of 6.67 ml/min and the recorded outlet temperature was 80-90°C. The spray dried capsules were collected and stored in a freezer at -18°C. Spray dried capsules were hydrated either at 5 %w/v eugenol in deionized water for 14 h at room temperature. Dispersions were measured for size distribution based on dynamic light scattering using Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc. Brea, CA),and volume-length mean particle diameter ($d_{4,3}$) was calculated using Equation 1:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad \text{.....(1)}$$

where n_i is the number of particles with a particle diameter of d_i.

6.2.3. Culture preparation

E. coli O157:H7 ATCC 43889 and ATCC 43894, *Lm* strains Scott A and 101 were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville. All cultures were grown in TSB and stored at -20°C in glycerol as stocks. Working cultures were obtained by inoculating 50 ml TSB with 100 µl stock cultures and incubating for 24 h at 35 or 32°C.

6.2.4. Antimicrobial susceptibility test in TSB

Bacterial inhibition in growth medium was studied using time-kill assays. The bacterial cultures were harvested at late logarithmic phase (overnight) and diluted to approximately 5.0-6.0 log CFU ml^{-1}. A total volume of 25 ml consisting of 12.5 ml of TSB, 10 ml of nano-dispersed eugenol (or deionized water as control) and 2.5 ml of inoculum was used. Spray dried capsules were hydrated to various eugenol concentrations to obtain nano-dispersions such that the overall final eugenol concentrations in the TSB mixture were 1.0, 1.25, 1.5, 1.75, 2.0 and 2.25 g/l eugenol . The mixture was adjusted to pH 6.8 and incubated at 35°C for *E. coli* treatments and 32°C for *Lm* treatments. At regular intervals (0, 3, 6, 12 and 24 h), a bacterial suspension Sample (1.0 or 0.1 ml) was serially diluted in 0.1 % peptone, plated in duplicate (1.0 or 0.1 ml) using tryptic soy agar (TSA), incubated for 24 h at optimum growth temperatures, and then enumerated for viable count as CFU ml^{-1}. The lowest concentration inhibiting visible growth of the test microorganisms above the original inoculums, approximately 4.0-5.0 log CFU ml^{-1} (inhibition) after 48 h was determined as the minimum inhibitory concentration (MIC), while the lowest concentration tested where bacterial death (inactivation) was observed after 48 h was determined as the minimum bactericidal concentration (MBC). The growth medium with and without bacterial culture was used as positive and negative controls in all treatments.

6.2.5. Antimicrobial susceptibility test in Milk

Inhibition of *E. coli* and *Lm* pathogens was studied in milk using time-kill assays as described above. The bacterial cultures were harvested at late logarithmic phase (overnight) and diluted to approximately 5.0-6.0 log CFU ml^{-1}. A total volume of 25 ml consisting of 12.5 ml of milk, 10 ml of nano-dispersed eugenol (or deionized water as control) and 2.5 ml of inoculum was used. The TSB mixture contained nano-dispersions with final overall concentrations of 3.5, 4.5, 5.5 and 6.5 g/l eugenol. After incubation at 35, 32, 25 or 4°C for 0, 3, 6, 12 and 24 h, a bacterial suspension Sample (1.0 or 0.1 ml) was drawn from the mixture and serially diluted in 0.1 % peptone, plated in duplicate (1.0 or 0.1 ml) using TSA, incubated for 24 h at optimum growth temperatures, and then enumerated for viable count as CFU ml^{-1}.

6.2.6. Data and statistical analysis

Duplicates of spray dried Samples were prepared for each formulation. Each spray dried Sample was tested in duplicate. Data from four replicate experiments were pooled to calculate the statistical mean and standard error of the mean for each condition tested. Data were analyzed by mixed model analysis of variance (ANOVA, $p<0.05$) using SAS 9.2 (SAS Institute, NC)

6.3. Results and Discussion

6.3.1. Nano-dispersion properties

Physical properties of nano-dispersions are detailed in Chapter 5. A representative particle size distribution is presented in Figure 6-1 for Sample prepared with WPI-MD40 conjugate at a 1:2 mass ratio. Approximately 20 % of particle diameter measurements were less than 100 nm, while up to 10 % were less than 50 nm. About 5 % of particles were from 100-160 nm. It may be possible that dispersions contained aggregated particles of varying sizes rather than individual droplets.

