

**Synthesis of some biobased surfactants, and their functionalities as emulsifiers and
antimicrobial agents.**

by

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ABSTRACT

Biobased surfactants, which are biochemicals derived from biological resources via various methods like enzymes and microbial fermentation, can achieve similar application functionality to petroleum-based surfactants. The conventional surfactants could face challenges in terms of limited fossil fuel availability, harsh processing conditions, low biodegradability and high aquatic toxicity. Biobased surfactants, on the other hand, can address some of these challenges; however, they also have their own challenges, for example, lower yield and production rate compared with conventional surfactant production method.

The overall goal of this work was to investigate the synthesis of specific biobased surfactants and evaluate their functionalities for potential food applications. This was accomplished by 1) optimizing the synthesis condition for glucose-fatty acid esters and evaluate their emulsification properties, 2) exploring the antimicrobial performance of some biobased surfactants and elucidate their mechanisms, and 3) exploring the emulsion-stabilizing effect of microbial biosurfactants and the emulsions' antimicrobial properties.

The method explored to synthesize glucose fatty acid esters biobased surfactants using lipase with the substrates of glucose of fatty acids. The emulsifying and antimicrobial properties of the glucose-fatty acid esters and two microbial biosurfactants - surfactin and fatty acyl glutamic acid (FA-glu) were also investigated. It was found that the conversion percentages of fatty acids and rates of reaction depended on the reaction substrate concentration the reaction medium. The glucose-fatty acid esters demonstrated some emulsification capabilities, but weaker than commercial sucrose esters compared. One of the glucose esters, glucose laurate inhibited growth of *E.coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Enteritidis during 24 h at the concentration of 6.5 mg/mL. The microbial biosurfactant FA-glu inhibited pathogens from

growing at the concentration of 25 mg/mL; the mechanism of inhibition was the disruption of bacterial cell membrane by FA-glu.

Surfactin, FA-glu and two other common food emulsifiers (lecithin and Tween 80) were also studied for their ability to stabilize nano- and coarse emulsions containing cinnamaldehyde (CM) and the emulsions' inhibition effect on pathogens. Although the minimum inhibitory concentrations were not reduced compared with non-emulsified CM, the dispersion of the CM in the emulsion strengthened the inhibition of pathogens at sub-minimum inhibitory concentrations. There was no definite relationship between the emulsion droplet size and antimicrobial effect.

In summary, our study provided important information on some biobased surfactants for their use in food and agriculture industries as the potential "clean label" emulsion and/or antimicrobial ingredients.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Research Premise:

Biobased surfactants are amphiphilic molecules that are produced from biological or renewable agricultural sources (Salimon, Salih, & Yousif, 2012); many studies have used enzymes or microbial fermentation to produce biobased surfactants. Biobased surfactants, compared with traditional petroleum based surfactants, possess enhanced biodegradability, and the methods to produce them are more sustainable. Based on structural characteristics of biobased surfactants, there are several types, such as glycolipids, lipopeptides and phospholipids. Sugar-fatty acid esters are a type of glycolipids that can be enzymatically synthesized by lipase, which catalyzes the formation of ester bond. Many researches have used disaccharides and free fatty acids to synthesize the esters via enzymes, but not many studies have used monosaccharide (glucose) to synthesize sugar-fatty acid ester and study their functionalities. Aside from being good emulsifiers, some sugar-fatty acid esters have demonstrated antimicrobial properties; however, the antimicrobial properties for glucose esters have also not been studied.

Glycolipid and lipopeptide types of biobased surfactants can be also produced from microbial fermentation with similar functionalities as emulsifiers and antimicrobial agents. Thus, they have potential applications in industries such as soil bioremediation, detergent, and pathogen control and disinfectants. Surfactin and its variant fatty acyl glutamic acid (FA-glu), which are lipopeptide biosurfactants produced from fermentation by *Bacillus subtilis*, are shown to have good surface activity. Surfactin has been studied extensively for its varied capability, for example, soil bioremediation (Bustamante, Duran, & Diez, 2012), biofilm inhibition (Rivardo, Turner, Allegrone, Ceri, & Martinotti, 2009; Sriram, Kalishwaralal, Deepak, Gracerosepap, Srisakthi, & Gurunathan, 2011), and antimicrobial activity (Zhao, Shao, Jiang, Shi, Li, Huang, et al., 2017).

Nevertheless, surfactin has not been reported in potential food systems for emulsion stabilizing effect, and their influence on pathogen in food system was not studied. FA-glu, a variant of surfactin with only one amino acid as polar head with enhanced aqueous solubility (Reznik, Vishwanath, Pynn, Sitnik, Todd, Wu, et al., 2010), has not been studied for emulsion formation and antimicrobial effects. Exploring these two biosurfactants functionalities as emulsifiers and antimicrobial agents will provide new information for food and agricultural industries, as these can be viewed as “clean label” ingredients. If the biosurfactants possess both emulsifiers and antimicrobial effects, their applicability in food and related applications will be enhanced.

1.2 Overall study goal and hypotheses

The overall goal of this work was to investigate the synthesis of specific biobased surfactants and evaluate their functionalities for potential food applications. This was accomplished in a series of studies to achieve three specific objectives, namely, 1) to optimize the synthesis condition for glucose-fatty acid esters and evaluate their emulsification properties, 2) to explore the antimicrobial performance of some biobased surfactants and elucidate their mechanisms, and 3) to explore the emulsion-stabilizing effect of microbial biosurfactants and the emulsions' antimicrobial properties. For Objective 1, the optimization of synthesis condition in terms of reactants' ratio, the purification methods, the confirmation of the products and the stabilizing ability of oil-in-water emulsion were studied. The hypothesis tested was that the glucose and free fatty acid can exclusively form monoesters in the mixed organic solvent system with emulsion stabilizing effects, and their yields is affected by substrate ratios. For Objective 2, the antimicrobial inhibition effect was studied and compared for different biobased surfactants, including synthesized glucose esters, commercial sucrose esters and microbial biosurfactants. The mechanisms of the antimicrobial behavior for one of the surfactants was also investigated using

artificial cell membrane components. The hypothesis was that the biobased surfactants tested demonstrate antimicrobial behavior, by disrupting and solubilizing of bacterial cell membrane. For Objective 3, the nanoemulsions/emulsions stabilized by two microbial biosurfactants were created and studied for their abilities to improve the antimicrobial effect of cinnamaldehyde against two pathogens in broth media. The hypothesis was that the emulsions stabilized by the biosurfactants containing cinnamaldehyde can improve the antimicrobial effect of non-emulsified cinnamaldehyde. The nanoemulsions would have better antimicrobial efficacy compared to free cinnamaldehyde and coarse emulsions.

1.3 Significance

As consumers are paying more attention to “clean-label” food products, food companies are eagerly looking for natural, biobased, and sustainably manufactured food ingredients. Biobased surfactants can be produced naturally either by enzymes or bacterial fermentation. Also, the biobased surfactant production can add value to the underutilized materials such as waste biomass and coproducts, as microbes can utilize them as growth medium. Therefore, it is important to optimize synthesis conditions for biobased surfactants and explore their functionalities as food emulsifiers and antimicrobial agents. Our study will provide important information on some biobased surfactants for use in food and agriculture industries as the potential “clean label” ingredients.

1.4 Dissertation organization

The dissertation is organized in 6 chapters. Chapter 1 describes the goals and significance of the research topics. Chapter 2 provides the background information on biobased surfactants synthesis using enzymes, their applications and functionalities as emulsifiers and antimicrobial

agents. Chapters 3, 4, and 5 present experimental research with pertinent results and discussion. These Chapters are presented as manuscripts that have been written, submitted or accepted as journal articles. The manuscript formats followed are for *Food Chemistry*, *Journal of Agriculture and Food Chemistry*, and *Food Chemistry* journals respectively. Finally, the Chapter 6 provides a general research conclusions and direction to possible future studies.

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CHAPTER 2. LITERATURE REVIEW: BIOBASED SURFACTANTS PRODUCTION AND THEIR USE AS EMULSIFIERS AND ANTIMICROBIAL AGENTS

2.1 Abstract

This literature review provides the information on biobased surfactants that can be produced by enzymes and microbial fermentation, and their characteristics and functionality. The synthesis characteristics of lipase-catalyzed sugar-fatty acid esters, regarding the regional esterification selectivity by lipase and various reaction systems are described in details. The biosurfactants produced via microbial fermentation are summarized regarding their applications and limitations. Finally, the antimicrobial performance of both microbial based and lipase synthesized biosurfactants and their mechanisms are discussed. The literature review reveals research gaps in producing biobased surfactants (yield improvement), and characterizing them for food related applications, namely, antimicrobial mechanisms against food-borne pathogens. This dissertation research aims to fulfil those research gaps.

2.2 Surfactants, biobased surfactants, their market value and applications

Surfactants are amphiphilic molecules having both hydrophilic and hydrophobic moieties that can adsorb onto the interface between different phases. Because of this characteristic, surfactants show various surface-active properties, including reducing interfacial tension, which enables them for a wide-range of uses in food, pharmaceutical, detergent, cosmetic, oil recovery, and soil bioremediation. Based on the charge on hydrophilic moieties, surfactants can be classified into four groups: anionic with negatively charged head group, cationic with positively charged head group, zwitterionic having both anionic and cationic center, and nonionic. Based on their adsorption properties, anionic surfactants are the most important group of surfactants

used in cleaning and detergent industry (Steber, 2007). Cationic surfactants are important in formulating fabric softeners and hair-conditioning products as they have great potency against microorganisms (Cross, 1994). Zwitterionic and nonionic surfactant also have various application in detergent products. Most of the surfactants used in various industry are chemically synthesized from petroleum-based resources. The petroleum-derived surfactants may not be readily biodegradable and possess toxicity to the environment. Biobased surfactants, unlike chemically synthesized surfactants, are derived from renewable agricultural or biological resources (Salimon, Salih, & Yousif, 2012), which can be produced by chemical or enzymatic catalyzed reactions or microbial fermentation using biological feedstocks (Hayes, 2009). Because of the nature of the biosurfactants, they are known to have enhanced biodegradability, safety, and sustainability (Hayes, 2009) with the production process yielding less carbon dioxide (Patel, 2003). Most biosurfactants are nonionic, because biological feedstocks provide non-charged groups for the bio-synthesized surfactants (Hayes, 2009).

Aside from being environmentally friendly process for biobased surfactants, many other factors contribute to the need and rationale to produce biobased surfactants, such as the prices and supply of petroleum crude oil and oleochemical feedstock (Hayes, 2009). The price for petroleum crude oil has fluctuated since 1974 (Macrotrends, 2017), and the price of oleochemical feedstock, such as palm oil and other vegetable oils, are closely linked with the petroleum products (ICIS chemical business, 2012). The increased demand for biofuels also led to the higher production for palm, rapeseed and soybean oils (Unnithan, 2015), which also has caused problems of deforestation and animal habitat loss (Gao, 2011). To meet the market demand, genetically engineered crops are expected to increase the yield of oil (Gressel, 2008). Although these may positively or negatively affect the biosurfactant industry, the global

biosurfactant market revenue was at 1.76 billion dollars in 2015 (Global market insights, 2017) and is expected to grow fast and the revenues may reach \$2.3 billion by year 2020 (Grand view research, Inc, 2015). Another driving force for biosurfactants growth is the increased need for non-ionic surfactant (Patel, 2003) for their synergistic effect with ionic surfactants in concentrated detergent products. The advantages of combining non-ionic and ionic surfactants include reduce critical micelles concentration (Jin, Garamus, Liu, Xiao, Eckerlebe, Willumeit-Römer, et al., 2016; Z.-G. Zhang & Yin, 2005) and avoid precipitation (Jin, et al., 2016).

Biobased surfactants mostly used in are rhamnolipids, sophorolipids, methyl ester sulfonates, alkyl polyglycosides, sorbitan esters, sucrose esters etc. in various industries, such as, household detergents, personal care, industrial cleaners, food processing, oilfield chemicals, agricultural chemicals, textiles etc. These industries totaled 1.6 billion revenue in 2013 (Global market insights, 2017). Some other potential applications are also described in section 2.4.

2.3 Technologies for biobased surfactants production

Biobased surfactant can be produced chemically, enzymatically or via microbial fermentation from renewable agricultural and biological sources (Hayes, 2009). The following sections will describe some production methods for biobased surfactants, but will mainly focus on enzyme and fermentation- based technologies.

2.3.1 Enzymes for synthesis of biobased surfactants

The enzymes that are used for biobased surfactants synthesis are lipase, glucosidase, proteinase and phospholipases. Lipase catalyzes the ester bond formation between hydroxyl group and carboxylic acid group. The acyl donors (hydroxyl groups) used were usually polyols (Janssen, Lefferts, & Van't Riet, 1990; D. Patil, De Leonardis, & Nag, 2011) or carbohydrate

(Degn & Zimmermann, 2001) and the acyl acceptors (carboxylic acid) were usually fatty acid esters (D. Patil, De Leonadis, & Nag, 2011) or free fatty acid (Ren & Lamsal, 2017).

Glucosidase can catalyze acetal bond formation between saccharides and fatty alcohols (Van Rantwijk, Woudenberg-van Oosterom, & Sheldon, 1999). Proteinase can also catalyze esterification/ transesterification reactions to form ester bond (De Medeiros, Souto, Fagundes, & Costa, 2011) or amide bond (Clapés, Morán, & Infante, 1999) depending on the substrate.

Phospholipases are enzymes that can hydrolyze at different phospholipid sites, thus, producing a wide range of products, including fatty acids, lysophospholipids, diacylglycerides, and phosphate esters; some of these products can be used as emulsifiers (Xie & Dunford, 2017).

2.3.2 Synthesis of sugar-fatty acid by lipase from *Candida antarctica*

2.3.2.1 Characteristics of lipase from *Candida antarctica*

Lipases are one of the most studied enzymes used for synthesis of biobased surfactants for their ability to catalyze a wide range of substrates, including naturally occurring (fats and oil) or synthetic (alkanolamines) ones (Zaks & Klivanov, 1984, 1985). Lipase is produced by many microorganisms, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Streptomyces sp.*, *Rhizomucor miehei*, *Candida Antarctica*, *Candida clindracea*, *Chromobacterium viscosus*, etc. (Kennedy, Kumar, Panesar, Marwaha, Goyal, Parmar, et al., 2006). Among all the lipases from different sources, lipase B, denoted as CALB, were used in various studies and were shown to be very effective and highly robust in different systems with specific regio- and enantio- selectivity (Anderson, Larsson, & Kirk, 1998). CALB has a molecular weight of 33 kDa and a pI of 6.0; it is very active on simple esters, amides, and thiols, rather than larger triglycerides (Rogalska, Cudrey, Ferrato, & Verger, 1993). Free lipase B is a very robust protein, stable in the range of pH 3.5-9.5, with the denaturation temperature varying between 50 °C and 60 °C (Anderson, Larsson, & Kirk,

1998). After immobilization, it is more thermostable and can be used in a continuous operation at 60-80°C (Arroyo & Sinisterra, 1994; Heldt-Hansen, Ishii, Patkar, Hansen, & Eigtved, 1989). The structure of lipase B has been reported mostly made of parallel beta-sheets surrounded by alpha-helices (Uppenberg, Hansen, Patkar, & Jones, 1994). The active serine residue is at the bottom of a narrow and deep pocket which is $10 \text{ \AA} \times 4 \text{ \AA}$ wide and 12 \AA deep. It is the physical restriction and the hydrophobic nature of the pocket that determines the substrate selectivity. The X-ray crystallography demonstrated that there are two channels in the active sites, responsible for the acyl- and alcohol- moieties. The acyl- channel is larger than the latter one, therefore, the lipase B is expected to have a broader selectivity for acyl donors than acyl acceptors.

2.3.2.2 Regioselectivity of the lipase for synthesis of sugar fatty acid esters

The selectivity of acylation reaction between acyl donor and acceptor can be affected by the substrate ratio, structure, and solvent hydrophobicity. For esterification of carbohydrate or polyol, the acylation usually occurs at the primary hydroxyl group. For example, acylation usually happened in the hydroxyl group in the 6th carbon of monosaccharides; Tsavas, Polydorou, Fafila, Voutsas, Tassios, Flores, et al. (2002) used lauric acid, vinyl ester and glucose to synthesize 6-O-lauroyl-glucose using lipase from *Thermomyces lanuginosus*. The purity of the compound was 99% and the structure was confirmed by HPLC and ¹H NMR. Ljunger, Adlercreutz, & Mattiasson (1994) used lipase from *Candida antarctica* for synthesis of glucose esters and found that a monoester was exclusively synthesized when the concentration of octanoic acid and glucose molar ratio was 10:1, whereas, at lower or higher concentrations of octanoic acid, reaction was slower and/or produced diesters. Ducret, Giroux, Trani, & Lortie (1995) used oleic acid and sorbitol or sugars to synthesize esters and reported a preference by lipase to primary hydroxyl group, and the monoesters contents were over 70%.

For esterification of disaccharides, sucrose, maltose, trehalose, and lactose have been used to obtain diesters. The regioselectivity varied depending on substrate and solvents types. Pedersen, Wimmer, Emmersen, Degn, & Pedersen (2002) tested the effect of fatty acid chain length on regioselectivity and reported sucrose monoesters (6'-O-acyl or 6-O-acyl) with butanoic acid (C-4:0) and decanoic acid (C-10:0); however, diester (6,6'-O-acyl) was formed only with C-4:0 in the solvent mix of t-butanol and pyridine. It is possible that increased steric hindrance caused by longer chain fatty acid or the form of diesters in the enzyme active pocket limited the acylation reaction. Woudenberg - van Oosterom, van Rantwijk, & Sheldon (1996) found that the 6 and 6' position of trehalose can be equally acylated with ethyl butanoate when using t- butanol as solvents. When sucrose, isomaltulose and maltulose were used, all of them formed diesters, whereas maltose only had monoester (6'-O-acyl). In both of these studies, maltose produced only monoesters and lactose esters were either not observed or low. They proposed the high crystal lattice energy as the possible reason leading to lower solubility. Rich, Bedell, & Dordick (1995) studied the regioselectivity of sucrose acylation in solvents with different hydrophobicity and suggested solvents have a role in accessibility of sugar molecule to the enzyme active sites that changes the acylation microenvironment. For example, if solvent is more hydrophilic, the glucose stabilizes in reaction medium and increases the 1' OH acylation; the addition of hydrophobic solvent would "push" the whole sucrose molecule into the binding site, thus, reducing the reactivity of 1'-OH, but increasing the reactivity of 6-OH.

2.3.2.3 Reaction systems and challenges for the synthesis of sugar-fatty esters using *Candida Antarctica* lipase

One of the most difficult challenges for sugar fatty acid esters synthesis is choosing a proper solvent that solubilizes both of the substrates (sugars and fatty acids). Laane, Boeren,

Vos, & Veeger (1987) summarized multiple studies and proposed a generalization that synthesis biocatalysis would have low activity in solvent with a partition coefficient ($\log [P]$) less than 2, moderate in a solvent with $\log [P]$ between 2 and 4, and high in polar solvents with a $\log [P] > 4$. The polar solvents were not suggested as good choices because they are able to strip water molecules from the enzyme active site. However, carbohydrates usually have very low solubility in non-polar solvents, so, simply choosing a solvent in which lipases have higher activity was not proper. Because of this, other reaction systems, such as mixed solvent systems, solvent free systems, and ionic solvent systems, need to be explored to address low-solubility of carbohydrates, as discussed below:

Mixed solvent systems

Mixed solvent systems comprise of combination of different organic solvents; however enzyme activities in such systems need to be evaluated. Degn & Zimmermann (2001) evaluated solubilities in a variety of combined solvent systems. They reported good enzyme activity and sugar solubility can be achieved in two mixed solvent systems: tert-butanol and DMSO mixture, and tert-pentanol and pyridine mixture. DMSO and pyridine are good solvents for glucose but lipase exhibited no activity in them. Tert-butanol and pentanol, although had lower solubility for water, were able to retain the highest enzyme activity. Therefore, combination of t-butanol/t-pentanol and DMSO/pyridine as mixed solvent systems were suggested for sugar esters' synthesis. (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999; Ferrer, Cruces, Plou, Bernabe, & Ballesteros, 2000; Pedersen, Wimmer, Emmersen, Degn, & Pedersen, 2002; Ren & Lamsal, 2017). The conversion of sucrose achieved during the synthesis of sugar esters in these systems were over 70% (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999; Ferrer, Cruces, Plou, Bernabe, & Ballesteros, 2000 or even 90% (Ren & Lamsal, 2017). However, the ratio of the solvents will affect

the product varieties as diesters were formed when DMSO content varied (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999).