6.3.2. Determination of MIC and MBC

Essential oils such as eugenol are potent antimicrobials, however they are difficult to incorporate in foods, because of their hydrophobic nature and low water solubility. The concentrations at which they are effective in food may well exceed levels that are acceptable to the consumer from perspectives of sensory appeal (Burt 2004). Ideally, the maximum microbial kill is achieved at a minimum antimicrobial usage level without compromising sensory acceptance of consumers. Thus, a balance between the efficacy and desirable organoleptic properties may be achieved by knowing the MIC and MBC of antimicrobials (Reiners and others 2000; Rivas and others 2010).

Table 6-1 presents the MIC and MBC values for eugenol contained in the nano-dispersions and as free oil, evaluated for growth inhibition and inactivation activity against different strains of *E. coli* O157:H7 and *Lm* in TSB and milk. Since results were similar between strains for each pathogen, this paper will restrict its discussion to *E. coli* O157:H7 ATCC 43889 and *Lm* strain Scott A. Overall, nano-dispersed eugenol demonstrated MIC of 1.25 g/l for *E. coli* and 1.75 g/l for *Lm*, with MBC of 1.75 g/l for both *E. coli* strains and 2.0 g/l for *Lm* strains in TSB adjusted to pH 6.8 (Figure 6-3). The present findings are similar to studies conducted by Gayinsky and others (2007), in which MIC values reported were approximately 1.5 g/l eugenol stabilized by surfactant micelles for both bacteria. Pei and others (2009) reported MIC of eugenol for *E. coli* to be 1.6 g/l in growth media. In milk, nano-dispersed eugenol was bacteriostatic at 4.5 g/l (almost fourfold when compared to TSB) and bactericidal at 6.5 g/l concentration for all strains (

Figure 6-4). It is well known that antimicrobial effectiveness is greatly reduced when applied to foods, due to interactions with food constituents such as proteins, or lack of solubility in aqueous environment. Gayinsky and others (2007) reported MIC of 5 g/l eugenol against *E. coli* and *Lm* when applied to semi-skimmed milk. It was found that increasing milk fat content proportionately increased antimicrobial MIC values.

In general, essential oils have been reported to be more effective against gram-negative microorganisms. Eugenol's antibacterial activity against gram-negative pathogens such as *E. coli* O157:H7 and gram-positive ones such as *Lm* (Pérez-Conesa and others 2011) is attributed to its interactions with cell membrane. Since food matrices are complex, it is important to understand the key factors that play a role in the final outcome of an antimicrobial delivery system. Being a hydrophobic phenol, eugenol is attracted by or even dissolved in lipophilic food components such as proteins and lipids. On one hand, studies have reported that the high flavor binding capacity of milk proteins can be a limiting factor for antimicrobial efficacy (Baranauskiene and others 2006; Gutierrez and others 2008; Gutierrez and others 2009), while on the other, hydrophobic interactions of proteins with essential oils have promoted better efficacy, by acting as effective vehicles and promoting dispersion into aqueous medium where bacteria are present. When eugenol, dispersed in an aqueous phase by proteins, is exposed to a lipid containing system such as milk, the partitioning process becomes even more complex. There may exist a competition between milk fat phases and phospholipid containing bacterial cell wall for the hydrophobic oil. Thus eugenol may be distributed between the milk fat phase and bacterial membrane interfaces, thus becoming less available for antimicrobial action. These interactions are then governed by the system surrounding and stabilizing eugenol droplets, in this case WPI–MD conjugates, and by the type of organism (gram-negative or gram-positive) involved. Although eugenol may be required in higher concentrations, it is now able to easily target the lipopolysaccharide (LPS) layer for penetration into gram-negative bacterial cells, as compared to the thick peptidoglycan layer in gram-positive organisms. Alternatively, it changes fatty acid composition of cellular structure, particularly important phospholipids, causing leakage of contents necessary for survival (Burt 2004). This increases its potency against the previously resistant gram-negative pathogens significantly, as seen in the present study.

While free oil demonstrated slightly better activity against *E. coli* and *Lm* strains in TSB, nano-dispersed eugenol showed a better activity in milk than free oil at concentrations higher than solubility of eugenol. Again, it may be possible that nano-dispersing eugenol allows better bacterial contact of antimicrobial. The free hydroxyl group, that eugenol possesses, imparts some polar characteristics, making it slightly soluble in aqueous environment, where bacteria exist, capable of contacting and passing through hydrophilic sections of bacterial cell envelope (García-García and others 2011; Karapinar and Esen Aktug 1987). The dispersion system may be potentially providing greater surface area, reduced surface tension and thus enhanced antimicrobial activity. This confirms that the use of nano-dispersions as new, natural antimicrobial ingredients in foods, which can deliver higher eugenol concentrations, without affecting appearance, is promising.

6.3.3. Antimicrobial activity of nano-dispersed eugenol in tryptic soy broth

The role of WPI:MD ratio (Figure 6-3A, C) and MD chain length (Figure 6-3B, D) in overall antimicrobial efficacy of nano-dispersion containing eugenol in TSB against *E. coli* O157:H7 ATCC 43889 and *Lm* strains Scott A, at pH 6.8 at optimum growth temperatures was evaluated in this study. Nano-dispersion containing 1:2 WPI:MD ratio showed significantly better ($p<0.05$) antimicrobial inhibition against *Lm* when compared to those containing 1:1 and 2:1 ratios . At an overall eugenol concentration of 1.75 g/l, there was a difference of 2.0 log CFU ml^{-1} after 48 h of bacterial exposure of nano-dispersed eugenol between formulations containing 1:2 and 1:1 ratios. It is interesting to note that, no such difference was seen for *E. coli,* however, an overall lower concentration of eugenol (1.25 g/l) was required to achieve similar reduction in population values for *E. coli*. For nano-dispersions containing MD of different chain lengths, there was no statistical significance observed in the antimicrobial activity against either pathogen. 1.25 g/l and 1.75 g/l eugenol yielded 4.0 log CFU ml^{-1} reductions from initial population values for *E. coli* and *Lm* respectively.

6.3.4. Antimicrobial activity of nano-dispersed eugenol in 2 % reduced fat milk

Dispersions prepared from conjugates with different WPI:MD ratios (Figure 6-5A, C) and MD chain lengths (Figure 6-5B, D) were similarly evaluated for antimicrobial efficacy against *E. coli* O157:H7 ATCC 43889 and *Lm* strains Scott A in 2 % reduced fat milk. Similar to observations in TSB, nano-dispersion containing 1:2 WPI:MD ratio showed significantly better ($p<0.05$) antimicrobial inhibition against *Lm* when compared to those containing 1:1 and 2:1 ratios. At an overall eugenol concentration of 4.5 g/l, there was a difference of 2.0 log CFU ml^{-1} after 48 h of bacterial exposure of nano-dispersed eugenol between formulations containing 1:2 and 1:1 ratios. For nano-dispersions containing MD of different chain lengths, formulations demonstrated non-statistical but, observable difference in bacterial population reduction after 48 h. These results indicate that nano-dispersed eugenol retained its antimicrobial activity in the presence of proteins, lipids and other food constituents and that its demonstrated effectiveness against both gram-negative and gram-positive bacteria seems promising in broad applications.

6.4. Conclusions

The present study confirms the efficacy of nano-dispersed eugenol against *E. coli* O157:H7 and *Lm* when applied to a simple, model broth system (TSB) and a complex, model food system (milk). Optimization of formulation ingredients such as WPI:MD ratio, MD chain length can further improve antimicrobial effectiveness. Nano-dispersed eugenol can be used alone or along with mild heat treatment as a hurdle technology in the food industry. Nanoscale systems can be used to deliver antimicrobials at concentrations much higher than solubility levels without affecting visual appearance, promising for application in various products.

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Appendix

Table 6-1. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of eugenol (g/l) required for inhibition and inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in tryptic soy broth (TSB) and 2 % reduced fat milk, at pH 6.8.

Bacteria	**Escherichia coli O157:H7**				**Listeria monocytogenes**			
Strain	**ATCC 43889**		**ATCC 43894**		**Scott A**		**101**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Nano-dispersed Eugenol[a] in TSB (g/l)	1.25	1.75	1.25	1.75	1.75	2.0	1.75	2.0
Free Eugenol in TSB (g/l)	1.0	1.5	1.0	1.5	1.5	1.75	1.5	1.75
Nano-dispersed Eugenol[a] in Milk (g/l)	4.5	5.5	4.5	5.5	5.5	6.5	5.5	6.5
Free Eugenol in Milk (g/l)	4.5	5.5	4.5	5.5	5.5	6.5	5.5	6.5

[a]Nano-dispersions were made using conjugates at 1:2 WPI:MD40 mass ratio.