Solvent-free systems

In view of the limitations in choosing best solvent system, use of the solvent free system under reduced pressure has also been studied. In solvent-free system, it is ultimately important to remove water or alcohol by non-reactive chemicals or reduced pressure. Kim, Han, Yoon, & Rhee (1998) studied the effect of a series salt hydrate pairs on the yield of sucrose caprite and found barium hydroxide $8\text{H}_2\text{O}$ resulted in highest yield (25.3%) and could effectively control the water activity (a_w , 0.44). Proper a_w could make enzyme molecules more flexible than in anhydrous conditions they have better access to bigger molecules such as disaccharides (Adlercreutz, 1992; Valivety, Halling, & Macrae, 1992). However, excessively high water activity would also cause hydrolysis. Another means to enhance miscibility of sugar with molten fatty acid were employing easily accessible sugar acetals and undergoing deacetalisation after esterification, which can avoid the diester synthesis (Sarney & Vulfson, 2001). Fig 2.1 demonstrates the acetonation process and removal of the modifying part: by preparation of the lactose acetals, lactose monoester was successfully synthesized. Fregapane, Sarney, & Vulfson (1991) used monosaccharide acetals and fatty acid methyl ester to make sugar fatty acid esters by lipase from *Pseudomonas sp* and *Chromobacterium viscosum*, with 50-90% product recovery reported under optimal conditions. Adelhorst, Bjokling, Godtfredsen, & Kirk (1990) used ethyl glucopyranosides and achieved 40-80% yield. Ward, Fang, & Li (1997) used xylose acetals to synthesize xylose 5-arachidonate and obtained 83-83% conversion rate. In comparison with mixed solvent systems, the yields were lower with disaccharides, but similar with monosaccharides; however, the extra step to pre-modify the carbohydrate to improve the

miscibility would add to the costs. The advantage of solvent-free systems is that some disaccharides, such as the lactose can be successfully synthesized to monoester.

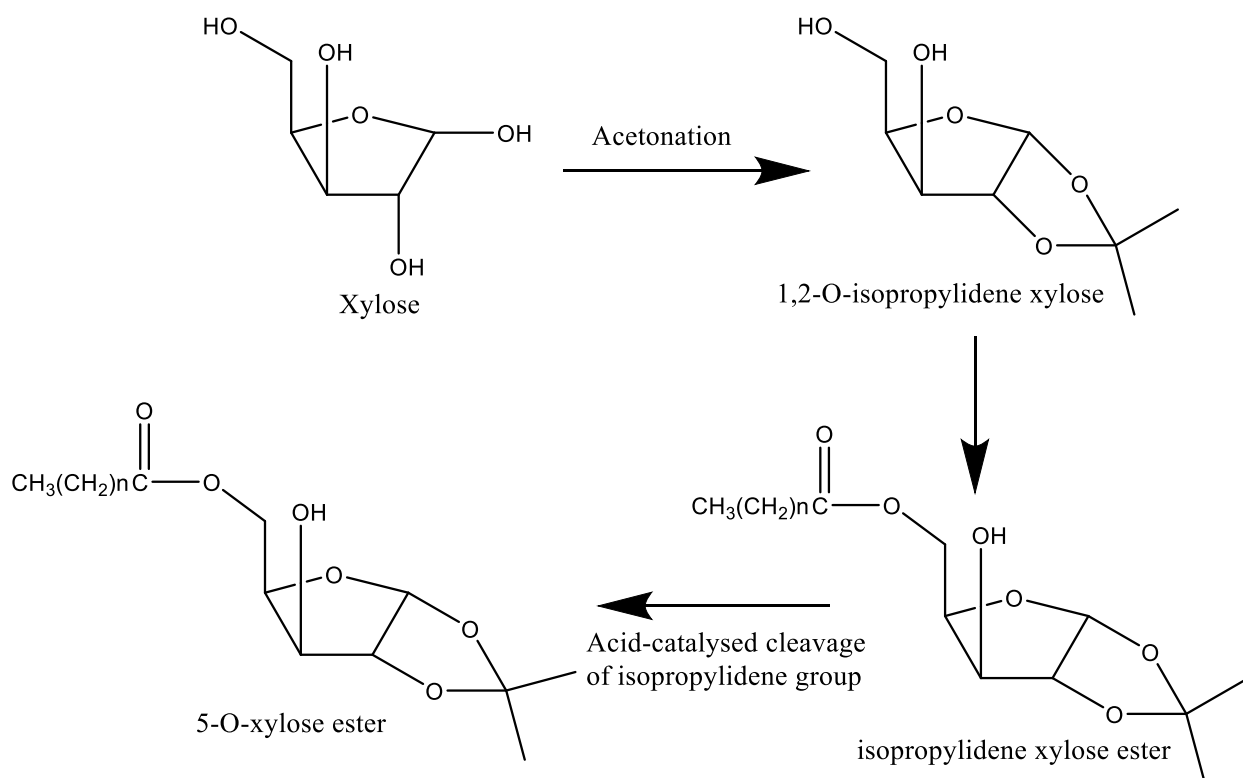


Figure 2.1 Principle of sugar modification followed by enzymatic esterification and removal of modifying groups in synthesis of sugar esters exemplified by xylose (Sarney & Vulfson, 2001)

Ionic solvent system

The use of ionic liquids (IL), which are organic liquid salts, has also been studied for biocatalysis of lipase with several advantages: low vapor pressure and many tunable properties such as solvent polarity, hydrophobicity and solvent miscibility. These properties can be modified by changing the cations and anions of the salts.

Lipase from *Candida antarctica* was shown to retain its activity in some ILs. However, there is not a standard to tell the efficacy of activation of lipase and the results were related to the purity of the IL (S. H. Lee, Ha, Lee, & Koo, 2006; van Rantwijk, Lau, & Sheldon, 2003). Table

2.1 lists the ionic liquids that have been used for the esterification of sugar fatty acid esters, conversion percentage and the enzyme reusability.

Table 2.1 Ionic liquid used in sugar-fatty acid esters synthesis

Substrates	Ionic liquid	Sugar conversion	Product yield	Enzyme stability	Reference
glucose and vinyl acetate	[EMIM][BF ₄]	50%			Park & Kazlauskas, 2001
	[MOEMIN][BF ₄]	99%			
	[PMIM][BF ₄]	28%			
	[BMIM][BF ₄]	78%			
	[sBMIM][BF ₄]	90%			
	[BMIM][PF ₆]	29%			
	[BPy][BF ₄]	42%			
	[PPy][BF ₄]	44%			
supersaturated glucose IL solution and vinyl laurate	[BMIM][TfO]	97%			S. H. Lee, Dang, Ha, Chang, & Koo, 2008
crystalline glucose	[BMIM][TfO]	8%			
glucose and palmitic acid	[BMIM][PF ₆]		0.205 mmol g ⁻¹	still kept activity at the 10th run	Findrik, Megyeri, Gubicza, Bélafi-Bakó, Nemestóthy, & Sudar, 2016
glucose and lauric acid vinyl ester	t-butanol (40%) and [BMIM][BF ₄](60%) (v/v)	62%			Ganske & Bornscheuer, 2005
	t-butanol (40%) and [BMIM][PF ₆](60%) (v/v)	62%			
glucose and palmitic acid	t-butanol (40%) and [BMIM][BF ₄](60%)(v/v)	45%			
	t-butanol (40%) and [BMIM][PF ₆](60%) (v/v)	45%			
glucose and vinyl laurate	[BMIM][TfO] and [BMIM][Tf ₂ N] (1:1 v/v)	70-90%		still kept activity at the 10th run	Mai, Ahn, Bae, Shin, Morya, & Koo, 2014

From the studies listed, it can be seen that that the BF_4^- and PF_6^- were the most frequently used anions in ILs. According to a review (Yang & Huang, 2012), the glucose solubility follows $\text{dca} > \text{TfO} > \text{BF}_4 > \text{PF}_6 > \text{Tf}_2\text{N}$, for ILs containing BF_4^- and PF_6^- ; the cations also influence the solubility too, with the trend $[\text{MOMMIM}] > [\text{MOEMIM}] > [\text{EOEMIM}] > [\text{BMIM}]$. However, like organic solvent system, solvents that have good solubility for glucose usually cannot activate enzyme, such as $[\text{BMIM}][\text{dca}]$ (Liu, Janssen, van Rantwijk, & Sheldon, 2005). Despite these reports for the synthesis of sugar-esters, the use of ILs is still in early stage of research; the stability of lipase in the ILs, the interaction of ionic liquid with enzyme structure, and functionality need to be explored further. The difficulties in these studies include complexed steps to synthesize and purify the ionic liquid themselves and purification of the final esters.

2.3.3 Chemical synthesis of biobased surfactants

Biobased surfactants, for example, esters, can also be synthesized chemically using acid or alkaline catalysts. Monoglycerides, one of the most commonly used emulsifiers in food industry, can be synthesized either by direct esterification of glycerol with fatty acids or by transesterification of glycerol with fatty acid methyl esters (Rarokar, Menghani, Kerzare, & Khedekar, 2017). The former process requires an acid catalyst (Sun, Hu, An, Zhang, Guo, Song, et al., 2017) and the latter one needs a strong base (Sonntag, 1982). Sucrose esters, as discussed above, can be prepared by base-catalyzed esterification with fatty acid esters (Cruces, Plou, Ferrer, Bernabé, & Ballesteros, 2001), however, the process involves organic solvents dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) due the immiscibility of the reactants. Another type of biobased surfactants, alkyl glucosides, which are known as nonionic surfactants, can be synthesized from carbohydrate and aliphatic alcohols with the aid of acidic catalyst. Nowicki, Woch, Mościpan, & Nowakowska-Bogdan (2017) synthesized a series of

alkyl glucosides from glucose and aliphatic alcohols using Fischer glycosylation in reverse micelle system. The catalyst, dodecylbenzenesulfonic acid, not only was the catalyst, but also a surfactant for the microemulsion that trapped the reaction byproduct (water) into micelles to improve glucose conversion.

2.4 Microbial biosurfactants and their applications

Many biosurfactants can be produced by microbial fermentation using cheap feedstocks medium, in which carbohydrate and lipids can be provided in the media. Some carbohydrates that were successfully utilized as energy sources were soy molasses (Solaiman, Ashby, Zerkowski, & Foglia, 2007), sugar cane molasses (Minucelli, Ribeiro-Viana, Borsato, Andrade, Cely, de Oliveira, et al., 2017), starch, date molasses (Al-Wahaibi, Joshi, Al-Bahry, Elshafie, Al-Bemani, & Shibulal, 2014), and fibrous feedstocks after pre-treatment such as switchgrass, alfalfa, bagasse, soy hulls, and distillers' dry grains with solubles (Sharma, Lamsal, & Colonna, 2016). Lipids used were various vegetable oils (Davila, Marchal, & Vandecasteele, 1994), free fatty acids (Ashby, Solaiman, & Foglia, 2008), diesel (Ndlovu, Rautenbach, Khan, & Khan, 2017), esters (Asmer, Lang, Wagner, & Wray, 1988), alkanes (Hu & Ju, 2001), and crude glycerin (Ashby, Nuñez, Solaiman, & Foglia, 2005). The biosurfactants that were produced by microorganisms were mostly small molecules such as glycolipids, lipopeptides, neutral lipids, or larger molecules such as lipoproteins, lipopolysaccharide-protein complexes, and polysaccharide-protein-fatty acid complexes (Ashby, Solaiman, & Zerkowski, 2009). This review focuses on two classes of biosurfactants, namely glycolipids and lipopeptides, examples of which include rhamnolipid, sophorolipids, and surfactin that were studied recently (Henkel, Geissler, Weggenmann, & Hausmann, 2017). Rhamnolipids are glycolipids; the hydrophilic groups consist of either one or two L-rhamnose, which is linked by a glycosidic bond to the

hydrophobic group made up of one or two β -hydroxy fatty acids (Chong & Li, 2017). Similar to rhamnolipids, sophorolipids consist of the disaccharide sophorose and hydroxyl fatty acid (Develter & Lauryssen, 2010). Surfactin is a lipopeptide composed of a seven-amino acids peptide loop and fatty acid chains that are 13-15 carbons long (Reznik, Vishwanath, Pynn, Sitnik, Todd, Wu, et al., 2010). Table 2 lists these three biosurfactants, microorganisms producing them, and their applications; it is important to note that the biosurfactants listed in the Table 2.2 were not a single pure molecule, rather, mixture of isomers, or mixture of monomer or dimers that differ in number of sugar molecules.

Table 2.2 Rhamnolipids, sophorolipids and surfactin's application studies

Biosurfactants	Working microorganisms	Applications	Reference
Rhamnolipids	Pseudomonas aeruginosa, P. chlororaphis, P. fluorescens. Burkholderia species. Acinetobacter species. (Li 2017)	The pseudomonas utilized the waste oil and crude oil growth medium, thus decreased the viscosity of the crude oil and the interfacial tension between oil, water and sand; The fermentation process also improved the oil solubilization, thus resulted in enhanced biodegradation and oil recovery.	Li 2005
		Rhamnolipid from fermentation reduced the surface tension of water to 29.4 mN/m and successfully removed some fractions of aromatic or paraffinic hydrocarbons from contaminated sandy soil (Anna 2008)	Anna 2008
		The fermentation extract from P.aeruginosa and purified mono- and di-rhamnolipids showed inhibition effect against plant pathogenic fungi (Sha 2012)	Sha 2012
		Using 1% rhamnolipids to pre-condition polystyrene reduced Listeria monocytogenes and Staphylococcus aureus adhesion because that rhamnolipids reduced the hydrophobicity of the polystyrene surface	Gomes 2012
		Pre-conditioning on the PVC (polyvinyl chloride) microplate with 100 ug of rhamnolipid inhibited Salmonella enterica biofilm formation .	Mireles 2001
		However, it was not able to reduce biofilm formation of L.monocytogenes and S enteritidis on polystyrene.	Nitschke 2008

Table 2.2 (continued)

Sophorolipids	Candida bombicola, C apicola (Nitschke 2007), Rhodotorula babjevae (Sen 2017)	Sophorolipid was less toxic to aquatic fleas compared with commercial surfactants alkyl polyglucoside, linear alkylbenzene sulfonate, lauryl ethersulfate and also had much better cleaning effect on hard surface. The surface tension was reduced to 32-34 mN/m depending on measurement method.	Develter 2010
		Possessed emulsifying activity and stability for liquid paraffin and sunflower oil at the range 20-80°C at pH 8. However, the emulsion stability decreased with increased salt concentration. The emulsion droplet size could be as small as 625 nm.	Patil 2017
		Demonstrated antifungal properties against plant and human pathogens, reduced the culture medium surface tension to 32.6 mN/m.	Sen 2017
		Can be produced in the medium of sugar cane molasses and chicken fat or sunflower oil. Sophorolipids reduced the water surface tension to 35 mN/m and enhanced the bioremediation of soils contaminated with lubricating oil.	Minucelli 2017
		Facilitate gel formation of silk fibroin by forming micelles that promote the intermolecular beta sheet formation.	Dubey 2016
		Reduced the water surface tension to 33.5 mN/m, demonstrate inhibitory effect for <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> and had comparable detergent effect than commercial detergent.	Joshi-Navare 2013

Table 2.2 (continued)

Surfactin	Bacillus amyloliquefaciens (Ndlovu 2017a, Alvarez 2015)	Inhibition against opportunistic and pathogenic microorganisms.	Ndlovu 2007b
		Surfactin maintained great emulsifying ability above the pH 7.4 for vegetable oil .	Long 2017
		Surfactin was able to co-precipitates with insulin and protect it from acidic and enzymatic attack in the gastrointestinal tract; it also facilitate the insulin to penetrate through the cell membrane of the intestinal tissues so it can be used as oral delivery agent.	Zhang 2016
		Purified surfactin from commercial product was not showing antimicrobial activity.	Nonejuie 2016
		Four surfactin analogs (994-1073 g/mol) and bacillomycin D were found to show broad spectrum to foodborne pathogens and some molds that cannot inhibit nisin .	Lee 2016
		Surfactin recovered petroleum oil from oil-contaminated sands, and amount of oil removed was comparable with SDS removed oil.	Alvarez 2015, AI-Wahaibi 2014,Liu 2015
		Using 0.25% surfactin to pre-condition the polystyrene surface reduced the biofilm formation by Listeria monocytogenes and Salmonella enteritidis.	Gomes 2012
		Pre-conditioning with 100 surfactin on PVC (polyvinyl chloride) microplate inhibited the Salmonella enterica biofilm formation.	Mireles 2001
		It was also able to reduce biofilm formation of L monocytogenes and S enteritidis on the polyethylene surface by making the surface more hydrophilic which increased the electrostatic repulsion between bacterial cell and the surface .	Nitschke 2008

In summary, because of the excellent surface activity of these three types of biosurfactants, they are used as detergents, emulsifying agents and soil cleansers for hydrocarbons. Because of their antimicrobial properties, they are also used for antimicrobial behavior against foodborne pathogens and plant fungus. Because of their capability to modify the surface hydrophobicity, they are able to inhibit or delay biofilm formation as well. Although they have potential usages in different applications, there are some issues with microbial biosurfactants. The chief one among them is low yields during fermentation, especially for surfactin whose production yield was only around 1 g/L (Ndlovu, Rautenbach, Vosloo, Khan, & Khan, 2017). Compared with rhamnolipids and sophorolipids, for which the yields were reported at around 13.2 g/L (Santa Anna, et al., 2007), and in the range of 20-40 g/L (Minucelli, et al., 2017; Sen, Borah, Bora, & Deka, 2017), respectively, surfactin's yield was the lowest. For any use in food application, toxicity studies are required for the biosurfactants. Some genetic engineering may also be required to transfer the biosurfactant-producing genes from pathogenic microorganisms to non-pathogenic ones. For example, the *Pseudomonas aeruginosa* for producing rhamnolipids is a pathogen so developing another engineered bacteria is needed (Chong & Li, 2017).

2.5 Sugar-fatty acids esters functionality studies

2.5.1 Sugar-fatty acid esters structures-surface properties

The critical micelle concentration (CMC) is one of the most important properties of surfactants as it determines how efficiently the surfactant can reduce surface tension and impart related functionalities. The effect of acyl chain length, sugar moiety and degree of acylation on CMC of sugar-fatty acid esters have been studied. Ferrer, Comelles, Plou, Cruces, Fuentes, Parra, et al. (2002) studied a variety of di- and tri- saccharide fatty acid esters with 12-18 alkyl

chain lengths and reported these esters' CMC ranged from 2-250 μM and the surface tension ranged from 24.5-36.5 mN/m. The CMC of the surfactants decreased as the hydrocarbon numbers increased. This is because longer alkyl chains have increased hydrophobic interaction, thus, promoting the formation of micelles. Sugar moiety types also affect the CMC in decreasing order following the trend: sucrose>maltose>leucrose>maltotriose (Ferrer, et al., 2002). The same authors also compared the CMCs of the di- and tri- esters with monosaccharide monoesters and found the latter ones had much lower CMC and exhibit higher solubility. As for the higher degree of acylation, they were not able to determine the CMC because of the low solubilities. However, another study reported diester of sucrose reduced less surface activity (foamability) compared with monoester due to different packing or formed aggregates affecting the adsorption at the surface (Husband, Sarney, Barnard, & Wilde, 1998). Abran, Boucher, Hamanaka, Hiraki, Kito, Koyama, et al. (1989) studied the rigidity of a series of sucrose esters with different alkyl chain lengths (C8-C18) and reported esters with longer chain lengths (C12-18) had better thermal stability in terms of stabilizing protein, and more rigid structures. By studying the esters' capability to reduce surface tension, they also proposed the relationship between the rigidity of structure and the ability to reduce surface tension - in a limited surface area under the same concentration, those having less rigid structure allows molecules occupy larger surface, thus the surface tension can be effectively reduced. While those with more rigid structure, molecules tend to form micelles rather than spread on the surface.