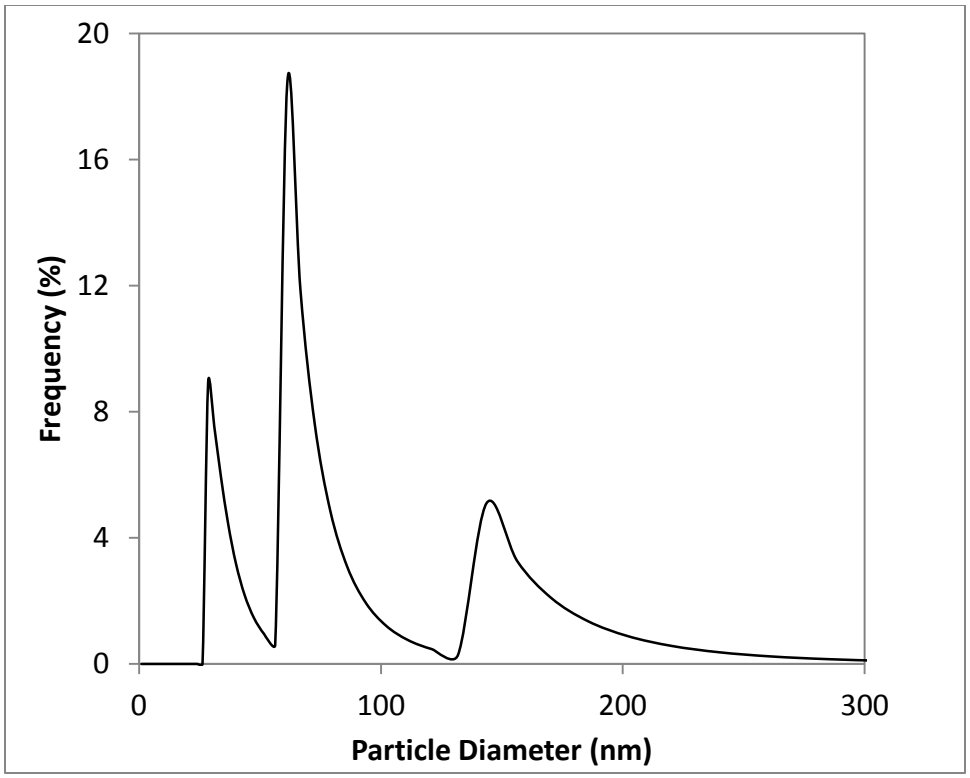

Figure 6-1. Particle size distribution of eugenol-containing nano-dispersion made using 1:2 WPI:MD40 conjugate, at pH 7.0. Values represent means from four measurements, two from each of two replicates.