The configuration of the carbohydrate moieties affects the surface activity, as different configurations result in different packing patterns. Garofalakis, Murray, & Sarney (2000) compared sucrose esters with same sugar head groups but with different stereochemistry. Interestingly, C12- β -D-glucoside had much higher CMC (0.13mM) than it of C12- α -D-

glucoside, as it decreased surface tension by 8 mN/m or more than others. The reason could be the β -anomer that had more number of intramolecular hydrogen bonds with each other and less hydration with water, which resulted in a more efficient packing and achieved a lower surface tension. In brief, the capability of the hydroxyl groups to form hydrogen bond among themselves and with the surrounding water depend on the number and positions of the hydroxyl groups that affect their packing pattern and influence the CMC and surface tension.

2.5.2 Sugar fatty acid esters' use in foods

Sugar-fatty acid esters have been used in food, cosmetic, detergent and pharmaceutical industry, among others. Sucrose esters were probably the earliest ingredients that have been approved as 'Generally Regarded As Safe' ingredients by FDA, such as emulsifiers in beta-carotene color preparation (GRN 129, FDA, 2017), fruit flavored beverages (GRN 248, FDA, 2017) and foaming agents (GRN 421; FDA, 2012). Sucrose fatty acid esters have been used in food for more than 20 years (GRN 514; FDA 2014). Safety studies demonstrated sucrose monoester to hydrolyze into sucrose and fatty acids by pancreatic lipase (Berry & Turner, 1960).

The applications in food system have been studied, but not many published studies are available. Neta, dos Santos, de Oliveira Sancho, Rodrigues, Gonçalves, Rodrigues, et al. (2012) used fructose, sucrose and lactose ester to coconut milk and tested surface tension and emulsion index. It was demonstrated lactose ester to be slightly more effective in reducing surface tension and achieving higher emulsion index. However, this study did not specify the degree of esterification of the disaccharide. Other sugar derivative esters, such as sugar alcohol esters were also studied in terms of surface tension reduction. It was found that biobased surfactant enzymatically synthesized by *Chromobacterium viscosum* from sorbitol and plant and animal lipid were more potent in reducing the surface tension between xylenes and water than

chemically synthesized sorbitan monoesters (monooleate, monostearate, monopalmitate) due to the higher hydrophilicity of sorbitol (Chopineau, McCafferty, Therisod, & Klibanov, 1988).

2.5.3 Antimicrobial properties of sugar-fatty acids.

Sugar-fatty acid esters are good antimicrobial agents that can be applied to food systems since they are nontoxic, nonirritant, odorless, and tasteless (Mitsubishi-Kagaku Foods Corporation, 2016). Minimal inhibitory concentration (MIC) is an important indication of antimicrobial efficacy of agents and is defined as the lowest bacteriostatic concentration for 24 h (Huang, Wei, Zhao, Gao, Yang, & Cui, 2008). Table 2.3 lists several studies for the antimicrobial effect of various sugar fatty esters and methyl sugar derivative esters. Many factors contribute to antimicrobial effect, such as food type and fat composition, storage temperatures, structures of sugar esters, and degree of esterification. Chen, Nummer, & Walsh (2014) reported lactose monolaurate had activity against five strains of *Listeria monocytogenes* in milk, yogurt, and cottage cheese. Surprisingly, the monolaurate only had inhibitory effect at 37°C rather than 4°C. The increased fat level reduced the antimicrobial function in food. Hathcox & Beuchat (1996) applied sucrose monolaurate in raw ground beef in the concentration range of 300 µg/mL-1000 µg/mL and reported no inhibition effects. Therefore, the nature of the food affects the antimicrobial properties, probably because the food state and composition interact with antimicrobials thus affecting their efficacy. For the effect of sugar esters structure, both sugar moiety and the levels of esterification were reported to affect the antimicrobial efficacy of biobased surfactants. Habulin, Šabeder, & Knez (2008) found that sucrose esters had stronger antibacterial activity compared to fructose fatty acid esters. Nobmann, Smith, Dunne, Henahan, & Bourke (2009) also proposed that the type of sugar moiety affected the efficacy, since methyl mannose esters derivative had lower MIC than those of methyl glucose esters. Diesters were not

as good as monoesters, which was attributed to their lower solubility in solvents: Ferrer, Soliveri, Plou, López-Cortés, Reyes-Duarte, Christensen, et al. (2005) reported sucrose dilaurates and 6-O-lauroylglucose did not show antimicrobial activity due to low aqueous solubility, while monoester of sucrose and maltose inhibited growth of *Bacillus* and *Lactobacillus* strains. Zhao, Zhang, Hao, & Li (2015) and Habulin, Šabeder, & Knez (2008) also reported that lauryl diesters were less effective in inhibiting bacteria growth. Gram-positive bacteria were reported to be more susceptible to mono and di-saccharide lauryl esters than gram-negative bacteria. For example, Nobmann, Smith, Dunne, Henahan, & Bourke (2009) tested the minimum inhibitory concentration of several carbohydrate fatty acid derivatives and found they were more efficient for *Listeria* (MIC less than 0.1 mM) than *Escherichia*, *Salmonella*, *Enterobacter* and *Pseudomonas* (the MIC more than 10 mM). Zhang, et al. (2016) reported the *E.coli* O157:H7 was more resistant to disaccharide esters than *Staphylococcus aureus*. The combination of other chemicals with sugar esters were also studied for their synergistic effect. The combination of nisin and sucrose fatty acid were reported to have enhanced antimicrobial affect against gram negative (*Stphylococcus aureus* and *Listeria monocytogenes*) and spores of *Clostridium sporogenes* rather than against gram positive bacteria (*Escherichia coli* and *Pseudomonoas fluorescens*) (Thomas, Davies, Delves - Broughton, & Wimpenny, 1998).

Table 2.3 Antimicrobial properties for various sugar-fatty acid esters against food-borne pathogens

Esters	Gram negative	Gram positive	Yeast	Reference
Fructose ester	<i>Escheriia coli</i>	<i>B. cereus</i> (sucrose	<i>Saccharomyces</i>	Habulin,
Sucrose ester	(low suppression	ester MIC 9.375	<i>cerevisisiae</i> (low	Šabeder, &
Commercial sucrose	from all the	mg/ml)	suppression with	Knez, 2008
ester	esters)		sucrose esters)	
Lactose monolaurate		Five strain of <i>listeria</i> <i>monocytogenes</i> (5mg/ml did not completely inhibited)		Chen, Nummer, & Walsh, 2014

Table 2.3 (continued)

Methyl 6-O-lauroyl- α -D-glucopyranoside	<i>Escherichia coli</i> (10-20 μ M) <i>Salmonella</i>	<i>Listeria innocua</i> (0.04-5 μ M) <i>Listeria</i>	Nobmann, Smith, Dunne, Henehan, & Bourke, 2009
Methyl 6-O-lauroyl- β -D-glucopyranoside	<i>enterica</i> (>20 μ M) <i>Enterobacter</i>	<i>monocytogenes</i> (0.04-2.5 μ M)	
Methyl 6-O- octanoyl- α -D- glucopyranoside	<i>aerogenes</i> (>20 μ M) <i>Pseudomonas</i>		
Methyl 6-O-lauroyl- α -D- mannopyranoside	<i>fluorescens</i> (>20 μ M)		
Methyl 6-O-lauroyl- α -D- galactopyranoside			
Methyl 4,6-di-O- lauroyl- α -D- glucopyranoside			
Nisin, sucrose palmitate, sucrose stearate	<i>Escherichia coli</i> <i>Pseudomonas</i> <i>fluorescens</i>	<i>Listeria</i> <i>monocytogenes</i> <i>Bacillus cereus</i> (both cells and spores) <i>Lactobacillus</i> <i>plantarum</i> <i>Staphylococcus</i> <i>aureus</i> Spores of <i>Clostridium</i> <i>sporogenes</i>	Thomas, Davies, Delves - Broughton, & Wimpenny, 1998
Sucrose monolaurate Maltose monolaurate Lactose monolaurate	<i>E.coli</i> O157:H7 (-)	<i>Staphylococcus</i> <i>aureus</i> (250-500 ug/mL)	Zhang, et al., 2016
Sucrose monododecanoate Sucrose monohexadecanoate		Spores of <i>Bacillus</i> <i>stearothermophilus</i> Spores of <i>Clostridium</i> <i>perfringens</i> S40	Moriyama, 1996

It is noted that although sugar-fatty acid esters demonstrated inhibition effect against some foodborne pathogens, their MICs were not usually comparable, and studies were not consistent with each other for the same sugar esters and pathogens, which may be because of

difference in experimental design, medium preparation and initial bacteria count. Also, some studies used methanol or ethanol as solvent to dissolve sugar esters and applied them in the medium without studying control microbial treatments of only with solvents. Caution is needed in comparing results in the literature.

2.6 Mechanisms of biobased surfactants' antimicrobial properties

The mechanism(s) of antimicrobial activity of sugar fatty acid esters have not been elucidated completely. Tsuchido, Yokosuka, & Takano (1993) found that sucrose palmitate tolerant mutant strain of *Bacillus subtilis* had lower uptake of sucrose monopalmitate from the growth medium. A higher amount of 41 kDa membrane protein was also observed. However, the amount of the autolytic enzyme (autolysin) was not significantly different between normal and mutant strains, which might indicate that the control of the autolytic enzymes was associated with the 41 kDa membrane protein affecting the intake of sucrose monopalmitate. However, this mutant strain only showed resistance to long-chain esters (palmityl and stearyl) rather than shorter-chain esters such as sucrose caprylyl ester, lauryl ester and myristyl ester. The inhibition of sugar-fatty acid esters to bacterial spores was also reported. For example, Moriyama (1996) found that sucrose esters had antimicrobial action on spores of *Bacillus stearothermophilus* and *Clostridium perfringens* S40. The mechanism involved the coatings of sucrose esters on the spores that prevented the spores from absorbing nutrients. However, Sugimoto, Tanaka, Moriyama, Nagai, Ogawa, & Makino (1998) reported that *Bacillus cereus* and their spores developed resistance to the sucrose esters due to the esterase secreted from the vegetative cells and spores.

The mechanisms of antimicrobial behavior reported for microbial biosurfactants are mostly from in-vitro interaction of biosurfactants with artificial membrane bilayers

(phospholipids). Thennarasu, Lee, Poon, Kawulka, Vederas, & Ramamoorthy (2005) studied the interaction of antimicrobial peptide subtilisin A with various phospholipids. They reported that a part of the subtilisin peptide induced the conformation change in the lipid head group. The subtilisin also affected the ordering of the lipid acyl chains in DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) to a small extent, which was reflected by the up field shift in ^{31}P NMR data and broadening of the phase transition peak of DSC (differential scanning calorimetry). Grau, Fernandez, Peypoux, & Ortiz (1999) also found that the addition of biosurfactant surfactin to various phospholipids resulted in different packing patterns of phospholipids, which was reflected by the DSC data. The addition of surfactin to DMPC (zwitterionic) caused a decline in gel-to-liquid transition temperature with slight decrease in enthalpy. The surfactin resulted in a broader phase transition peak and significantly lower enthalpy for DMPG (1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt, negative charged), and unchanged gel-to-liquid crystalline phase transition temperature for DEPE (1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine, zwitterionic), but broadened the lamellar-hexagonal-HII transition temperature range. Also, enthalpies for the two-phase transition process decreased, indicating surfactin's destabilization of the hexagonal structure of DEPE. They concluded that surfactin perturbed the cooperativity between the acyl chains in the gel state by inserting itself into the acyl chains with polar amino acids laying near the lipid-water interface. They also observed that at the highest concentration, surfactin was able to form clusters with phospholipid, which can give rise to membrane pore formation and leakage through bacterial membranes. These studies indicated that surfactin interacted differently depending on the type of the phospholipids in membranes. The common observations were that surfactin loosened the phospholipid bilayer compaction that resulted in a more fluid state. Leakage from phospholipids

unilamellar vesicles would occur at higher biosurfactant concentrations due to formation of surfactant-phospholipid clusters (Thennarasu, Lee, Poon, Kawulka, Vederas, & Ramamoorthy, 2005); membrane solubilization later on would occur due to the formation of mixed micelles with the phospholipid (Helenius & Simons, 1975). Usually, the surfactant concentration that induced the leakage is higher than the critical micelle concentration (CMC). Heerklotz & Seelig (2007) found that the cellular leakage started at surfactin-to-phospholipid ratio of 0.22 and completed at 0.43 (solubilization), the concentrations that were much higher than CMC. The surfactin also showed influence on the spores of some plant pathogenic fungus, in that, surfactin combined with other lipopeptide from *Bacillus subtilis* made the spores more permeable (Liu, Hagberg, Novitsky, Hadj-Moussa, & Avis, 2014).

2.7 The antimicrobial properties of emulsions/nanonemulsions stabilized by various emulsifiers

Although biobased surfactants were shown to have antimicrobial properties by themselves, their use in food applications as both emulsifiers and antimicrobials has not been reported. If the biobased surfactants can serve as both emulsifiers and effective antimicrobial agents in the food system, they could find broader applications in food systems, and presumably, costs could be reduced. Many other emulsifiers-stabilized food systems, with or without antimicrobial lipid phase, has been reported. These studies investigating emulsions/nanoemulsions without antimicrobial lipid phase claimed that the oil droplets can fuse with, and then disrupt, the bacterial cell membranes because emulsion droplets possessed positive charge and interacted with negatively charged bacterial membranes (Hamouda & Baker, 2000). The studies with emulsifiers and antimicrobial lipid phase proposed that the lipid phase is better dispersed or solubilized into the aqueous phase so the contact area of the lipid and bacteria

will be enlarged (Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012; Wilkinson, 2015; Xue, Davidson, & Zhong, 2017). This part of review will focus on the studies that involve using antimicrobial lipid phase and discuss the effect of several factors that influenced the inhibition behaviors.

Essential oils (EO) are one of the most studied antimicrobial lipid phase because of their strong antimicrobial activity. However, it has limited use in food for their strong aroma not being very acceptable for consumers. Adding emulsifiers to the EO systems not only improves the dispersion of essential oil in the food, so that the antimicrobial property could be enhanced, but also may reduce the EO concentrations to acceptable levels to consumers. The factors important for emulsion systems involving EO were are essential oil's concentration, emulsifiers' concentration and droplet sizes.

Usually the higher EO concentrations lead to stronger inhibition effects. Chang, McLandsborough, & McClements (2015) studied incorporating thyme oil in the emulsifier mixture of Tween 80 and lauric arginate with the Ostwald ripening inhibitor vegetable oil; the maximum amount of thymol oil (800 $\mu\text{g/mL}$) completely stopped the growth of the spoilage yeast within 24 h while other lower concentrations did not. The increased amount of the vegetable oil, while can inhibit the Ostwald ripening, decreased the minimum inhibitory (MIC) of the thymol oil (Chang, McLandsborough, & McClements, 2012, 2015). Terjung, Löffler, Gibis, Hinrichs, & Weiss (2012) studied Tween 80 stabilized emulsion containing eugenol and reported only the concentrations higher than 800 ppm attaining 5-log reduction for *E.coli* in 24 h.

There were no general trends reported for the effect of emulsifier concentration on antimicrobial behavior of resulting emulsions. Wilkinson (2015) reported only the intermediate concentration (0.01 wt%) of lecithin-stabilized eugenol emulsion increased the effectiveness, but

lower or higher concentration did not differ from the control. Li (2011) observed the emulsion formulated with eugenol or carvacrol with lower concentration (0.0025 wt%) of lecithin decreased the D-value at 37°C for *E.coli* O157:H7 more than other higher concentrations. They proposed the reason to be concentrations of lecithin being lower than the critical micelle concentration (CMC) that were able to bring antimicrobial effects higher concentrations above CMC could not, due to the formation of micelles.

The role of emulsion droplet sizes is not very clear in enhancing inhibition effects of antimicrobials; although it is generally hypothesized that smaller droplets lead to enhanced inhibition effects, there are no clear trends to describe the relationship between droplet sizes and antimicrobial activities. Terjung, Löffler, Gibis, Hinrichs, & Weiss (2012) found larger droplets led to better antimicrobial activity to kill *Listeria innocua* than smaller droplet sizes in Tween 80 emulsions containing phenolic antimicrobials. By measuring the concentrations of the phenolic compounds in the aqueous phases, higher amount of antimicrobials in the aqueous phase in coarse emulsion (3000 nm) were found than those in nanoemulsion (80 nm). Therefore, they proposed the possible reason as more antimicrobial locating inside in the emulsion droplets limiting the access of antimicrobials to bacteria. However, another study reported nanoemulsions (<200 nm) having better inhibition effects to foodborne pathogens than coarse emulsions (>500nm): Topuz, Özvural, Zhao, Huang, Chikindas, & Gölükçü (2016) used lecithin to emulsify anise oil and found that nanoemulsion inhibited to larger extent than the coarse emulsion and non-emulsified treatments. The contradictory conclusions not only existed for droplet sizes' effect, the emulsions themselves did not necessarily result in increased antimicrobial activity. Burt & Reinders (2003) found the lecithin stabilized essential oil (oregano oil and thyme oil) had the MIC twice as high as free essential oil; they proposed lecithin to orient

itself between oil and water phase, which physically hindered the interaction between essential oil and bacterial cells. However, some other studies (Donsì, Annunziata, Vincensi, & Ferrari, 2012; Liang, Xu, Shoemaker, Li, Zhong, & Huang, 2012) reported the nanoemulsions containing essential oil showing significantly increased bacteriostatic effect than non-emulsified essential oil (peppermint oil, carvacrol, limonene and cinnamaldehyde). Donsì, Annunziata, Vincensi, & Ferrari (2012) measured the concentration of essential oil in the aqueous phase and concluded the nanoemulsion increased the solubility of essential oil thus resulted in improved effect. In light of seemingly contradictory reports, it is hard to generalize the effect of emulsified antimicrobials and their droplet sizes without evaluating the system on hand. One of the studies described in this dissertation aims to study this question for emulsion/nanoemulsion stabilized by different emulsifiers containing antimicrobial agent cinnamaldehyde.

The food composition also affects the antimicrobial properties for the emulsified essential oil. For example, Xue, Davidson, & Zhong (2017) used the gelatin and lecithin to disperse thymol oil in milk with different fat content. Although these two agents did not decrease the MIC, they achieved 5-log reduction of *E. coli* O157:H7 within 24 h in skim milk, which were shorter than non-emulsified treatments. However, in the 2% fat and whole milk, the time to achieve 5-log reduction was increased to 50 h, indicating fat protected bacteria. When the researchers applied the same emulsion in the cantaloupe juice (pH 6.81), *E. coli* O157:H7 and *Listeria monocytogenes* was inhibited in 24 h but recovered growth after that. These results indicated the different compositions of milk and cantaloupe juice affected the antimicrobial effect of the nanoemulsion.

2.8 Summary

The published literature discussed in above sections compared biobased surfactants production methods and applications as emulsifying and antimicrobial agents, and revealed some gaps in research areas. In terms of production, utilizing different types of biomass, increasing the biobased surfactants production yields by properly selecting synthesis conditions or genetically modifying non-pathogenic bacteria need additional research. In terms of biobased surfactants application as antimicrobials, the use of biobased surfactants as both emulsifiers and antimicrobials should be studied and the mechanisms of emulsions for antimicrobial activities investigated.

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CHAPTER 3. SYNTHESIS OF SOME GLUCOSE-FATTY ACID ESTERS BY LIPASE FROM CANDIDA ANTARCTICA, AND THEIR EMULSION FUNCTIONS

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3.1 Abstract

The synthesis of glucose esters with palmitic acid, lauric acid and hexanoic acid using lipase enzyme were studied and their emulsion functionality in oil-in-water system were compared. Reactions at 3:1 molar ratio of fatty acids-to-glucose had the highest conversion percentages (over 90% for each of the fatty acid). Initial conversion rate increased as substrate solubility increased. Ester bond formation was confirmed by nuclear magnetic resonance technique that the chemical shifts of glucose H-6 and α -carbon proton of fatty acid in the esters shifted to the higher fields. Contact angle of water on esters' pelleted surface increased as the hydrophobicity increased. Glucose esters' and commercial sucrose esters' functionality as emulsifiers were compared. Glucose esters delayed, but did not prevent coalescence, because the oil droplets diameter doubled during 7 days. Sucrose esters prevented coalescence during 7 days since the droplets diameter did not have significant change.

Key words: glucose ester; synthesis; lipase; contact angel; emulsifier; sucrose ester.

3.2. Introduction

Surfactants are amphiphilic molecules with both hydrophilic and hydrophobic moieties that can adsorb at the interface between different polarity phases and reduce interfacial tension. Thus, they have functionality in detergency, emulsifying, dispersion, foaming industry (Greek, 1991, 1990). Traditional surfactants are mainly derived from petroleum industry products, which requires unfavorable reaction conditions such as high temperature, high acidity, alkalinity, organic solvent, and have low biodegradability and high aquatic toxicity (Deleu & Paquot, 2004). However, surfactants can also be produced via enzymatic reactions or microbial fermentation utilizing biological feedstocks. These environmental compatible surfactants, also called biobased surfactants, are biodegradable and environmental friendly. Some of the microorganisms that produce surfactants during fermentation are *Pseudomonas*, *Rhodococcus*, *Mycobacterium*, *Toruplopsis*, *Bacillus*, *Thiobacillus*, etc (Desai & Banat, 1997). The surfactants derived from microorganisms are glycolipid, lipopeptides, lipoproteins, fatty acids and phospholipids, etc (Desai & Bannat 1997). In enzyme-catalyzed production of biobased surfactants, the common enzymes utilized are lipase, proteinase and glucosidase (Hayes, 2011). Lipase catalyzes ester bond formation between fatty acyl groups and hydroxyl group of alcohols or polyols; amino acid also can act as acyl donor and form ester or amide bond if proteinase are used; glycosidases catalyze the acetal bond formation between saccharides and fatty alcohols (Van Rantwijk, Woudenberg-van Oosterom, & Sheldon, 1999). Lipase-catalyzed reactions were studied in terms of reaction solvent, substrate ratio, reaction time etc. Commonly used acyl acceptors are carbohydrate, sugar alcohol. Acyl donors are various fatty acids or fatty acid esters. Enzymatic synthesis of esters is one of the major methods due to the higher selectivity, relatively lower temperatures (lower than 70°C), lower solvent toxicity, and easier separation of products.

Enzyme that have been used for synthesis of esters are subtilisin from *Bacillus amyloliquefaciens* (Rich, Bedell, & Dordick, 1995), lipase from *Candida antarctica* (Pedersen, Wimmer, Emmersen, Degn, & Pedersen, 2002), *Candida rugosa* (Zaidan, Abdul Rahman, Othman, Basri, Abdulmalek, Abdul Rahman, et al., 2012) *Mucor miehei* (Degn, Pedersen, & Zimmermann, 1999), *Humicola lanuginosa* (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999), *Thermomyces lanuginosus* (Tsavas, Polydorou, Fafila, Voutsas, Tassios, Flores, et al., 2002), and alkaline protease from *Streptomyces* spp (Kitagawa, Tokiwa, Fan, Raku, & Tokiwa, 2000). Sugar- fatty acid esters are non-ionic surfactants with a wide range of hydrophilic-lipophilic balance (HLB) values. Since they are biodegradable, non-toxic (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999), non-irritant to skin (Plat & Linhardt, 2001) and odorless, they are widely used in food, pharmaceutical, cosmetic and detergent industries. Sucrose esters have been approved by Food and Drug Administration and are widely used in food industry, such as wheat products, confectioneries, and dairy products, etc. The functions are various, such as increasing dough resistance to kneading, increasing cake volume, prevent stickiness to the machine, make stable emulsion, improve mouthfeel, prevent staling, etc. (Mitsubishi-Chemical Foods Corporation).

The challenge to synthesize sugar-fatty acid ester enzymatically is to find good solvent(s) to solubilize the substrates that have different polarities, at the meantime, not deactivating enzymes. It has been extensively studied in different medium, for example, single phase organic solvent systems (Degn, Pedersen, & Zimmermann, 1999; Ljunger, Adlercreutz, & Mattiasson, 1994;), two organic solvent systems (Kitagawa, Tokiwa, Fan, Raku, & Tokiwa, 2000; Reyes-Duarte, López-Cortés, Ferrer, Plou, & Ballesteros, 2005), non-solvent systems (Martin-Arjol, Isbell, & Manresa, 2015), ionic liquid systems, supercritical carbon dioxide (Habulin, Šabeder, & Knez, 2008) and deep eutectic systems (Pohnlein, Ulrich, Kirschhofer, Nusser, Muhle-Goll,

Kannengiesser, et al., 2015). Mixed organic solvents were preferred than single solvent since by varying the ratio of each solvents, solubility of acyl acceptor and enzyme activity can be controlled. To avoid the use of organic solvent and address the solubility issues, solvent-free system were used (Fregapane, Sarney, & Vulfson, 1991; Ward, Fang, & Li, 1997). However, the reaction system had high viscosity and low miscibility (Wei, Yu, Song, & Su, 2003). Ionic solvents were used because of their advantage of low vapor pressures and tunable chemical structure that can solubilize different substrates (Park & Kazlauskas, 2001) while they have disadvantage of extra steps to synthesize and purify of ionic solvents, and some ionic liquid were reported to deactivate the enzyme (Schöfer, Kaftzik, Wasserscheid, & Kragl, 2001). Dimethyl sulfoxide (DMSO) and 2-methyl-2-butanol (2M2B) were chosen as reaction media to synthesize glucose esters due to relatively high solubility of sugars and being benign to enzymes.

Although the synthesis of sugar fatty acid esters has been studied extensively, their functionality as emulsifiers in the basic oil-in-water systems has not been looked in-depth. Glucose is a cheap carbohydrate with only one primary hydroxyl group, therefore, the high selectivity is expected. Also, not many research (Arcos, Bernabe, & Otero, 1998; Degn, Pedersen, & Zimmermann, 1999; Ljunger, Adlercreutz, & Mattiasson, 1994) studied the synthesis condition and their functionalities. The objectives of this study are to: 1) optimize the synthesis of glucose esters with respect to substrate ratio and fatty acid types, and 2) evaluate the functionality of glucose esters as emulsifiers and compare with commercial sucrose esters with different HLB values.

3.3. Materials and Methods

3.3.1 Reagents

DMSO, 2M2B, D-glucose, HPLC-grade methanol, and molecular sieves (3Å) were purchased from Fisher Scientific (Fair Lawn, NJ). Palmitic acid (98%), lauric acid (>98%), hexanoic acid (>99.5%), and immobilized lipase from *Candida Antarctica* were purchased from Sigma-Aldrich (St. Louis, MO). Pure canola oil was purchased from a local grocery store. Commercial sucrose esters SP30 (sucrose distearate, HLB 6, monoester content 30%), SP50 (sucrose stearate, HLB 11, monoester content 50%), PS750 (sucrose palmitate, HLB 16, monoester content 75%) were donated.

3.3.2 Synthesis of glucose esters

The lipase-catalyzed synthesis of glucose esters was carried out in 50-mL Erlenmeyer flasks following published method with some modifications (Ferrer, Cruces, Bernabe, Ballesteros, & Plou, 1999). Palmitic acid, lauric acid, and hexanoic acid were used as acyl donor and glucose was used as acyl acceptor for esterification reactions. Molar ratios of fatty acid and sugar were 0.3 mM: 0.1 mM, 0.2 mM: 0.1 mM, 0.1 mM: 0.1 mM, and 0.1 mM: 0.3 mM. One g molecular sieves and 0.25 g immobilized lipases were added in 10 mL solvent mix (80% DMSO and 20% 2M2B). The flasks were incubated at 55°C in a water bath with shaking at 96 rpm for 48 h.

After 48 h of reaction, reactants were centrifuged to obtain the supernatant. The supernatants were placed under a fume hood overnight to evaporate 2M2B. Water (approximately 10:1 v/v of solvent) was added to the medium to precipitate the fatty acid residue and esters. The viscous white slurry were filtered to obtain white solid. The solids were washed with 10 volumes of methanol for 3-4 times to dissolve free fatty acid residue and obtain highly

pure esters. The purity of glucose esters was determined with NMR 1D proton test to obtain the area ratio of proton of α carbon ($-\text{CH}_2\text{-COOH}$) of bonded fatty acid and free fatty acid.

3.3.3 Quantitation of fatty acid conversion

The *quantitation* of fatty acids by HPLC method followed a previous study (Reyes-Duarte, López-Cortés, Ferrer, Plou, & Ballesteros, 2005) with slight modification. At 12th, 24th, 36th and 48th h, aliquots of reactant mix were withdrawn and measured for residual free fatty acid by high performance liquid chromatography (Thermo Scientific, ACCELA 1250 HPLC) using a C18 column (Hypersil Gold, 50×2.1 mm, 1.9 μm), a PDA detector (at 200 nm), and EZChrom Elite software (Agilent, Version3.2.1). For palmitate acid detection, methanol: water 70/30 (v/v) with 0.1% v/v acetic acid was used as mobile phase A for the first 2 min, then a gradient from this eluent to pure methanol (B) was continued for 5 min, after which the gradient was changed back to the original mobile phase for 5 min. The flow rate was 0.5 mL/min and the temperature was 45°C. The method to detect lauric acid was the same as it was for palmitic acid. For hexanoic acid detection, the mobile phase A was methanol: water 80/20 (v/v) with 0.1% acetic acid for 4 min, then a gradient to pure methanol was last for 5min, then it changed back to A. The flow rate of mobile phase was 0.5 mL/min for all fatty acids. The conversion of fatty acid was calculated as:

$$\text{Conversion of fatty acid} = (X_0 - X_1) / X_0 \times 100\%$$

X_0 - fatty acid concentration at the start of reaction

X_1 -residual fatty acid concentration in reaction mix at different time points.

X_0 - theoretical concentration of fatty acid that can be fully converted to ester.

From stoichiometry of reaction systems, X_0 is the 1/3 of initial concentration at 3:1 acid: sugar ratio, and 1/2 of initial concentration for 2:1 ratio.

3.3.4 Identification of esters

Agilent Quadrupole Time-of-Flight (QTOF) 6540 liquid chromatography mass spectrometry (LC/MS) was used to identify reaction products. A XDB C18 column (4.6×50 mm, 1.8 μ m) and an electrospray ionization detector were used. The products were scanned in the negative mode from 100-1000 Daltons. A gradient from 95% mobile phase A (water, 100%) and 5% B (methanol, 100%) to 95% B and 5% A was applied for 20 min and kept for another 5 min. The flow rate of mobile phase was 0.8 mL/min. Nuclear magnetic resonance (NMR) (Bruker Avance III 600, Billerica, MA and Karlsruhe, Germany) was used to confirm the formation of ester bonds and chemical shifts of important carbon and hydrogen atoms. The products were dissolved in deuterated DMSO to achieve a concentration range of 50-800 mg/mL. Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectroscopy for ^1H and ^{13}C were used to determine the ester bond formation. The data were analyzed with the TopSpin software (Bruker, Billerica, MA).

Chemical shifts, splitting patterns, J-coupling and positions of hydrogen and carbon for reactants and products are as follows:

Glucose, the spectrum showed it was a mixture of α - and β -anomers. ^1H NMR (600 MHz, DMSO- d_6) δ 4.91 (t, J = 4.2 Hz, 1H, H-1), 3.42 (dt, J = 9.1, 4.3 Hz, 1H, H-2), 3.11 (ddd, J = 10.0, 6.7, 3.6 Hz, 1H, H-3), 3.04 (td, J = 9.3, 5.4 Hz, 1H, H-4), 3.58 – 3.52 (m, 1H, H-5), 3.48 – 3.44 (m, 1H, H-6a), 3.60 (dd, J = 11.5, 5.9 Hz, 1H, H-6b). ^{13}C NMR (151 MHz, DMSO- d_6) δ 92.67 (C-1), 72.809 (C-2), 73.541 (C-3), 71.03 (C-4), 72.403 (C-5), 61.676 (C-6).

Palmitic acid. ^1H NMR (600 MHz, DMSO- d_6) δ 11.93 (s, 1H, -COOH), 2.166 (t, J = 7.5 Hz, 2H, -CH₂CO-), 1.486 (t, J = 7.2 Hz, 2H, -CH₂-CH₂-CO-), 1.205-1.294 (s, 24H, chain), 0.853 (m, 3H, -CH₃). ^{13}C NMR (151 MHz, DMSO- d_6) δ 174.75 (C=O), 34.119 (-CH₂-CO-), 31.87 (-

CH₂-CH₂-CO-), 29.661-29.661, 29.31, 29.16, 24.997, 22.617 (-CH₂- palmitic acid backbone), 14.263 (-CH₃).

Lauric acid. ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.95 (s, 1H, -COOH), 2.176 (t, *J* = 7.5 Hz, 2H, -CH₂-CO-), 1.495 (t, *J* = 7.4 Hz, 2H, -CH₂-CH₂-CO-), 1.244-1.297 (s, 16H, chain), 0.86 (d, *J* = 7.5 Hz, 3H, -CH₃). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 174.85 (C=O), 34.12 (-CH₂-CO-), 31.816, 29.541, 29.528, 29.449, 29.285, 29.243, 29.079, 24.979, 22.597 (-CH₂- lauric acid backbone), 14.344 (-CH₃).

Hexanoic acid. ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.889 (s, 1H, -COOH), 2.172 (t, *J* = 7.6 Hz, 2H, -CH₂CO-), 1.502-1.264 (6H, chain), 0.859 (t, *J* = 7.1 Hz, 3H, -CH₃). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 174.86 (C=O), 34.05 (-CH₂-CO-), 31.25, 24.64, 22.31 (-CH₂- hexanoic acid backbone), 14.16 (-CH₃).

6-*O*-Palmitoylglucopyranose. ¹H NMR (600 MHz, DMSO-*d*₆) δ 4.91 (t, *J* = 4.4 Hz, 1H, H-1), 3.137 (m, 1H, H-2), 3.44 (td, *J* = 9.1, 4.5 Hz, 1H, H-3), 3.042 (td, *J* = 9.3, 5.5 Hz, 1H, H-4), 3.78 (dd, *J* = 9.5, 6.5 Hz, 1H, H-5), 4.28 (d, *J* = 11.5 Hz, 1H, H-6a), 4.00 (dd, *J* = 11.7, 6.2 Hz, 1H, H-6b), 2.28 (t, *J* = 7.4 Hz, 2H, -CH₂-CO-), 1.51 (q, *J* = 7.2 Hz, 2H, -CH₂-CH₂-CO-), 1.25 (s, 24H, chain), 0.87 (t, *J* = 6.9 Hz, 3H, -CH₃). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 92.755 (C-1), 72.66 (C-2), 73.336 (C-3), 71.035 (C-4), 69.601 (C-5), 64.355 (C-6), 173.363 (C=O), 33.906 (-CH₂-CO-), 31.778 (-CH₂-CH₂-CO-), 29.534 – 28.931, 24.94, 22.569 (-CH₂- palmitoyl backbone), 14.341 (-CH₃).

6-*O*-Lauroylglucopyranose. ¹H NMR (600 MHz, DMSO-*d*₆) δ 4.909 (d, *J* = 4.7 Hz, 1H, H-1), 3.137 (m, 1H, H-2), 3.439 (td, *J* = 9.2, 4.3 Hz, 1H, H-3), 3.043 (m, 1H, H-4), 3.774 (dd, *J* = 9.7, 6.8 Hz, 1H, H-5), 4.277 (d, *J* = 11.9 Hz, 1H, H-6a), 4.006 (dd, *J* = 11.7, 6.2 Hz, 1H, H-6b), 2.28 (t, *J* = 7.3 Hz, 2H, -CH₂-CO-), 1.52 (t, *J* = 7.1 Hz, 2H, -CH₂-CH₂-CO-), 1.25 (s, 16H,

chain), 0.87 (t, $J = 6.8$ Hz, 3H, $-\text{CH}_3$). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 92.754 (C-1), 72.659 (C-2), 73.334 (C-3), 71.027 (C-4), 69.602 (C-5), 64.362 (C-6), 173.272 (C=O), 33.909 ($-\text{CH}_2\text{-CO-}$), 31.814, 29.541, 29.526, 29.434, 29.268, 29.24, 28.964, 24.94, 22.592 ($-\text{CH}_2\text{- lauroyl backbone}$), 14.42 ($-\text{CH}_3$).

6-O-Hexanoylglucopyranose

^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 4.911 (d, $J = 3.9$ Hz, 1H, H-1), 3.132 (m, 1H, H-2), 3.43 (m, 1H, H-3), 3.055 (dd, $J = 17.2, 7.4$ Hz, 1H, H-4), 3.771 (s, 1H, H-5), 4.29 (dd, $J = 19.7, 11.6$ Hz, 1H, H-6a), 3.997 (m, 1H, H-6b), 2.293, 1.51 (dt, $J = 14.7, 7.9$ Hz, 2H, $-\text{CH}_2\text{-CO-}$), 1.576-1.191 (m, 6H, caproyl backbone), 0.873 (d, $J = 8.3$ Hz, 3H, $-\text{CH}_3$). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 173.444 (C=O), 34.177 ($-\text{CH}_2\text{-CO-}$), 31.097, 24.643, 22.287 ($-\text{CH}_2\text{- hexanoyl backbone}$).

3.3.5 Initial sugar solubility

Initial sugar solubility of ester was tested using an HPLC. After mixing reactants except lipase in the solvents as described in synthesis method above, flasks were incubated in the water bath at 55°C at 3.8 rpm for 30 min. After cooling down, aliquots from each flask were run through HPLC. A carbohydrate column (HyperRez XP Carbohydrate H+, 300×7.7 mm, $8\ \mu\text{m}$), a guard column (HyperRez XP Carbohydrate H+, 50×7.7 mm, $8\ \mu\text{m}$) and a RI detector were used. The temperature for guard column and carbohydrate column were 65°C and 70°C , the flow rate was 0.4 mL/min.

3.3.6 Emulsion stability Index

0.01% w/w, 0.1% w/w and 0.5 % w/w 100g ester solutions were prepared and mixed with 10g canola oil, control treatment did not contain any esters. The mixtures were sonicated for 10 min to improve ester dispersion. The mixture was homogenized for 2 min at 15,000 rpm using

a blender (Bamix Type M 150) and 10 μL - 20 μL aliquots of emulsion were diluted with 2 mL deionized water in the spectrophotometer cuvette. Absorbance of emulsion at 500 nm was measured at 0, and 20 min. The emulsion stability index (ESI) was calculated as:

$\text{ESI} = A_0 \cdot 20 / [A_0 - A_{20}]$. A_0 and A_{20} were the absorbance obtained at 0 min and 20 min (Pearce & Kinsella, 1978). The higher ESI values indicated higher emulsion stability.

3.3.7 Emulsion droplet size distribution

The emulsion droplet size and distribution was measured by Malvern Particle Size analyzer (Mastersizer Hydro 2000). Emulsions with 0.5% w/w level of esters were prepared as above and were introduced into the instrument until a laser obscuration of 10-20% was achieved. Measurements were taken at time 0 h, 6th h, 24th h, 3rd d and 7th d.

3.3.8 Contact angle measurement

Contact angel measurement followed a previous research (Crowley, Desautel, Gazi, Kelly, Huppertz, & O'Mahony, 2015). Pellets of each of the esters were prepared with 0.08g powdered ester, placing them on a 13 mm pellet die and pressing under a force of 5000 kg for 2 min in Carver Press (model 3619, Carver Inc, Wabash, IN). Contact angle measurement was conducted using a goniometer (Rame-Hart Model 250 Standard Goniometer). Approximately 4 μL water droplet was dispensed on the pellet's surface placed on a stage. Side view pictures were taken immediately after the water droplet left the syringe tip using a high-resolution camera.

3.3.9 Statistical analysis

Statistic test was conducted using SAS 9.4 software (SAS Institute Inc., Cary, NC). Proc GLIMMIX test was used to determine significant difference between treatments ($P < 0.05$). At least three observations for each treatment were measured for conversion, sugar solubility, contact angel, emulsion stability and droplet size analysis.