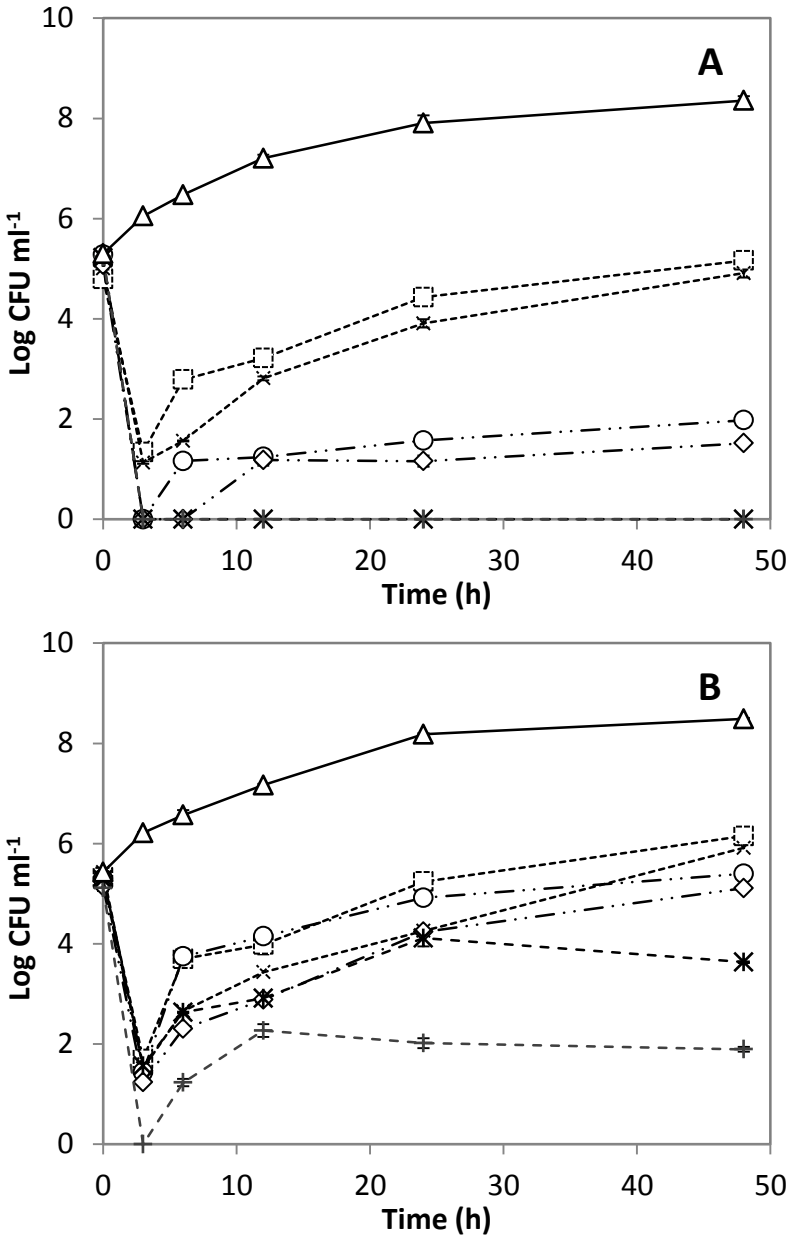

Figure 6-2. Antimicrobial activity of free eugenol and eugenol-containing nano-dispersion made using 1:2 WPI:MD40 conjugate, used at an overall eugenol concentration of 1.0, 1.25 and 1.75 g/l in tryptic soy broth adjusted to pH 6.8, against *Escherichia coli* O157:H7 ATCC 43889 at 35°C (A) and *Listeria monocytogenes* strain Scott A at 32°C (B), respectively. Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed eugenol: (□) 1 g/l, (○) 1.25 g/l, (✳) 1.75 g/l; Free eugenol: (×) 1 g/l, (◇) 1.25 g/l, (+) 1.75 g/l and Control of bacterial culture with no eugenol (△).