3.4. Results and Discussion

3.4.1 Fatty acid conversion

Figure 3.1 (a), (b) and (c) show the conversion percentages of palmitic acid, lauric acid and hexanoic acid over 48 h, respectively. The fatty acid/glucose molar ratios were compared for optimum ratio for the highest conversion. For each glucose-fatty acid ester studied, the highest conversion of fatty acid was achieved when the ratio was 3:1 (97.17%, 76.57%, 113.11% for glucose palmitate, laurate and hexanoate, respectively). The second highest conversions were at the ratio of 2:1 (75.96%, 62.82%, 73.66%, respectively). The 1:1 and 1:3 ratios did not have any significantly differences; the conversion was around 50%, 40%, 30%, respectively for palmitic acid, lauric acid and hexanoic acid. Higher concentrations of FA favored formation of the products as explained by the equilibrium constant:

$$k = \frac{[\text{ester}][\text{water}]}{[\text{fatty acid}][\text{glucose}]} \quad (\text{Equation 1});$$

$$\text{or, } [\text{ester}] = k \times [\text{fatty acid}][\text{glucose}]/[\text{water}] \quad (\text{Equation 2}).$$

At a given temperature (55°C in this study), k is a constant. At higher molar ratios of fatty acid to glucose (2:1 or 3:1), the limiting reactant is glucose, thus, only 1/3rd of available fatty acid is converted to ester and water. Therefore, the increase in fatty acid concentration in reaction mix would be larger than the increase in water amount. Meanwhile, the glucose concentration remained at a similar level compared to the reaction when the ratio was 1:1. Therefore, the increase of fatty acids amount increased the ester amount according to Equation 2, as reflected by the conversion percentage. For the reactants ratio of 1:1 and 1:3 of fatty acid to glucose, glucose solubility was limited in the medium as glucose crystals could be seen throughout the reaction, and fatty acid were completely dissolved. For these conditions, terms in Equation 2 did not have significant changes, indicating similar conversions for these two ratios.

Among various fatty acids, the hexanoic acid had the highest conversion percentage at 3:1 ratio (113%). This could be due to the formation of diesters, along with monoesters, but at low levels since they were not detected by LCMS and NMR. Two reasons could contribute to the higher conversion of hexanoic acid: shorter carbon chains making the solvent more polar resulting in higher glucose solubility (data will be shown in later section) than other two fatty acids, and possible stearic hindrance- the smaller molecule would have easier access to enzyme active sites and result in more esterification.

3.4.2 Initial substrate concentration and initial conversion rates

Influence of reactant concentrations on the initial conversion rates (linear range for first three hours) is presented in Table 3.1. For the molar ratios of 3:1, 2:1, 1:1 (fatty acid/glucose) in esterification of palmitic acid and lauric acid, fatty acids were completely soluble in solvent, so, the fatty acids concentration increased as molar ratio increased leading to higher initial fatty acid conversion rates. For molar ratio increase from 1:1 to 3:1 (fatty acid concentration increase from 74.3 mM to 243.7 mM), the initial conversion rates also increased threefold from 3.3 $\mu\text{mol}/(\text{min} \cdot \text{g})$ to 9.6 $\mu\text{mol}/(\text{min} \cdot \text{g})$. Glucose did not completely solubilize for these molar ratios of 1:1 and 1:3 (fatty acid/glucose), because sugar crystals were seen in the medium, thus we examined the soluble glucose concentration in reactant. For fatty acid: glucose molar ratios of 1:1 and 1:3 with increase in glucose concentration from 21 mM to 28.2 mM, the initial conversion rates increased from 3.3 $\mu\text{mol}/(\text{min} \cdot \text{g})$ to 5.2 $\mu\text{mol}/(\text{min} \cdot \text{g})$. From these two comparisons, it is seen that the concentration of both fatty acid and glucose had direct and proportional relationship with the initial conversion rate, indicating the reaction to be a first-order reaction in terms of either fatty acid or glucose (Degn & Zimmermann, 2001). For hexanoic acid, the increase of fatty acid concentration from 146.5 mM to 235.2 mM almost quadrupled the rate (Table 3.1), and the

glucose solubility did not change over the three ratios and did not affect the initial rate. The highest conversion rate obtained was $11.83 \mu\text{mol}/(\text{min}\cdot\text{g})$, which is similar to reported $15.2 \mu\text{mol}/(\text{min}\cdot\text{g})$ for synthesis of glucose myristate (Degn & Zimmermann, 2001) that was conducted in t-butanol: pyridine system. Although the glucose solubility was higher in our system, the deactivation of lipase in DMSO is stronger than pyridine. They reported that below molar ratio of 10:1 up to 20 mg/mL soluble glucose concentration, the initial reaction rate increased as the ratio increased, which was consistent with our study when we kept the sugar level constant. The effect of lauric acid and glucose concentration on initial conversion rates in the single solvent 2M2B was reported to increase when either of the two substrate concentrations increased, however, lauric acid was saturated at 140 mM, glucose was not saturated up to 50 mM (Flores, Naraghi, Engasser, & Halling, 2002). In our study, we did not find saturation levels for both substrates even at much higher fatty acid concentration of 259.6 mM. The reason for the difference in solubility could be that our bi-solvent system dissolved more fatty acids boosting the initial rates.

Initial sugar solubility can also be associated with medium hydrophobicity (Pedersen, Wimmer, Emmersen, Degn, & Pedersen, 2002; Reyes-Duarte, López-Cortés, Ferrer, Plou, & Ballesteros, 2005). Glucose solubility increased as the chain length of the fatty acid decreased at each substrate ratio. The solubility is also associated with the amount of sugar that was put into the medium, as it increased when the ratio of sugar increased, since they themselves created a more polar environment. Though not measured in our study, previous studies indicated as the esters were being produced, glucose solubility would increase through hydrophobic interaction (Degn & Zimmermann, 2001; Tsavas, et al., 2002).

The initial conversion rates may or may not relate to the length of the acyl donor, as variously reported. Some studies reported the reaction rate with was faster for shorter chain length of fatty acid (C4-C12) that were esterified with disaccharide (Pedersen, Wimmer, Emmersen, Degn, & Pedersen, 2002). Adelhorst, Bjokling, Godtfredsen and Kirk (1990) reported the enzyme showed faster reaction with longer fatty acids (C12-C18) than shorter acids (C8-C10) in solvent free condition. However, Degn, Pedersen, & Zimmermann, (1999) found that the initial reaction rate was independent within chain length C2-C20 of acyl donors for glucose. Our study also indicates that the initial rates and chain length were independent. The difference could be due to the difference in substrate and reaction conditions.

3.4.3 Product identification and complete ¹H and ¹³C assignment for reactants and products.

The formation of glucose palmitate, glucose laurate and glucose hexanoate were confirmed by LCMS (data not shown) and NMR techniques. In the HMBC graph (Figure 3.2) of reaction mixture of glucose and palmitic acid, the sixth protons (4.01 and 4.27 ppm) of glucose were seen to have interacted with carbonyl carbon indicating the formation of ester bonds. The chemical shifts of H-6 of glucose and α -carbon proton of fatty acid in the esters to the higher fields indicated the chemical environment change due to esterification that caused de-shielding effect (Kitagawa, Tokiwa, Fan, Raku, & Tokiwa, 2000; Pedersen, Wimmer, Emmersen, Degn, & Pedersen, 2002; Walsh, Bombyk, Wagh, Bingham, & Berreau, 2009). We had two chemical shifts for the sixth proton in glucose indicating the alpha and beta conformation of the D-glucose (Roslund, Tähtinen, Niemitz, & Sjöholm, 2008). Similar NMR graph for glucose laurate and glucose hexanoate esters were obtained.

We successfully obtained the purity of 95.50%, 98.97% of glucose palmitate and glucose laurate, respectively. Due to relatively high solubility of glucose hexanoate in both hydrophobic

and hydrophilic environment, it was not possible to purify the ester by solvent extraction. NMR data for glucose heanoate was obtained from reaction mixture rather than pure product.

3.4.4 Contact angle on product surface

Due to the low solubility of glucose palmitate in water, it was hard to measure the critical micelle concentration and surface tension; therefore, contact angle was measured for the esters to compare the relative hydrophobicity. The hydrophobicity is generally positively related to contact angle (Daffonchio, Thaveesri, & Verstraete, 1995) and reversely related to HLB value (Griffin, 1949). The contact angles and HLB values for glucose palmitate, glucose laurate, SP30, SP50 and PS750 were 98.6 (HLB 8.6), 93.6 (HLB 9.9), 76.9 (HLB 6), 44.8 (HLB 11) and 28.1 (HLB 16) respectively. The contact angles of our products followed the trend: the longer alkyl chain of palmitic acid made glucose palmitate more hydrophobic. The HLB values for these two products were calculated respectively according to the method of Griffin (1955). The HLB value indicates that both of the esters can perform as emulsifiers and wetting agents for oil-in-water system (value from 7-18, according to Griffin (1946)). Commercial SP50 and PS750 esters may have more function in detergent and solubilizing application because they had higher HLB value (Griffin, 1949). Overall, the larger the contact angle the ester had, the lower the HLB value they were, except that SP30 had lower HLB value than glucose palmitate but it had lower contact angle than the other.

3.4.5 Emulsion stability of esters

The emulsion stability index (ESI) for esters are shown in Figure 3.3. Glucose esters were not water-soluble and they stayed on top of aqueous phase; whereas, sucrose esters were

dispersible but not soluble. Glucose esters demonstrated stabilizing effect compared to control treatment. The ESI increased as the concentration increased for all esters, which indicated lower concentrations of esters did not completely cover the oil surface to prevent creaming, the addition of more esters covered more surface area so the creaming process were retarded. At low concentrations of 0.01% for glucose palmitate and glucose laurate, the ESI values (171.4, 178.0 respectively) were slightly higher than control treatment (138.8). At medium concentration of 0.1%, glucose palmitate ESI (315.3) was twice as much as control, whereas glucose laurate ESI (163.4) did not increase compared to 0.01%. At 0.5%, glucose palmitate again showed stronger stabilizing effect (ESI 664.7) than glucose laurate (460.8). These data show that glucose palmitate had better stabilizing effect compared to glucose laurate. One reason for this could be the stronger hydrophobic interactions by longer alkyl chains in the molecule with each other and with oil droplets, which can form a more compact structure than glucose laurate (Ferrer, Comelles, Plou, Cruces, Fuentes, Parra, et al., 2002).

For commercial sucrose esters, similarly, the ESI increased as the concentration increased. Compared with glucose esters, the sucrose esters had better stabilizing effect since the ESI were higher than those of in-house glucose esters at every concentration. Particularly, at 0.5%, the ESI were significantly higher (1351.8, 1212.5, 1492.3 for SP30, SP50, PS750 respectively) than glucose esters.

3.4.6 Emulsion droplet size distributions

Droplet distribution and diameter parameters for control, glucose palmitate, glucose laurate, and sucrose esters SP30 are presented in Figure 3.4 and Table 3.2 respectively. It was obvious for control and glucose esters that the distribution had undergone from single-modal to bio-modal or tri-modal change during 7-day storage, indicating the size were diverging into

bigger or smaller. The appearance of the peaks to the right side of the distribution indicated the presence of droplets that have not been completely covered by the surfactants have experienced coalescence (McClement, 2004a). The sucrose esters emulsified systems were relatively stable as the distributions were bio-modal or tri-modal throughout. Only one for the sucrose esters is presented because the distribution of the patterns were similar.

Mean and standard deviation of the droplet diameters for each treatment for 7-day time points are reported in Table 3.2; for multiple comparison, data were transformed to a natural log scale to fit the normal distribution. For all the diameters, both fixed effects (type of esters and time) had significant effects on change in droplet diameters ($P < 0.05$). The interaction of treatment and time was also significant ($P < 0.05$), meaning the diameters changed differently among all the treatments at different times. From 0 h to 7th day, $D[0.1]$ decreased gradually for control (5.2 μm to 2.0 μm), glucose palmitate (1.7 μm to 0.3 μm) and glucose laurate-stabilized emulsions (2.0 μm to 0.3 μm), respectively; multiple comparison of log-transformed data indicated that the change was significant ($P < 0.05$). However, droplet size almost did not change for sucrose esters. This indicated that smaller droplets were decreasing in control and glucose esters and were undergoing coalescence. This can be confirmed by $D[0.9]$ and volume mean diameter $D[4,3]$ that the size of droplets for these three esters were increasing. However, they increased differently – for the volume mean diameter, control group experienced the greatest change (from 37.2-74.7 μm), glucose palmitate stabilized droplet changed from 18.2 μm to 32.7 μm , glucose laurate stabilized droplet changed from 16.6 μm to 47.4 μm . This indicated that glucose palmitate and glucose laurate generated smaller droplets overall and they had stabilizing effect on droplet size, but could not completely prevent coalescence. Compared with glucose laurate, glucose palmitate is relatively more effective in stabilizing the oil-in-water emulsions.

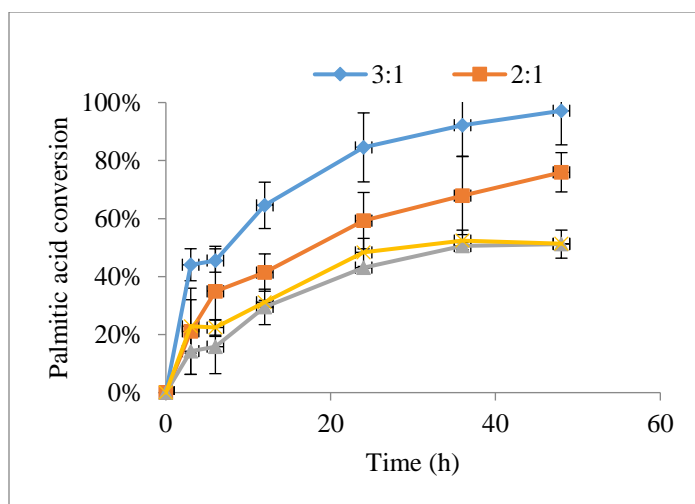
The commercial sucrose esters can effectively prevent coalescence, as indicated by no significant changes for diameter parameters over time. Three mechanisms can explain the phenomenon we observed: hydrophobic interaction (Ferrer, et al., 2002), steric stabilization (Nilsson & Bergenståhl, 2007) and adsorption on the interfacial surface (McClement, 2004b). Between glucose palmitate and glucose laurate, the former has longer alkyl chain, likely with the stronger hydrophobic interaction with oil droplets resulting in larger area coverage on the droplet to prevent coalescence. Also, the bigger molecule of glucose palmitate has stronger steric hindrance that prevent droplet from aggregating. For sucrose esters, both steric hindrance and adsorption contribute to the better stabilization effect. Sucrose esters has bigger size because the presence of a fructose moiety in addition to glucose, meanwhile the presence of more hydroxyl group made it easier to solubilize in the continuous phase and easier to adsorb at the interface, whereas glucose esters had much lower solubility in the continuous phase, their adsorption at the interface were much slower.

3.5 Conclusion

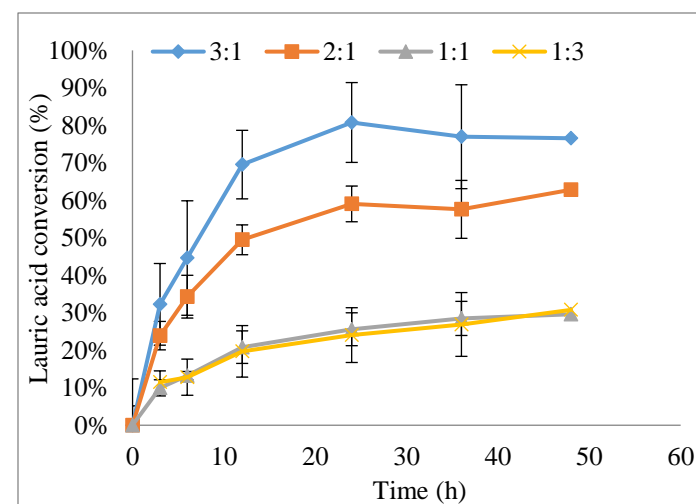
Glucose-fatty acid monoesters were successfully synthesized in tert-amyl butanol and DMSO mixture solvent system in lipase catalyzed reactions with high level of conversion. Products were purified with solvent extraction. Synthesized and commercial esters were compared for emulsion capabilities. Glucose esters stabilized oil droplets to some extent, but could not completely prevent coalescence compared to commercial sucrose esters. The relatively smaller sizes of glucose esters and low aqueous solubility can explain their difference of emulsifying property.

Acknowledgement

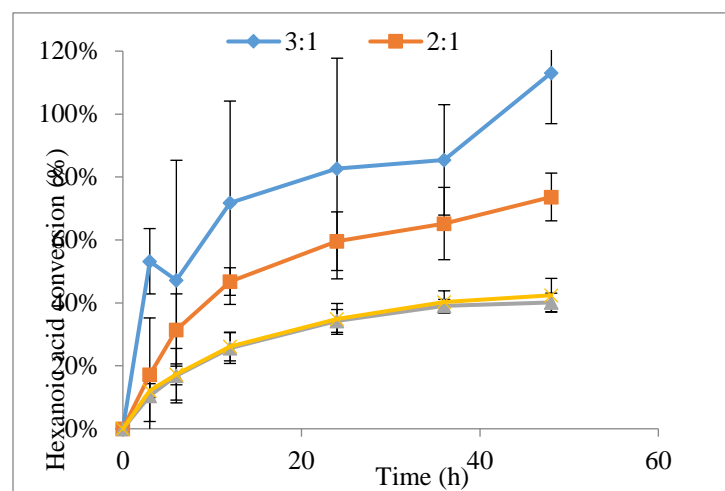
Iowa State University Agriculture Experiment Station and USDA/NIFA multistate project S1041 partially contributed to the project. We express sincere appreciation to Iowa State University Chemical Instrumentation Facility staff member Sarah Cady for training and assistance to interpret the AVIII-600 results included in this publication.



a



b



c

Fig 3.1 Conversion percentage of palmitic acid (a), lauric acid (b) and hexanoic acid (c) during 48 h. Lines with diamond, square, triangle and cross represents molar ratio of 3:1, 2:1, 1:1 and 1:3 of fatty acid/glucose respectively. Standard deviations are shown

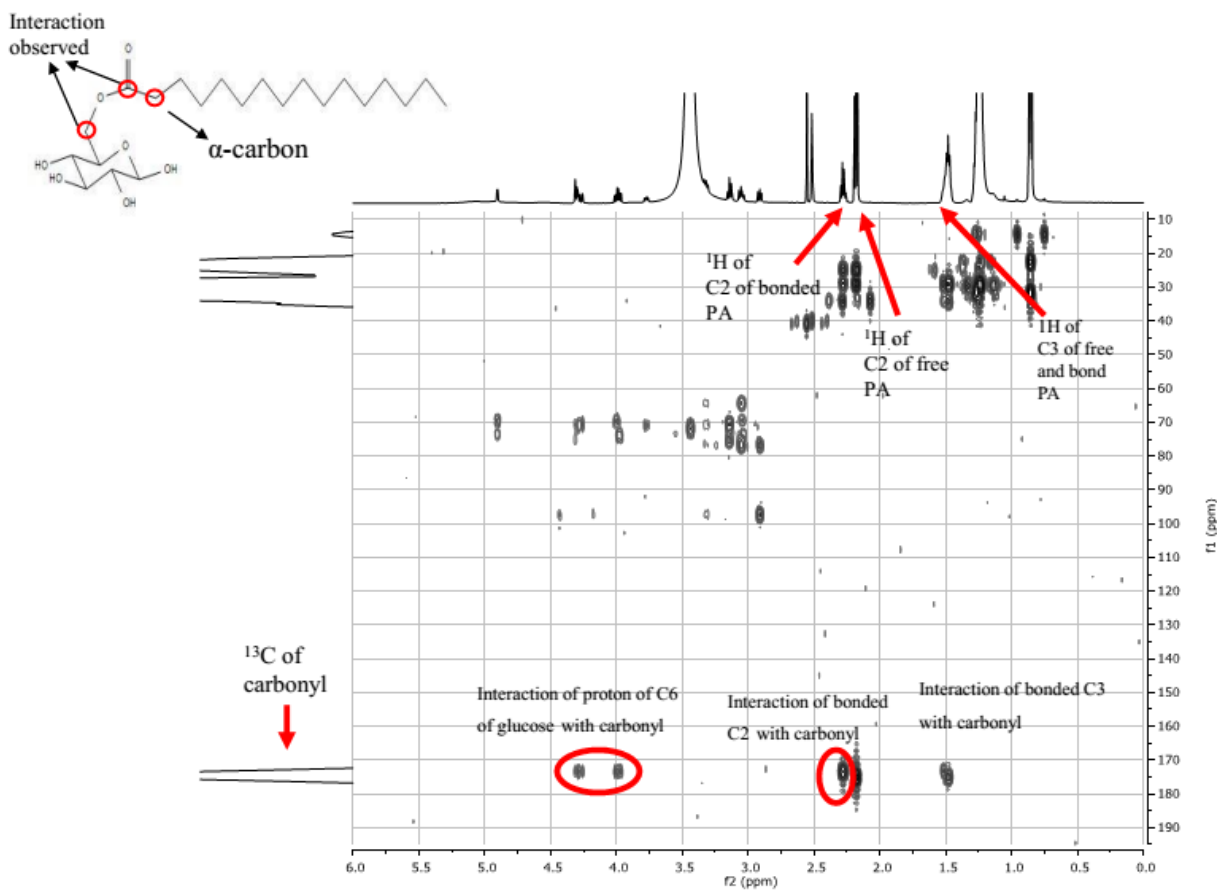


Fig 3.2 Heteronuclear multiple bond correlation (HMBC) of reaction mixture of palmitic acid (PA) and glucose. The horizontal and vertical axis indicate ^1H proton and ^{13}C chemical shift (ppm) respectively. The interaction of proton of C6 of glucose ring with the carbonyl carbon demonstrated ester bond has been formed. The ester bond also caused the α -carbon (the one next to the carbonyl carbon) chemical shift to the higher field.

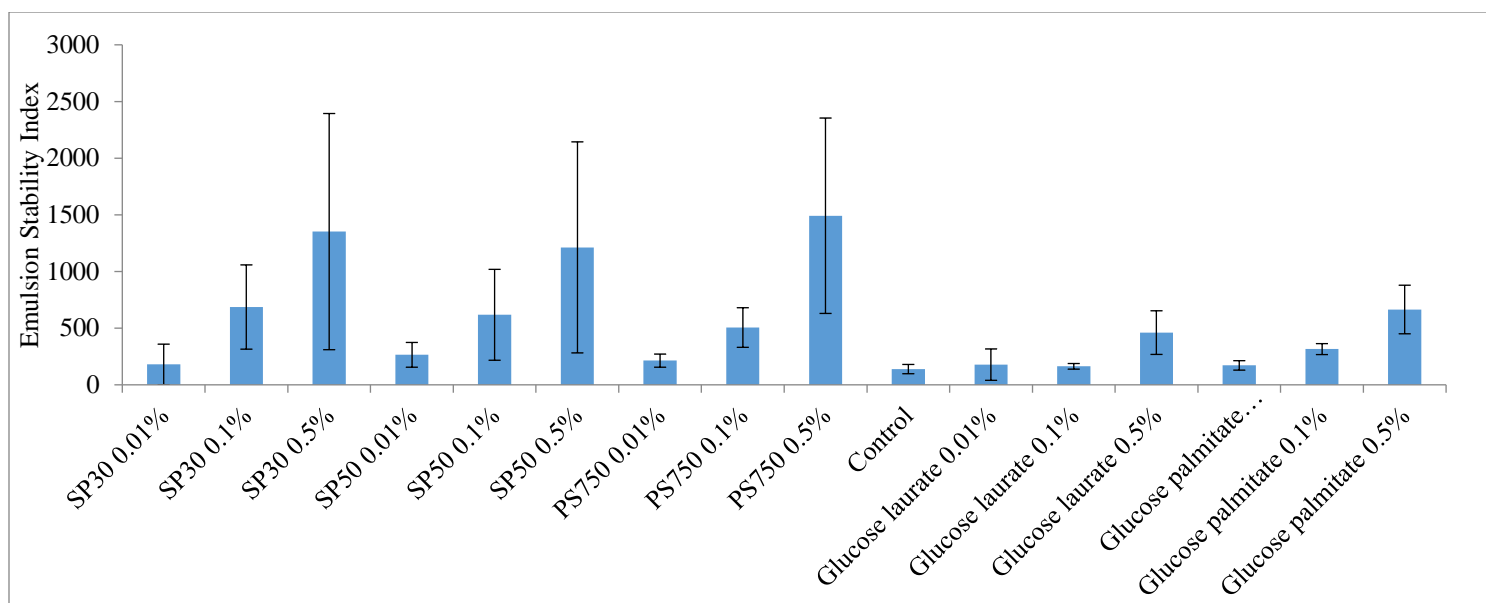
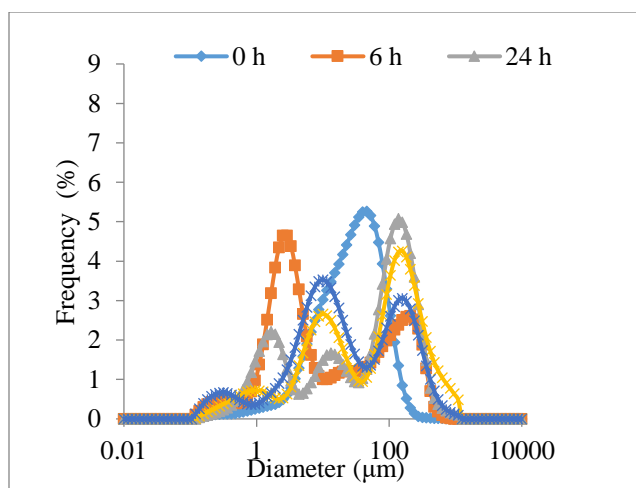
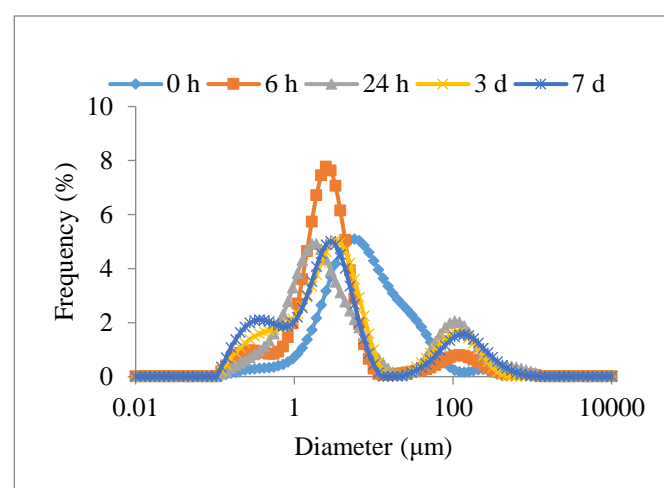


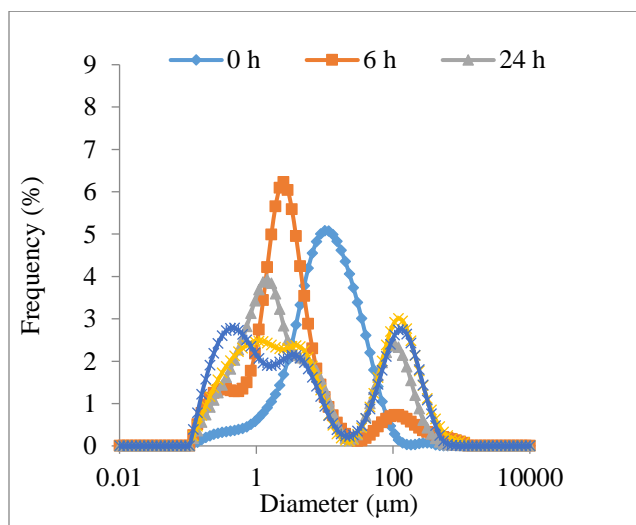
Fig 3.3 Emulsion stability index of glucose esters and sucrose esters. Mean value and standard deviation are shown.



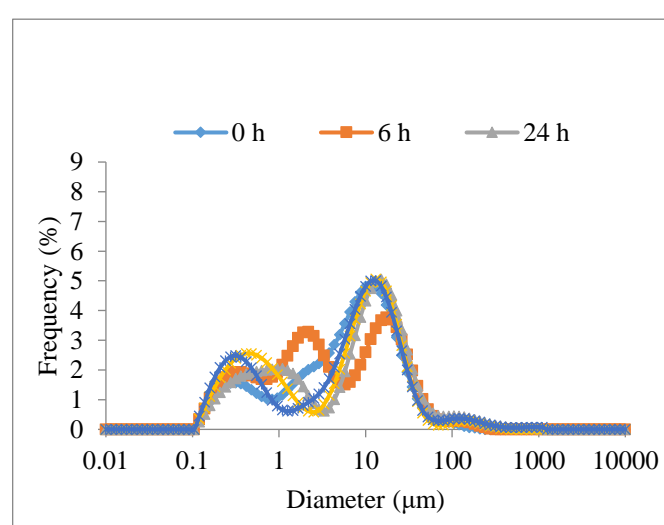
a



b



c



d

Fig 3.4 Droplet size distribution of emulsions of control treatment (a), glucose palmitate (b), glucose laurate (c), sucrose ester SP30 (d) at 0 h, 6th h, 24th h, 3rd day, 7th day

Table 3.1 Initial reaction rate ($\mu\text{mol}/\text{min}\cdot\text{g}$) and substrate solubility (mM)

Ratio of fatty acid/glucose	PA initial conversion rate ($\mu\text{mol}/(\text{min} \cdot \text{g})$)	Initial sugar solubility (mM)	PA concentration (mM)	LA initial conversion rate ($\mu\text{mol}/(\text{min} \cdot \text{g})$)	Initial sugar solubility (mM)	LA concentration (mM)	HA initial conversion rate ($\mu\text{mol}/(\text{min} \cdot \text{g})$)	Initial sugar solubility(mM)	HA concentration (mM)
3:1	9.6 ^a	16.9 ^c	243.7 ^a	7.2 ^a	21.1 ^b	259.6 ^a	11.8 ^a	26.2 ^a	235.2 ^a
2:1	4.8 ^b	18.3 ^{bc}	158.0 ^b	5.3 ^a	22.5 ^b	178.3 ^b	3.8 ^b	22.8 ^a	146.5 ^b
1:1	3.3 ^b	21.0 ^b	74.3 ^c	2.2 ^b	21.6 ^b	103.8 ^c	2.3 ^b	28.1 ^a	75.5 ^c
1:3	5.2 ^{ab}	28.2 ^a	72.9 ^c	2.6 ^b	33.6 ^a	103.6 ^c	2.7 ^b	30.5 ^a	74.4 ^c

PA- palmitic acid, LA- lauric acid, HA- hexanoic acid. Different letters indicate significantly difference in a row ($P<0.05$).

Table 3.2 Diameter summary of droplet size distribution

	D[0.1]					D[0.5]				
	0h	6h	24h	3d	7d	0h	6h	24h	3d	7d
Control	5.2±1.2	1.2±0.3	1.2±0.1	2.3±1.2	2.0±2.0	26.8±4.6	10.4±6.5	58.0±28.3	65.5±41.9	17.1±2.2
GP	1.7±0.1	0.6±0.1	0.6±0.0	0.4±0.1	0.3±0.0	6.6±0.5	2.4±0.1	2.5±0.1	2.8±0.3	2.6±0.8
GL	2.0±0.3	0.4±0.1	0.4±0.1	0.4±0.0	0.3±0.0	9.8±0.8	2.4±0.3	2.6±1.0	5.9±4.2	3.1±1.2
SP30	0.4±0.1	0.3±0.1	0.4±0.0	0.3±0.1	0.3±0.0	6.4±1.8	4.7±2.4	8.3±2.7	6.9±2.3	7.1±2.0
SP50	0.3±0.1	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.1	5.4±0.8	2.8±1.4	4.3±2.1	2.1±1.4	4.5±2.8
PS750	0.5±0.2	0.3±0.0	0.3±0.1	0.3±0.0	0.2±0.0	4.7±2.1	1.3±0.4	4.5±1.4	8.1±3.5	4.1±1.0

	D[0.9]					D[4,3]				
	0h	6h	24h	3d	7d	0h	6h	24h	3d	7d
Control	83.2±15.3	201.0±3.6	232.0±24.1	314.1±91.4	210.7±55.3	37.2±6.8	58.5±6.7	94.6±17.3	124.1±34.2	74.7±17.5
GP	34.1±4.1	20.6±22.1	148.5±93.5	91.3±23.6	99.5±96.7	18.2±4.8	17.8±6.4	42.3±29.2	23.7±5.8	32.7±30.4
GL	36.8±2.8	35.5±41.5	117.6±30.0	172.5±17.0	160.5±34.2	16.6±1.5	21.7±10.1	32.5±9.2	57.2±10.6	47.4±12.3
SP30	23.2±4.0	26.1±5.6	30.9±4.8	24.0±6.6	25.9±3.1	8.1±1.6	5.2±3.3	6.9±2.0	5.5±5.0	6.4±5.9
SP50	19.3±5.1	12.9±6.0	17.4±0.6	15.3±1.6	15.2±1.6	10.3±1.7	11.1±2.0	17.7±0.8	12.0±0.8	17.0±1.6
PS750	24.4±7.6	22.5±6.8	29.8±3.1	64.1±57.2	34.5±10.1	10.1±4.8	6.6±2.4	10.7±1.0	21.9±17.9	12.9±3.9

Mean value and standard deviation of diameters are shown in the table. For data analysis, data were transformed to a log scale to fit a normal distribution.

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CHAPTER 4. ANTIMICROBIAL EFFECT OF BIOBASED SURFACTANTS AND MECHANISM OF ACTION OF A NOVEL BIOSURFACTANT, FATTY ACYL GLUTAMIC ACID, AGAINST SELECT FOOD-BORNE PATHOGENS

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4.1 Abstract

The antimicrobial effects of some biobased surfactants sugar fatty acid esters, surfactin, and fatty acyl glutamic acid (FA-glu) on two food-borne pathogens were investigated. The possible mechanism of antibacterial action of FA-glu on bacterial cells was studied in-vitro. Of all the biosurfactants tested, FA-glu was the most water-soluble. The minimum inhibitory concentration (MIC) of glucose laurate monoester and FA-glu for *L. monocytogenes* and *E. coli* O157:H7 were 6.5 mg/mL and 25 mg/mL, respectively. FA-glu caused cellular leakage and surface damage in *E. coli* O157:H7, and rough surfaces in *L. monocytogenes*. Further investigation with artificial cell membrane phospholipids (PLs) indicated that interaction with FA-glu decreased PLs' gel-to-fluid phase transition temperature and disrupted the cooperativity of the bilayer structure. Exposure of bacteria to FA-glu resulted in release of cellular phospholipids. These findings suggested that antimicrobial effect of glucose fatty acid esters was

limited by their low aqueous solubility and FA-glu inhibits bacteria by disrupting their cell membranes.

4.2 Importance

The biobased surfactants, which were made from renewable biological sources and produced by lipase and microbial fermentation (by *Bacillus subtilis*), were studied for their inhibitive properties against three foodborne pathogens. Two types of biosurfactants: glucose laurate and fatty acid glutamic acid (FA-glu) at the concentrations of 22.1 mg/mL and 25 mg/mL, respectively, were found to inhibit the bacteria growth during 24 h. The mechanism of FA-glu's antimicrobial properties is related to its interaction with the bacterial cell membrane- at low concentrations, it can disorganize the cell membrane structure, and at higher concentrations, it can dissolve important components in the cell membrane to cause cellular leakage. The study revealed mechanisms of biosurfactants' antimicrobial properties, and will provide useful information to food and detergent industry regarding cleaning/ disinfectant development.

Key words: biosurfactant, fatty-acyl glutamic acid; surfactin, antibacterial mechanism

4.3 Introduction

Bio-based surfactants are chemicals derived from biological or renewable agricultural sources ¹ and have potential for use in food, detergent, cosmetic, paints, coatings and pharmaceutical industry ^{2,3}. The use of bio-based products lowers the risk of the environment pollution and reduces petroleum usage ². There are several types of bio-based surfactants: glycolipid and lipopeptide produced by living cells via fermentation, sugar-, polyol- and amino-based surfactants produced by enzymatic synthesis, and pulmonary surfactants that play important roles in physiological process ⁴. Apart from excellent surfactant activity, antimicrobial behavior was also reported for various biosurfactants. For example, sugar fatty acid ester such as

fructose laurate synthesized by *Candida antarctica* B lipase was shown to suppress the growth of *Streptococcus mutans* in brain heart infusion broth ⁵, sucrose esters inhibited growth of *E. coli*, *Bacillus cereus* and *Saccharomyces cerevisiae* in liquid media ⁶, and lactose monolaurate inhibited several strains of *L. monocytogenes* in dairy products ⁷. The mechanism for sugar-fatty acid esters' antimicrobial properties is not reported extensively due to their limited aqueous solubility ⁸, even though their chemical structure might be an important factor for observed antibacterial effects ⁹. Other biosurfactants such as glycolipid (rhamnolipid) and lipopeptide (surfactin) have been studied for their antibacterial effect on some foodborne pathogens. Rhamnolipid exhibited bacteriostatic effect on *L. monocytogenes* at the concentration range of 78.1 µg/mL to 2500 µg/mL ¹⁰. Rhamnolipid and surfactin reduced pathogen biofilm formation by influencing bacterial surface hydrophobicity, electron donor properties and food-contact surface hydrophobicity ¹¹. Several inhibition mechanisms for antibacterial properties of biosurfactants were studied such as disruptive interaction with artificial membrane lipids ^{12, 13} and cell-leakage ¹⁴. Other possible mechanisms for inhibition, such as interaction with cell wall and proteins from different cell fractions have not been explored.

FA-glu is a novel fatty acyl biosurfactant with only one amino acid esterified to hydrophobic fatty acid chain, and a variant of surfactin that has 7 amino acids (in a cyclic peptide) as Fig 4.1 shows ¹⁵. It is produced by a genetically modified strain of *Bacillus subtilis* that normally produces surfactin. FA-glu is composed of a β-hydroxyl fatty acid (chain length ranging from C11-17, usually myristic) linked to a glutamate molecule ¹⁵. FA-glu has much better water solubility (312 mM) and a very low critical micellar concentration (1.3 mM) ¹⁵ due to the presence of just one amino acid. Since surfactin inhibits bacterial biofilm formation ¹¹, we hypothesized that FA-glu, based on the better aqueous solubility than surfactin, may also possess

antibacterial properties. In our previous study with enzyme-synthesized glucose fatty acid esters (glucose palmitate and glucose laurate), which have shown to possess emulsion stabilizing properties¹⁶. Since the above reported sucrose esters showed antimicrobial properties, glucose fatty acid esters with similar structures might also possess antimicrobial characteristics which broaden their applicability in various food systems, including clean labels.

Accordingly, the objectives of this study were to 1) evaluate and compare the antimicrobial effect of some sugar-fatty acid esters, surfactin, and FA-glu on three common food borne pathogens, and 2) to study, in-vitro, the mechanisms of antibacterial action of FA-glu against bacterial cell membranes. The hypothesis tested was that suppression of bacterial growth by these biobased surfactants results from their interaction with the cell membranes and leakage of cellular constituents. Biosurfactant effects on artificial cell membrane phospholipids were evaluated to explain membrane interactions.

4.4 Materials and Methods

4.4.1 Bacterial cultures and reagents

Three common food-borne pathogen strains, *Listeria monocytogenes* Scott A NADC 2045 serotype 4b, *Salmonella* Enteritidis ATCC 13076, and *E. coli* O157:H7 FRIK125 were obtained from USDA/ National Animal Disease Center (Ames, IA), American Type Culture Collection, and Food Research Institute University of Wisconsin-Madison, respectively. Brain heart infusion (BHI) broth was purchased from Fisher Scientific (Fair Lawn, NJ). Glucose palmitate monoester (6-O-Palmitoylglucopyranose) and glucose laurate monoester (6-O-Lauroylglucopyranose) were synthesized in-house (Section 2.2). Sucrose ester (PS750, HLB 16) with 75% monoester content were donated by a commercial company. Lysozyme, mutanolysin and benzonase nuclease were purchased from Sigma-Aldrich (St. Louis, MO). Fatty acyl

glutamic acid was donated by Modular Genetics Inc. (Wooster, MA). Some of the surfactin (95% purity) were prepared in-house via fermentation with *Bacillus subtilis* T1651 on sugar-based media 17, whereas, others were purchased from Sigma ($\geq 98\%$ purity). Phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama).