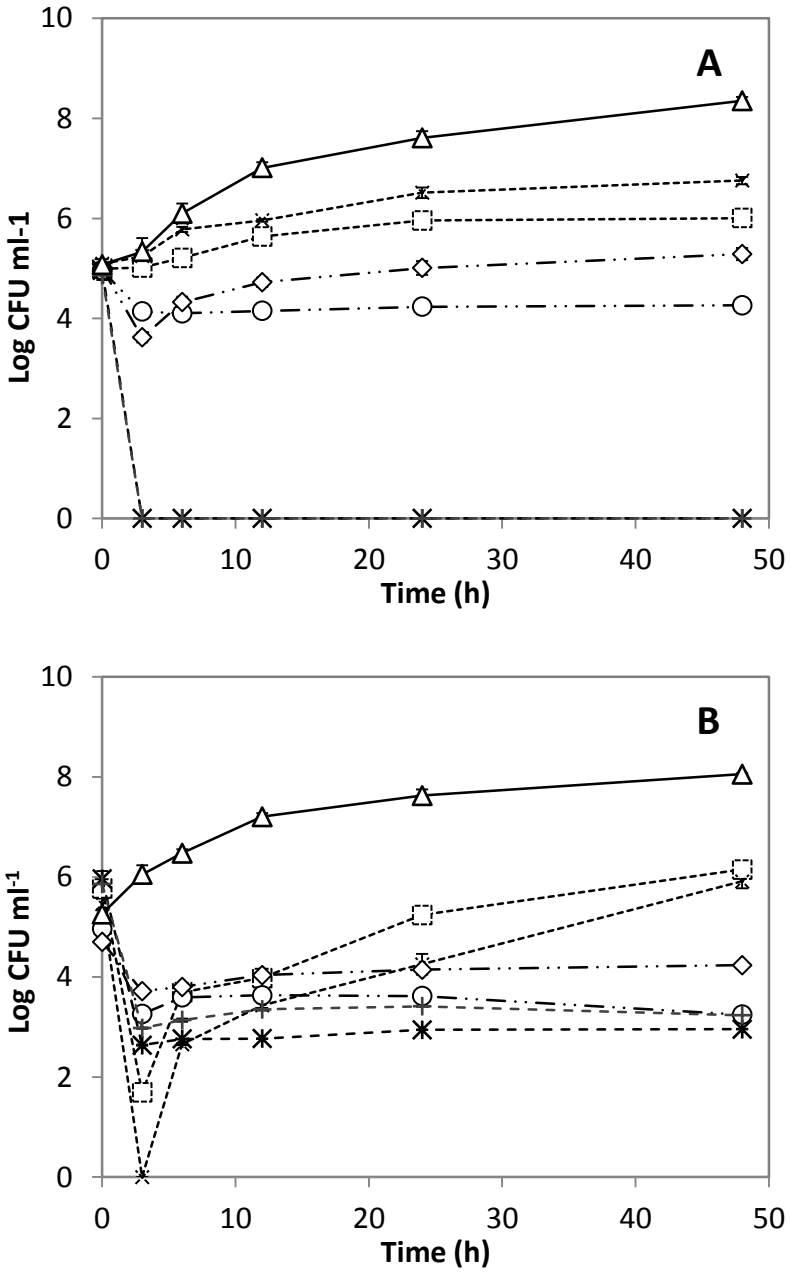

Figure 6-3. Antimicrobial activity of free eugenol and eugenol-containing nano-dispersion made using 1:2 WPI:MD40 conjugate, used at an overall eugenol concentration of 3.5, 4.5 and 5.5 g/l in 2 % reduced fat milk, against *Escherichia coli* O157:H7 ATCC 43889 at 35°C (A) and *Listeria monocytogenes* strain Scott A at 32°C (B), respectively. Error bars represent standard error of mean from four measurements, two from each of two replicates. Nano-dispersed eugenol: (□) 3.5 g/l, (○) 4.5 g/l, (✳) 5.5 g/l; Free eugenol: (×) 3.5 g/l, (◇) 4.5 g/l, (+) 5.5 g/l and Control of bacterial culture with no eugenol (△).