4.4.2 In-house preparation of bio-based surfactants sugar-fatty acid esters and surfactin

Glucose palmitate and glucose laurate monoesters were synthesized with lipase in tert-butanol and DMSO mixture solvent and purified by solvent extraction following procedures previously reported ¹⁶. Palmitic acid and lauric acid were reacted with glucose at the ratio of 0.3 mM: 0.1 mM in 10 mL solvent mix (80% dimethyl sulfoxide and 20% tert-amyl alcohol). One gram of molecular sieves and 0.25 g immobilized lipase were added into the system. After reacting for 48 h, the system was centrifuged to obtain the supernatant, which was placed in a fume hood to evaporate the tert-amyl alcohol. Water (approximately 10:1 v/v of solvent) was added to the remaining solvent to precipitate the fatty acid residue and esters. The resulting slurry was filtered to obtain white solid. The solid was washed with methanol for 3-4 times to dissolve free fatty acid and obtain purified glucose esters. In-house surfactin was prepared from 15-L fermentation broth using glucose as growth medium for *Bacillus subtilis* T1651¹⁷. The in-house surfactin purification process briefly was as follows: 3 M hydrochloric acid was added to permeate of ultrafiltered (500,000 kDa) fermentation broth to decrease the pH to 2 to precipitate surfactin, the resulting slurry was mixed at 4°C for 3-4 h and was centrifuged at 12,000 g for 15 min at 4°C. The precipitate was then washed by excess amount of water and was dried properly.

The dried precipitate was dissolved in methanol, followed by the addition of ammonium hydroxide to adjust the pH to 8.5-9.0. The solution was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was collected. This was repeated two more times, and the supernatant containing surfactin was then freeze-dried and stored at 4°C until used.

4.4.3 Bacterial growth inhibition percentage and minimum inhibitory concentration.

Growth curves for three common food-borne pathogens *L. monocytogenes*, *S. enteritidis* and *E. coli* O157:H7 FRIK125 were obtained using a Bioscreen C turbidometer (Growth Curves USA, Piscataway, NJ). Bacterial stock cultures were stored frozen (-80°C) and activated separately in BHI broth (35°C, 24 h). Two consecutive 24-h transfers of each working culture in fresh BHI (35°C) were prepared prior to each experiment. Each bacterial culture was diluted 100-fold in fresh BHI to obtain a cell concentration of 10^7 CFU/mL and 30-μL aliquots of that diluted culture were used to inoculate 3-mL volume of BHI broth containing different concentrations of surfactants. Thoroughly mixed aliquots (250 μL each) of the inoculated broth were transferred to a 100-well microtiter plate and incubated (37 °C) in the Bioscreen C turbidometer. The absorbance (at 600 nm) of the broth was recorded every 30 min for 24 h. Since the glucose esters were only partially soluble in BHI broth, all the bio-based surfactants were dissolved in dimethyl sulfoxide (DMSO) first and then placed in BHI broth to improve biosurfactants' solubility. The blank control contained broth only (B), the negative control 1 (NC1) was broth and 5% DMSO, negative control 2 (NC2) was broth with added 5% DMSO and biosurfactants. Positive control 1 (PC1) was inoculated broth, positive control 2 (PC2) inoculated broth with 5% DMSO, and positive control 3 (PC3) was inoculated broth with 5% DMSO and biosurfactants. The average absorbance difference between PC1 and B, PC2 and NC1, PC3 and NC2, respectively, gave the relative influence of broth, 5% DMSO, and surfactants on bacterial

growth. Each treatment had 3 microplate wells for measurements. To compare the surfactant treatment groups with non-treated groups, the growth inhibition effect was calculated based on Equation 1⁶. To compare the growth at 24 h with 0 h for a given treatment, another parameter: growth absorbance difference was used, which was calculated based on Equation 2.

$$\text{Inhibition effect (\%)} = \frac{OD(DMSO_{24}) - OD(surfactant_{24})}{OD(DMSO_{24})} \quad \text{Equation 1}$$

where, OD (DMSO₂₄): difference in optical density at 600 nm between PC2 and NC1.

The values for PC2 and NC1 were averaged from three plate well measurements.

OD (Surfactant₂₄): difference in optical density at 600 nm between PC3 and NC2. The values for PC3 and NC2 were averaged from three plate well measurements.

$$\text{Growth absorbance difference} = OD(24 \text{ h}) - OD(0 \text{ h}) \dots\dots\dots \text{Equation 2}$$

where, OD (24) is the OD of a surfactant treatment at 24 h, and OD (0) is the OD of the surfactant treatment at 0 h.

The minimum inhibitory concentration (MIC) was defined as the lowest biosurfactant concentration that prevented bacterial growth for first 24 h at 37 °C in BHI¹⁸.

4.4.4 Solubility of biosurfactants in broth:

MIC of biosurfactants in broth would be influenced by their solubility, as they were not 100% pure, and solubility varied. The actual amount of glucose palmitate, glucose laurate, and PS 750 solubilized in the BHI broth was measured for their partial solubilities. Different amount of biosurfactants were dissolved in DMSO and were added into the broth. The broths were then incubated at 37°C for 24 h²⁰, centrifuged, residual solids were collected, and dried at 60°C in a vacuum oven until the subsequent final weights were within 0.0001 g. The differences of the

weights between the solid residue after drying and the amount that was added into the broth were calculated as the solubility. The dissolved concentration of the sugar-fatty acid esters in the broth was reported as biosurfactants concentrations in the discussions.

4.4.5 Mechanistic study of biosurfactant-pathogen interaction:

Mechanistic study of interaction between biosurfactants and pathogens was carried out using FA-glu with *E.coli* O157: H7 and *L. monocytogenes* due to FA-glu's excellent aqueous solubility. The analytical tests are described below.

4.4.5.1 Cell morphology after biosurfactant treatments.

The bacterial cell surface morphology after FA-glu treatment was observed using transmission electron microscopy (TEM) as described by Moghimi and others²¹. Briefly, *L. monocytogenes* or *E. coli* O157: H7 cells (10^{10} CFU) in 10 mL of BHI broth were harvested by centrifugation (4,000 x g for 10 min, 4 °C) and washed twice in phosphate buffer solution (PBS, pH 7.3). The pelleted cells were collected and incubated with 10 mL of 5 mg/mL FA-glu (treatment) and PBS buffer (control) for 4 h at 37°C. Cells were then washed with PBS buffer and harvested by centrifugation as previously described. The pelleted cells were washed twice with PBS solution and fixed in 0.1 M cacodylate buffer (containing 2% paraformaldehyde and 3% glutaraldehyde) and stained by 2% uranyl acetate in preparation for observation by TEM. An aliquot (3 µL) of that solution was applied to a carbon film grid and stained with 2% uranyl acetate. Images were captured using a JEOL 2100 200Kv scanning and transmission electron microscope (Japan Electron Optics Laboratories, USA, Peabody, MA).

4.4.5.2 Leaked cell constituents

Absorbance at 260 nm and at 280 nm were used to measure nucleic acid (A₂₆₀) and proteins (A₂₈₀) that leaked from the bacterial cells treated with aqueous FA-glu., respectively²².

Cells were incubated with FA-glu solution at 5 mg/mL and 10 mg/mL for 4 h at 37°C and were harvested by centrifugation as described in Section 2.3 and the absorbance of the supernatant was measured. Controls for them were cells incubated with PBS solution (blank control) and FA-glu solutions at 5 mg/mL and 10 mg/mL that were not inoculated with cells. The absorbance of these controls were also measured at these two wavelengths.

4.4.5.3 Cell protein fractionation and protein electrophoresis

The protein from centrifuged (cell-free) solution of FA-glu incubated with cells, cell wall protein, cell membrane protein and cytoplasmic protein were fractionated according to methods described in a previous study²². Briefly, *L. monocytogenes* and *E. coli* O157:H7 were sub-cultured in 50 mL BHI broth for 24-36 h to obtain a final cell concentration of $\sim 10^9$ CFU/mL. Cells were harvested by centrifugation (6,200 x g, 10 min, 23°C), washed with PBS buffer and incubated with 50 mL of 5 mg/mL FA-glu in PBS buffer for 4 h at 37°C. The cells from surfactant-free control were incubated in 50 mL PBS solution. After incubation, the supernatants were collected by centrifugation (6200 x g, 15 min, 23°C), and filter-sterilized using 0.22 μ m syringe filter. Leaked cellular constituents were isolated by freeze-drying the supernatant and hydrated with 400 μ L of 1 M Tris-HCl (pH 8.8). Protoplasts or spheroplasts were generated by incubating pelleted cells in 0.1 mL sucrose wash buffer (SWB, 10 mM Tris-HCL pH 6.9, 10 mM MgCl₂ and 500 mM sucrose) containing 10 mg/mL lysozyme and 2,500 U/mL mutanolysin for 2 h at 37°C. The cell wall protein and protoplasts/spheroplasts were then separated by centrifugation at 6,200 x g for 15 min at 4°C to obtain supernatant for protein analysis. The pelleted protoplasts/spheroplasts were washed in 1 mL SWB and suspended in 200 μ L lysis buffer (100 mM Tris-HCl pH 7.5, 10 mM MgCl₂, and 362 U benzonase nuclease). Cell lysis was achieved by exposure of cells to three freeze-thaw cycles and the samples were subjected to

centrifugation as previously described. The supernatant and pellet with cytoplasmic protein and cell membrane proteins, respectively, were suspended in 0.1 mL TRIS-EDTA buffer (10 mM TRIS-HCL pH 7.9 and 1 mM EDTA Na₂). All protein fractions were stored at -20°C and estimated by Bradford assay. Around 11 to 60 µg protein samples were loaded for protein electrophoresis and silver staining was employed.

4.4.5.4 Biosurfactant interaction with artificial cell membrane phospholipids

The interaction of FA-glu with in-vitro membrane phospholipids was determined by differential scanning calorimetry¹². DMPC, DMPG and DPPE were chosen as representative cell membrane phospholipids. Three micromoles of phospholipid were mixed with FA-glu at 0%, 1%, 2.1%, 2.5%, 5.1% and 12.5% (mol/mol), dissolved in methanol/chloroform (2:1 v/v), dried overnight under fume hood and dried further under vacuum for 2 h. The mixtures were hydrated with 40 µL 150 mM NaCl, 0.5 mM EDTA, 20 mM TRIS (pH 7.4) above their phase transition temperature (24°C for DMPC, 23°C for DMPG and 63°C for DPPE). For DPPE, the mixtures were heated (70°C) for 1 hr and were sonicated at 20% amplitude for two 3-min periods with a 3-min resting period²³. Phospholipid vesicles (15 µL or 20 µL) were transferred to aluminum calorimetry pan sealed and set for 24 h to reach equilibrium. Scans were conducted in a Perkin-Elmer DSC7 differential scanning calorimeter. For DMPC and DMPG, samples were heated from 10°C to 50°C, DPPE were heated from 10 to 80°C, with heating rate of 10°C/min. For each treatment, at least three samples were scanned.

4.4.5.5 Identification of phospholipid by ³¹P nuclear magnetic resonance (NMR) and mass spectrometry

The ³¹P NMR and mass spectrometry were used to detect the presence of phospholipid and their types in the FA-glu treated cell-free supernatants. Controls that were extracted by

chloroform and methanol were prepared according to the method described by Bligh and Dyer²⁴. Bacterial cells collected after 18-24 h incubation in 50 mL BHI broth (37 °C) were suspended in 5 mL PBS solution and 4 mL chloroform and 2 mL methanol were added. The mixture was placed in shaker incubator for 20 min and then subjected to centrifugation (6,200 x g, 15 min at room temperature) to obtain the lower organic phase. For treated cells, 3 mL chloroform and 1.5 mL methanol were added to 2 mL cell-free supernatant and after 20 min of incubation, the lower organic phase were collected. The samples were scanned in the negative mode from 1000-1500 Daltons using an Agilent Quadrupole Time-of-Flight (QTOF) mass spectrometry. For ³¹P NMR test (Bruker Avance III 600, Billerica, MA and Karlsruhe, Germany), samples for mass spectrometry were dried in the fume hood and dissolved in deuterated chloroform.

4.4.5.6 Statistical analyses

Statistical tests were conducted using SAS 9.4 software (SAS Institute Inc., Cary, NC). Proc GLIMMIX test was used to determine significant difference between treatments ($P < 0.05$). At least three measurements were taken for Bioscreen tests, DSC and leakage content determination.

4.5 Results and Discussion

4.5.1 Microbial growth and inhibition by biosurfactants

Since some of our biobased surfactants had limited solubility in the BHI broth, DMSO was used as a co-solvent to improve the solubility. Preliminary experiments indicated that 5% (v/v) DMSO in BHI broth did not adversely affect bacterial growth. Although increase in absorbance of all three bacterial cultures in broth with added DMSO was slightly less than that of controls (Fig 4.2a, 4.2b), absorbance in broth cultures with DMSO reached a comparable level with the non-DMSO controls at 24 h. Moreover, DMSO is used as a cell preservative²⁵,

therefore, regarded as a non-detrimental medium for bacteria while acting as a solvent for hydrophobic substances. The biosurfactants were dissolved in pure DMSO and diluted in BHI to achieve the 5% (v/v) DMSO as well as the desired surfactant concentration. Figure 2a and 2b shows growth of the pathogens in BHI with or without 5% (v/v) DMSO, and with select biosurfactants. Only representative growth patterns are presented since several graphs for the three pathogens showed similar trends.

Gram-positive (*L. monocytogenes*) and Gram negative (*S. Enteritidis* and *E. coli* O157:H7) exhibited differences in resistance to glucose palmitate. Due to the limited solubility of glucose palmitate, the highest concentration tested while minimizing insoluble material was 0.56 mg/mL. None of the concentrations of glucose palmitate tested fully inhibited the growth of the three microorganisms as shown in Table 4.1. It was obvious that the inhibition percentages did not reach 100%. The concentrations 0.51 mg/mL and 0.56 mg/mL achieved the highest inhibition, with lower concentrations showing lower inhibitions during the incubation period, thus the growth curves are not presented.

Glucose laurate was effective against *L. monocytogenes* at 6.5 mg/mL, in that, it inhibited the growth of this pathogen after 5 h with absorbance remaining unchanged for 24 h (Fig S4.1a). Similar results were obtained for *S. Enteritidis* and *E. coli* O157:H7 (growth curves not shown). Although the microbial growth inhibition was less than 100% (91%, 85%, and 74% for *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, respectively; Table 1), these results only demonstrate the effectiveness of glucose laurate treatments compared to controls in suppressing bacteria at the 24th h, which did not completely reflect how it grew since the beginning of inoculation. In this respect, the absorbance differences between 0 h and 24 h for a treatment were used to evaluate the biosurfactants' antimicrobial effectiveness (Table 4.1). The smaller the

difference in absorbance, the stronger the inhibitory effect on bacterial growth, indicating effective antimicrobial behavior by biosurfactants. Glucose laurate at 6.5 mg/mL completely prevented bacterial growth (difference being -0.03, -0.05 and -0.01 for *L.monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, respectively). In this regard 6.5 mg/mL was the MIC of glucose laurate for all three pathogens tested, because it inhibited the bacteria for the first 24 h. Exposure of *L. monocytogenes* to the lower glucose laurate concentrations of 0.13 mg/mL and 0.085 mg/mL resulted in an initial increase in absorbance at 5 h ($A = 0.82$) and 10 h ($A = 1.09$), respectively, followed by a drastic decrease in absorbance to 0.02 and 0.19, respectively, at 24 h (Fig S4.1b). This general trend was also observed for *S. Enteritidis* and *E. coli* O157:H7. Results showed inhibition percentage for *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, respectively, as 108%, 133% and 130% (Table 4.1), and 24 h absorbance differences as -0.27, -0.22 and -0.51 (Table 4.1, values in parenthesis) when treated with glucose laurate at 0.13 mg/mL. This concentration, however, was not chosen as the MIC, although theoretically it fit the definition of MIC stated in previous reports. Those same reports referred to MIC as the lowest concentration at which the absorbance did not rise significantly compared with negative control, and it only described the absorbance difference between the start and end point of incubation time. In this regard, no consideration was given to substantial changes in absorbance that can occur between those two time points. From a practical food safety standpoint, the MIC should be the lowest concentration of the antimicrobial agent that inhibits bacterial growth throughout the entire incubation period. Higher concentrations of glucose laurate (2.8 mg/mL and 0.62 mg/mL) suppressed growth of all three pathogens after about 7 h; however, the organisms continued to grow slowly later on. Other lower concentrations were not effective.

A comparison between growth profiles for glucose palmitate and glucose laurate treated cells reveals that glucose laurate was more effective biosurfactant against all three bacteria. This may be related to the fatty acid moiety in the sugar esters, lauric acid, which has been demonstrated to be the most effective C6-C18 free fatty acids against gram-negative and positive bacteria²⁶. It also inhibited a bacterium that causes skin inflammation²⁷, and was effective against other three Gram-positive cocci²⁸.

For the commercial sucrose ester PS750, soluble concentration of 2.26 mg/mL (amount put in the broth 4.15 mg/mL) inhibited bacterial growth (70%, 52% and 51% inhibitions for *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, respectively), that were lower at lower concentrations (Table 4.1). Since it was not very effective in inhibiting bacteria compared to our in-house synthesized glucose laurate, we did not pursue further MIC studies on this biosurfactant.

Surfactin has been reported to inhibit biofilm formation^{11, 29}, however, reported studies on its direct inhibitory effect on foodborne pathogens are scarce. Contrary to the results expected, in-house surfactin at 5, 10 and 15 mg/mL led to an unexpected increase in absorbance between 5 h and 10 h, followed by a dramatic drop, possibly indicating surfactin-induced cell lysis (Fig S4.1b). The surfactin used at higher concentrations (5, 10, 15 mg/mL) were from our in-house preparation and was lower in purity (95%); we speculate that possible impurities likely to be residual metabolites from bacterial fermentation, might have supported bacterial growths during 9-10 h for unexpected increase. Lower concentrations (0.005-0.1 mg/mL) did not show that pattern. The surfactin used for lower concentrations (0.005-0.1 mg/mL) were from Sigma (purity over 98%). In general, both in-house and commercial surfactin were much less effective by 24 h than glucose laurate, because the inhibition percentages in all the concentrations used were less

than 40% (Table 4.1). Consistent with our result, Nonejuie (2016)³⁰ reported the commercial surfactin did not show any antibacterial activities. However, some other studies report surfactin to be quite effective. For example, Nobmann and others reported surfactin from various strains of *Bacillus subtilis* inhibited *L.monocytogenes* at relatively low concentrations (less than 0.3 mg/mL). Magalhães and Nitschke¹⁰ demonstrated that the MIC to inhibit spores of *B. cereus* was only 156.25 µg/mL. These concentrations were much lower than those used in the present study. Various reasons could have contributed to the different outcomes: one reason is that surfactin is a composite mixture of its isomers, it is possible that not every structure of surfactin possessed antibacterial property. Therefore, different surfactin-producing bacteria may produce differently active surfactin. Moreover, the environmental parameters such as (such as pH, salt and methanol presence) varied among reported studies, which preclude meaningful comparison of results with present study. Due to the expensive price of surfactin, higher concentrations of surfactin were not tested for its MIC for 3 bacteria studied.

Since the FA-glu had much better aqueous solubility compared to other bio-based surfactants tested, its inhibitory effect in 5% DMSO broth was studied. At 25 and 20 mg/mL, FA-glu in broth itself had better inhibition effect than in broth with 5% DMSO. The FA-glu concentration at 25 mg/mL in aqueous solution strongly inhibited the growth of *L. monocytogenes* and *E. coli* O157:H7, as indicated by the inhibition percentage 100% and 99%, respectively, and very small absorbance difference between 0 h and 24 h (0.04 and 0.07). The concentration at 20 mg/mL inhibited growth for up to 16 h; however, bacteria started to grow afterwards (Fig 4.2b). At 15 mg/mL, 10 mg/mL and 5 mg/mL, FA-glu in 5% DMSO broth had better growth inhibition effect than in broth alone, which could be due to the presence of DMSO, but none of these concentrations was bacteriostatic. For other concentrations, the lower they

were, the less inhibition they showed: 0.05 mg/mL and 0.1 mg/mL had almost no effect on the growth for 24 h. *S. Enteritidis* was the most resistant among the three pathogens studied, since its growth was not inhibited at the FA-glu concentrations studied. It was apparent that FA-glu had better antibacterial effect than surfactin (at 5, 10, 15 mg/mL) against all three pathogens.