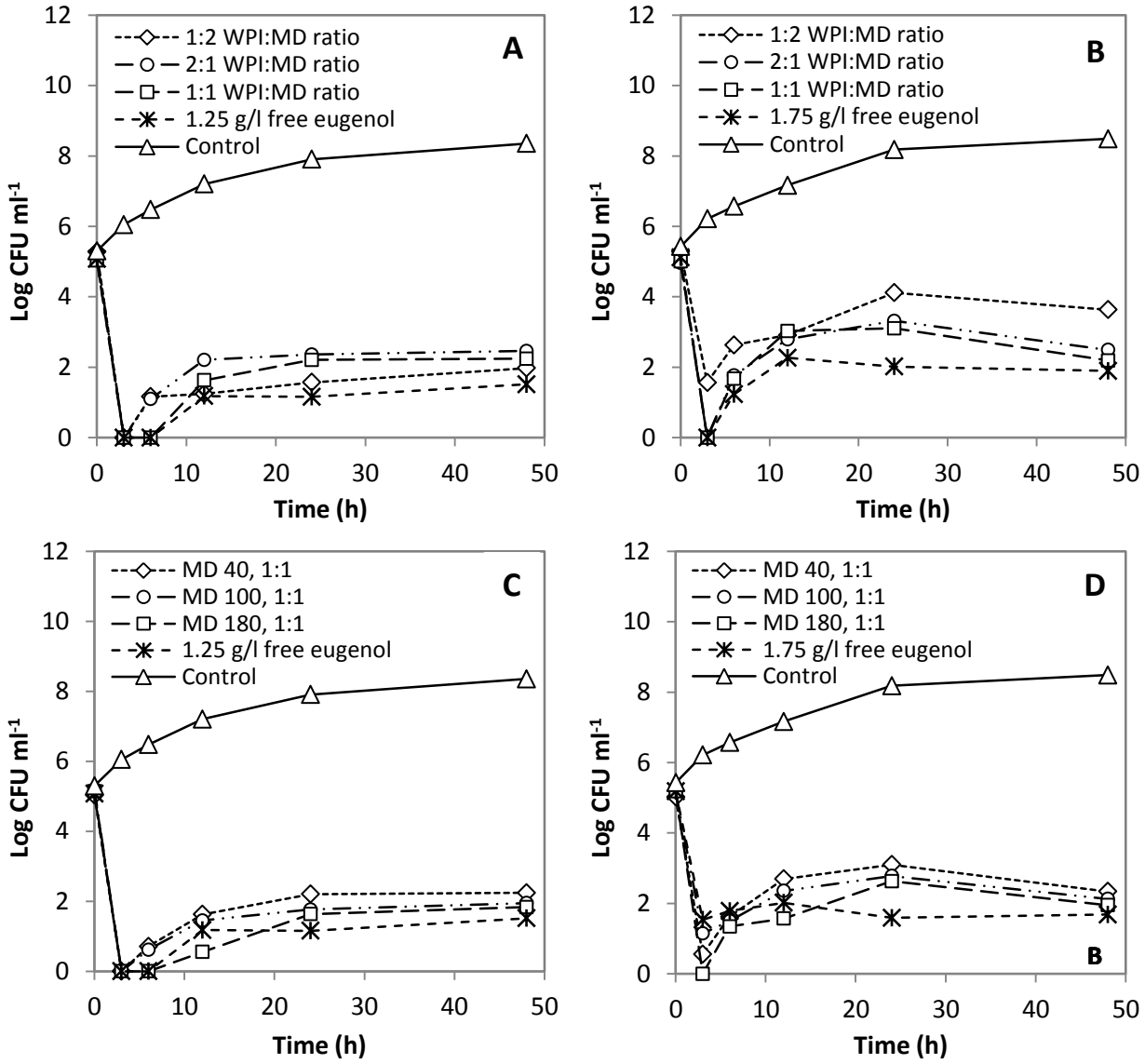

Figure 6-4. Inhibition of *Escherichia coli* O157:H7 ATCC 43889 (A and C) and *Listeria monocytogenes* strain Scott A (B and D) by 4.5 g/l eugenol, as affected by WPI:MD ratio (A and B) and maltodextrin chain length (C and D), respectively, used in tryptic soy broth adjusted to pH 6.8, at 35 and 32 ˚C for *E. coli* and *Lm strains,* respectively. Error bars represent standard error of mean from four measurements, two from each replicate.

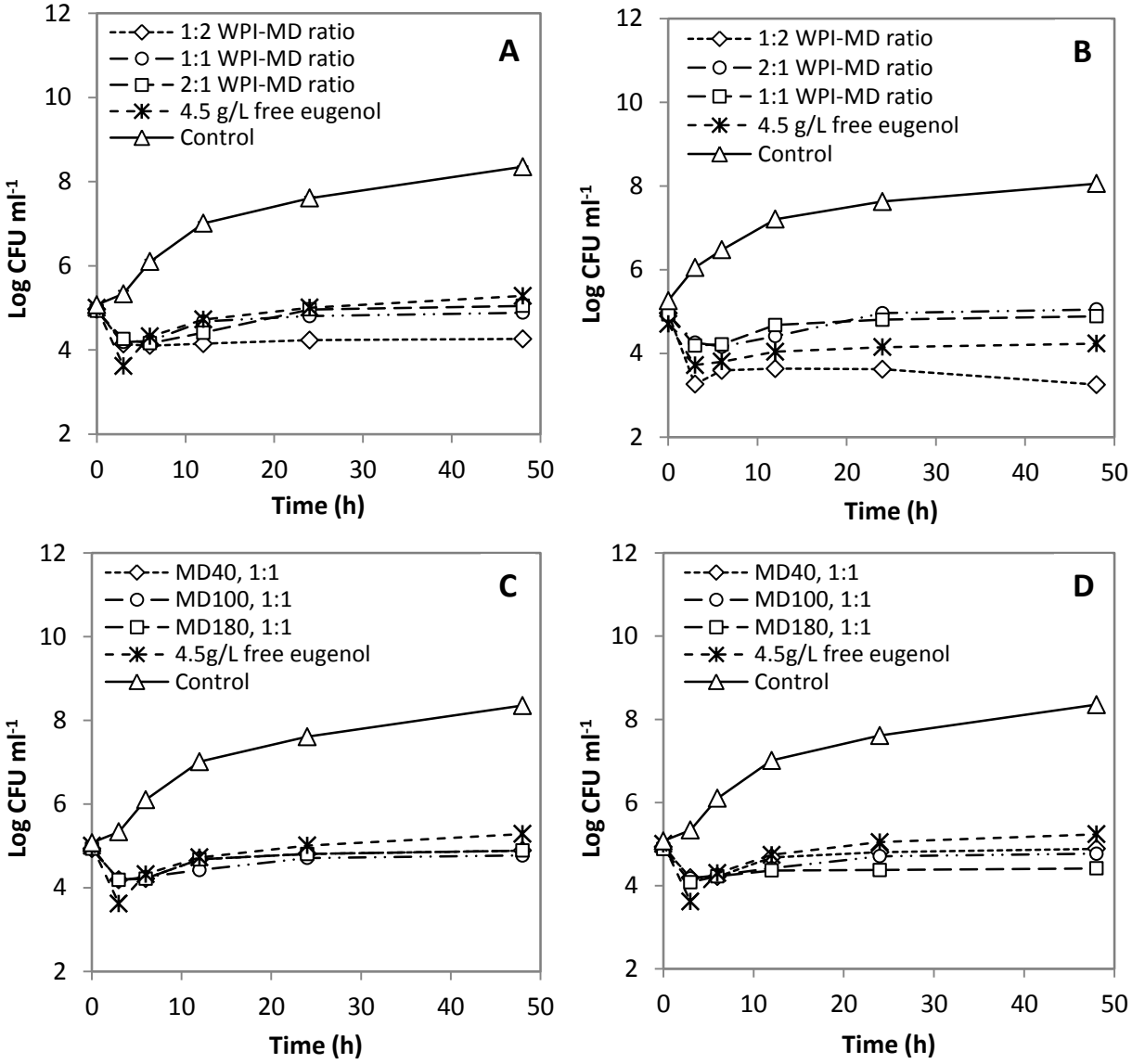

Figure 6-5. Inhibition of *Escherichia coli* O157:H7 ATCC 43889 (A and C) and *Listeria monocytogenes* strain Scott A (B and D) by 4.5 g/l eugenol, as affected by WPI:MD ratio (A and B) and maltodextrin chain length (C and D), respectively, used in 2 % reduced fat milk adjusted to pH 6.8, at 35 and 32 ˚C for *E. coli* and *Lm strains,* respectively. Error bars represent standard error of mean from four measurements, two from each replicate.

7. Concluding remarks and future work

This dissertation demonstrated that the researched novel antimicrobial nano-dispersion systems are clear, heat stable and not only significantly better in dispersing lipophilic essential oils from thyme, 'thymol' and from clove, 'eugenol' but, also inhibitory against both gram positive and gram negative foodborne pathogens such as *E. coli* O157:H7*, Listeria monocytogenes, Salmonella typhimurium* and *Staphylococcus aureus*.

Nano-dispersions were prepared from spray dried capsules of oils encapsulated in conjugates of whey protein isolate (WPI) and maltodextrin (MD). Conjugates, with water soluble MD covalently linked to amphiphilic WPI, anchored at the surface of capsules in dispersions improve dispersibilty of capsules because of an improved overall hydrophilicity of capsules compared to controls. Different mass ratios of WPI:MD and MD chain lengths in formulations influenced the encapsulation performance of spray dried capsules containing different essential oils and their dispersion properties. Clear dispersions for thymol and eugenol-containing capsules were produced at 1:2 WPI:MD mass ratio, but with MD180 and MD40 respectively. These differences may have occurred, because, emulsions were not prepared under controlled conditions; thus, fundamental physical bases of observations will require extensive future work using advanced homogenizers and precise conditions. Nevertheless, the optimized emulsion compositions, from the present study, can be used as a basis for designing systems for other essential oils with similar physical chemistries.

This work demonstrated antimicrobial activity of nano-dispersed thymol and eugenol in microbiological media, tryptic soy broth and model food systems, apple cider and 2 % reduced fat milk, against *E. coli O157:H7* and *Listeria monocytogenes,* at different pH and temperatures. nano-dispersed eugenol showed higher antimicrobial efficiency in milk than free oil, while inhibitory activities were similar for both nano-dispersed and free eugenol and thymol in media, and apple cider. Since *Lm* strains did not

grow at low pH in apple cider, other suitable gram positive bacteria must be tested for susceptibility to the antimicrobial nano-dispersions, along with more complex foods systems including high solutes such as salt and sugar. Further investigations on the effect of milk fat levels on efficiency of antimicrobial nano-dispersions can give a better understanding of the antimicrobial performance of the nano-dispersions in challenging food matrices. The nano-dispersions, in the current study, showed excellent antimicrobial efficacy, however, being highly aromatic and volatile, it is important to consider undesirable sensory attributes associated with their application in foods at concentrations. Sensory threshold tests must be conducted for concentrations at which nano-dispersed thymol and eugenol are effective for food preservation and pathogen control.

Finally, the simple, scalable, food grade/GRAS and low-cost technology presented in this research can be used to disperse various other lipophilic ingredients in foods, without adversely affecting their visual appearance, thus contributing to the growing functional food and beverage market.

VITA

Bhavini Dipak Shah was born in Mumbai, Maharashtra, India, on July 30, 1985. After graduating from St. Teresa's Convent High School in Mumbai in 2001, she entered College of Home Science, Nirmala Niketan, University of Mumbai to pursue a Bachelor of Science degree in Foods, Nutrition and Dietetics. In June 2006, she continued to work on her Master of Science degree in Foods, Nutrition and Dietetics in the same institution, while conducting research for her thesis project at Bhabha Atomic Research Center in Mumbai. In July 2008, she came to Knoxville, TN, to begin her doctoral study in the Food Science and Technology Department at the University of Tennessee, Knoxville under the direction of Dr. Qixin Zhong. She initially worked on a project involving the development of enteric formulations for a water-soluble amino acid, L-glutamine. Her dissertation research was focused on the development of novel, nanoscale delivery systems containing essential oils such as thymol, carvacrol and eugenol to provide antimicrobial efficacy against foodborne pathogens such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 in beverages such as apple cider and milk. She will soon be working at Mead Johnson Nutrition as Sr. Product Development Scientist, in Evansville, Indiana.