The minimum inhibitory concentrations evaluated for glucose laurate and FA-glu were 6.5 mg/mL and 25 mg/mL, respectively. Although glucose laurate exhibited a greater antibacterial effectiveness, its lower solubility in aqueous solution restricts its use in practical applications; therefore, the mechanistic study for microbial inhibitory effect was only conducted for more soluble biosurfactant FA-glu. *L. monocytogenes* and *E. coli* O157:H7 were treated with 5 mg/mL FA-glu in studying the possible inactivation mechanism(s) and results compared with those of with PBS-treated control. These two organisms were chosen for being foodborne pathogens that are Gram positive and Gram negative bacteria, respectively.

4.5.2 Cell surface morphology

Figures 4.3a and 4.3b show the TEM images of the surface of FA-glu treated and control cells of *L. monocytogenes* and *E. coli* O157:H7, respectively. PBS-treated control *E. coli* O157:H7 cells (Fig 4.3a(1) and 4.3a(3)) had smooth surface and uniform color, while the biosurfactant treated cells seemed very distorted in shape with markedly rough surface and some electron dense material between the cells (Fig 4.3a(2) and 4.3a(4)), possibly indicating leaked materials from the cells. Electron-dense particles or precipitates were reported around damaged bacterial cells in comparison to undamaged cells. Our results are consistent with those of a recent study on naturally derived surfactants, which showed that sophorolipid- and thiamine dilauryl sulfate-treated *E. coli* O157:H7 cells were distorted with uneven surfaces. Those authors ascribed the observed alterations to cell membrane damage caused by biosurfactants treatments. Similar

morphological changes in bacterial cells were reported by other researchers. For *L. monocytogenes* control groups (Fig 4.3b(1) and 4.3b(3)), the cell surfaces were rather smoother and uniform, although some staining on cell the surface caused a bit darker appearance. The *L. monocytogenes* treated cells (Fig 4.3b(2) and 4.3b(4)) remained intact but the surface seemed to have several black specks, which might indicate that FA-glu caused morphological alterations to make the surface non-uniform. Unfamiliar triangles and round shapes were seen in TEM pictures, which most likely occurred for PBS salt crystals caused by vacuum effect prior to TEM examination.

4.5.3 Spectrophotometry study of the leaked cellular constituents.

The FA-glu solutions at 5 and 10 mg/mL had some absorbance by themselves without any cells; the increase in its concentration had increase in absorbance. For *E. coli* O157:H7 (Fig 4.4a), the cell-free supernatant treated with FA-glu at 5 mg/mL had increased absorbance at both 260 nm and 280 nm compared with no-cell blank control and FA-glu solutions. This indicated that those 260 nm – and 280 nm- wavelength absorbing substances were released from the cells exposed to FA-glu at 5 mg/mL, thus, strongly suggesting damage to the outer lipopolysaccharide membrane and the cytoplasmic membrane of *E. coli* O157:H7. Our results correspond another study which demonstrated the damaging effect of sophorolipids against the *Pseudomonas aeruginosa* membranes based on copious release of protein from treated cells. For cells treated with 10 mg/mL FA-glu, there was a significant increase in A_{280} (but not A_{260}) value of the supernatant compared to control 10 mg/mL FA-glu. Comparing the absorbance for cells treated at two concentrations, no significant increases at both wavelengths was observed, indicating increased absorbance at higher FA-glu concentration did not result from leakage of nuclei acid and proteins.

For *L. monocytogenes* (Fig 4.4b), the data had similar trend as those for *E. coli* O157:H7. Cells treated with 5 mg/mL FA-glu produced slightly higher absorbance in the cell-free supernatant at both wavelengths than control 5 mg/mL FA-glu solution; however, supernatant from cells treated with 10 mg/mL FA-glu did not result in significant absorbance increase when compared with FA-glu 10 mg/mL solution. Additionally, higher FA-glu concentrations did not cause more leakage. It is possible that the leaked constituents were in very small quantity and thus challenging to discern since the FA-glu by itself exhibited some absorbance. Based on these observation, silver staining was performed to determines the protein leakage from cells.

4.5.4 Cell leakage and silver staining protein from different cell fraction

Transmission electronic microscopy and spectrophotometry qualitatively confirmed the leakage from treated cells; however, constituent types and quantity was not verified. Therefore, fractionation of proteins was conducted for 1) protein in cell-free supernatant after incubation with cells with FA-glu, and 2) proteins from cell wall, cell membrane, and cytoplasm of bacteria with and without FA-glu treatment (5 mg/mL). These proteins were subject to silver-staining electrophoresis for their presence. The electrophoresis procedure was repeated three times, and representative figures that show common characteristics are presented in Fig 4.4a and 4.4b.

For *E. coli* O157:H7, significant band profile differences were observed in cell-free supernatant, cell wall, and cytoplasmic fraction of treated and untreated microbial cells. Smearing occurred from the supernatant of treated cells, while almost no proteins were observed in untreated cells (data not shown). Considering the high absorbance at 280 nm from the spectrophotometry, the smearing was possibly caused by significant amounts of cytoplasmic components. More protein bands were observed in the cell wall fraction of treated cells than those from untreated cells (Fig 4.5a). Protein bands at around 55 kD, 73kD, 76 kD and 100 kD

that were observed in the control were not observed in fraction from treated cell wall. Sotirova et al.³¹ postulated that certain biosurfactants can interact with bacterial surface proteins and may cause removal of those proteins by solubilization. In the cell membrane fraction, no differences were seen in band types between control and treated cells, but the intensity of the band in the treated group was a little fainter, possibly indicating FA-glu binding of some of the membrane-embedded proteins and causing them to detach from the membrane prior to fractionation. Proteins larger than 57.5 kD were not seen in the cytoplasmic fraction of treated cells suggesting that the larger proteins leaked out of the cells. In the smeared lane of cell leaked-content (not shown), some bands from 57.5-150 kD were observed, although not very distinct (indicated with arrows, Fig 4.5a), which likely represented those leaked cytosol proteins.

For *L. monocytogenes* cells, several bands (around 75 kD, 55kD and 45 kD) were present in the supernatant from FA-glu treatment (Fig 4.5b). The leaked content was not as much as those observed for *E. coli* O157:H7, which can explain small and not significant absorbance difference observed in cell free FA-glu solution incubated with *L. monocytogenes* cells (Fig 4.5b). For cytoplasmic protein content, proteins with larger than 75 kD were not observed in treated cells. As for cell wall fraction, no significant differences in protein were observed in treated and control cells.

Based on the results of silver-staining electrophoresis, it can be said that FA-glu caused more leakage of cellular content for *E. coli* O157: H7 than *L. monocytogenes*. This difference might be explained by differences in cell wall (peptidoglycan) thickness of Gram positive and Gram negative bacteria which have 10 to 20, and 1 to 2 layers of peptidoglycans, respectively, with the thin Gram negative peptidoglycan located between the outer and inner membrane³². The thicker peptidoglycan in Gram positive bacteria with a large, rigid, mesh-like structure can

protect the cytoplasmic membrane and most likely acted as a barrier in minimizing FA-glu's full interaction with the cytoplasmic membrane.

4.5.5 In-vitro Interaction of FA-glu with artificial cell membrane

The outer-membrane of *E. coli* O157:H7 has phospholipids (PL) that can be easily accessed by FA-glu. Two approaches were taken to determine how FA-glu interacted with cell membranes. In one, the DSC was used to determine the interaction of FA-glu with artificial cell membrane PLs; it was then followed by the detection of phospholipids in the treated cell-free supernatants. DPPE are the predominant PLs in *E. coli* cell membrane³³ and phosphatidylglycerol is in higher amounts in *L. monocytogenes* than other bacteria²²; Phosphocholine is a general essential PL in membranes thus DMPC was also chosen. The DSC transition temperature, peak broadness and enthalpy of the membrane phospholipids were measured and compared to demonstrate the influence of FA-glu incorporation on phospholipid.

Interaction with FA-glu decreased the phase transition temperature of DMPC and resulted in a broader peak, as indicated by the $\Delta T_{1/2}$; the melting temperature was around 23°C (Table 4.2, Fig S4.2). Further addition (2.1%-12.5% mol/mol) of FA-glu decreased the phase transition temperature. Our results are consistent with similar research reporting the interaction of DMPC with surfactin¹³, antimicrobial peptide³⁴ and α -tocopherol³⁵. The decrease in melting temperature indicated perturbation in the acyl chain cooperativity by FA-glu; in other words, the outside molecule or "impurity" (FA-glu) distributed within the bilayer, affecting the van der Waals forces between the hydrocarbons, resulting in phase change of tightly packed hydrocarbon core into free-rotational chain at lower temperature^{13, 36}. As reported in the literature, a small molecule like FA-glu could get buried in the hydrocarbon core, interacting with C2-C8 methylene region. As a consequence of such interaction, melting temperature decreased, peaks

became broader, and enthalpy remained relatively unaffected. The interaction of FA-glu with another phospholipid DMPG exhibited similar results (Table 4.2, Fig S4.2). As for DPPE (Table 4.2, Fig S4.2), the phase transition temperature slightly decreased with FA-glu addition; however, the width of the peak was not significantly affected. Although the mean values of enthalpy decreased with the increase in FA-glu concentration, a statistical significance was not observed. This could be due to the location of FA-glu, which could be at C9-C16 in PL hydrocarbon chains, according to some reports³⁷. The enthalpy slightly decreased, indicating amino acid interaction with the PL head group, caused by the head-to-head repulsion¹². The DSC study indicated that FA-glu disrupted the cooperativity of the PL packing, and could therefore modify the organization of bacterial cell membrane. The charge on PL did not seem to have a significant impact on the pattern of interaction with FA-glu; DMPC and DPPE are zwitterionic and DMPG has negative charge. For the concentration range studied, no mixed micelle of PL and FA-glu formed because only a single phase transition peak in all treatments was observed.

4.5.6 FA-glu interaction with phospholipid of bacterial cell membrane.

The silver staining electrophoresis clearly illustrated that FA-glu caused significant perturbation to cell membranes to cause leakage of cytoplasmic proteins. At lower surfactant concentrations, PLs are only disrupted cooperatively without changing the bilayer structure as the DSC results showed; whereas at higher concentrations, PLs will form mixed micelle with the surfactant, causing bilayer damage. Solubilization of PLs has been reported to occur when the mixed micelle is formed³⁸.

To confirm whether the 5 mg/mL FA-glu solubilized some PLs in bacterial cell membranes, mass spectrometry and ³¹P NMR were conducted. Table 3 shows the phospholipid profiles present in bacterial cell membranes that were extracted by chloroform and methanol

mixture solvent (control)²⁴ and FA-glu. The data were compared with reported phospholipid studies for *E. coli*³³ and *L. monocytogenes*³⁹. Three types of PLs ([M-H]⁻ 579, 733 and 773) were present in the FA-glu supernatant from *E. coli* O157:H7, while only one type of PL ([M-H]⁻ 773) was present in *L. monocytogenes* FA-glu supernatant (Table 4.3). Due to limited data in the published literature, not many PLs were identified for *L. monocytogenes*; the ones identifiable in the organic solvent extract were not many as they mostly were for the *E. coli*. Chemical shifts -1.4 ppm and -13.4 ppm in ³¹P NMR were observed both in organic solvent extract of *E. coli* O157:H7 and FA-glu supernatant. The chemical shift of -1.4 ppm indicated the presence of PL^{40, 41}, while shift at -13.4 ppm might indicate the other forms of phosphorous, such as adenosine thiamine diphosphate⁴², a nucleotide sugar that is associated with response to cellular stress, or uridine diphosphoglucose⁴³. However, in the organic solvent extract and supernatants from FA-glu treated *L. monocytogenes*, only the chemical shift of -13.2 ppm was observed, which meant that PLs were not detected by this technique. This suggests that the single cell membrane in *L. monocytogenes* may not be enough to generate a signal in ³¹P NMR while *E. coli* O157:H7 have a double cell membrane; therefore, the PLs are more concentrated. The presence of PLs in the bacterial FA-glu supernatant confirmed our hypothesis that the FA-glu solubilized some PLs in the cell membranes.

Acknowledgment:

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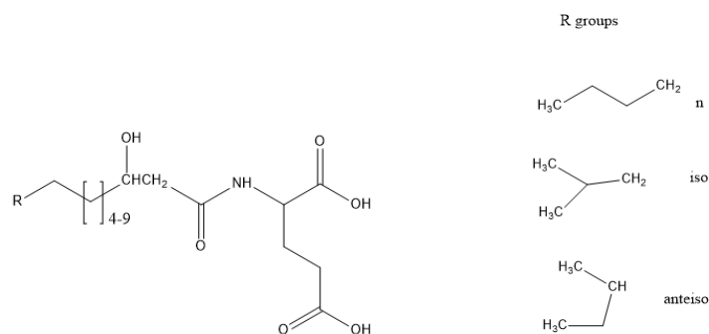
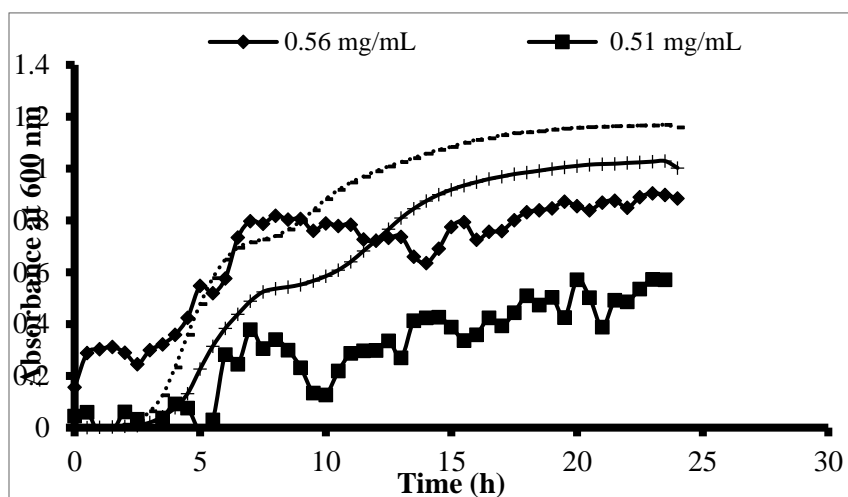
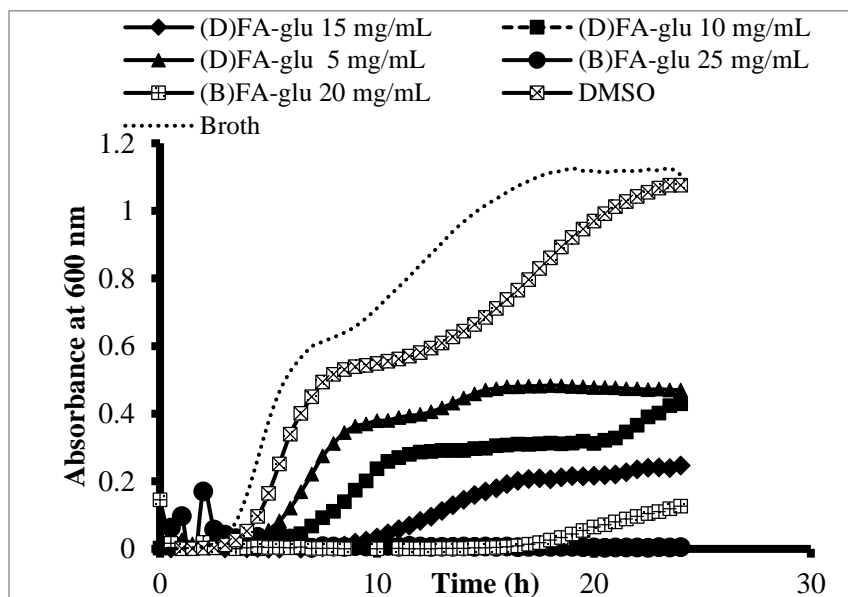


Figure 4.1 Structure of FA-glu

Figure 4.2a Glucose palmitate monoester (PA)'s inhibition effect on *Salmonella* EnteritidisFigure 4.2b FA-glu's inhibition effect on *Listeria monocytogenes*. “(D)” indicates the 5% DMSO broth, “(B)” indicates the broth

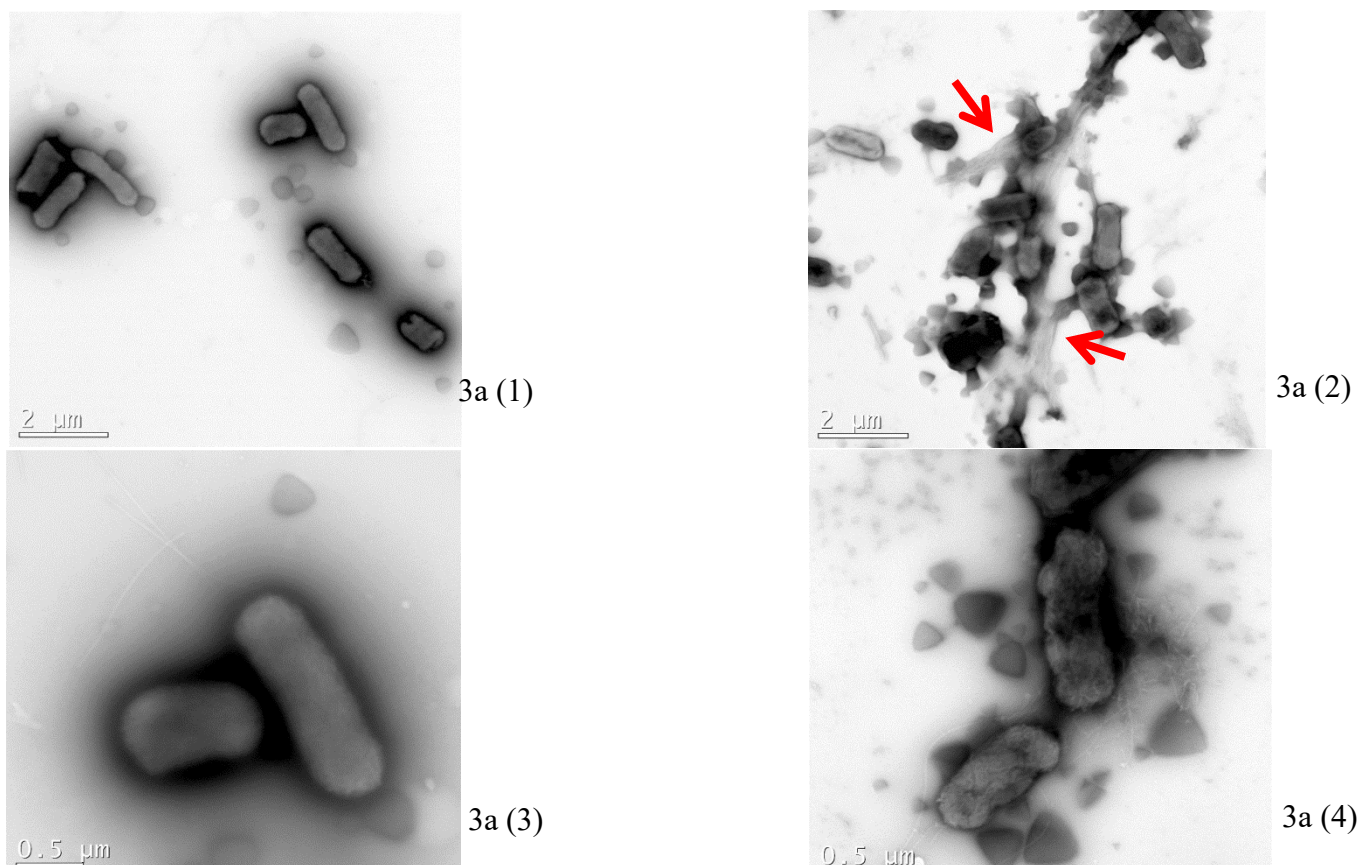


Fig 4.3a Transmission electronic microscopy image of negative stained *E.coli* O157:H7. 2a (1) and 2a (3) were untreated *E.coli* O157:H7, 2a (2) and 2a (4) were *E.coli* O157:H7 treated with 5 mg/mL FA-glu

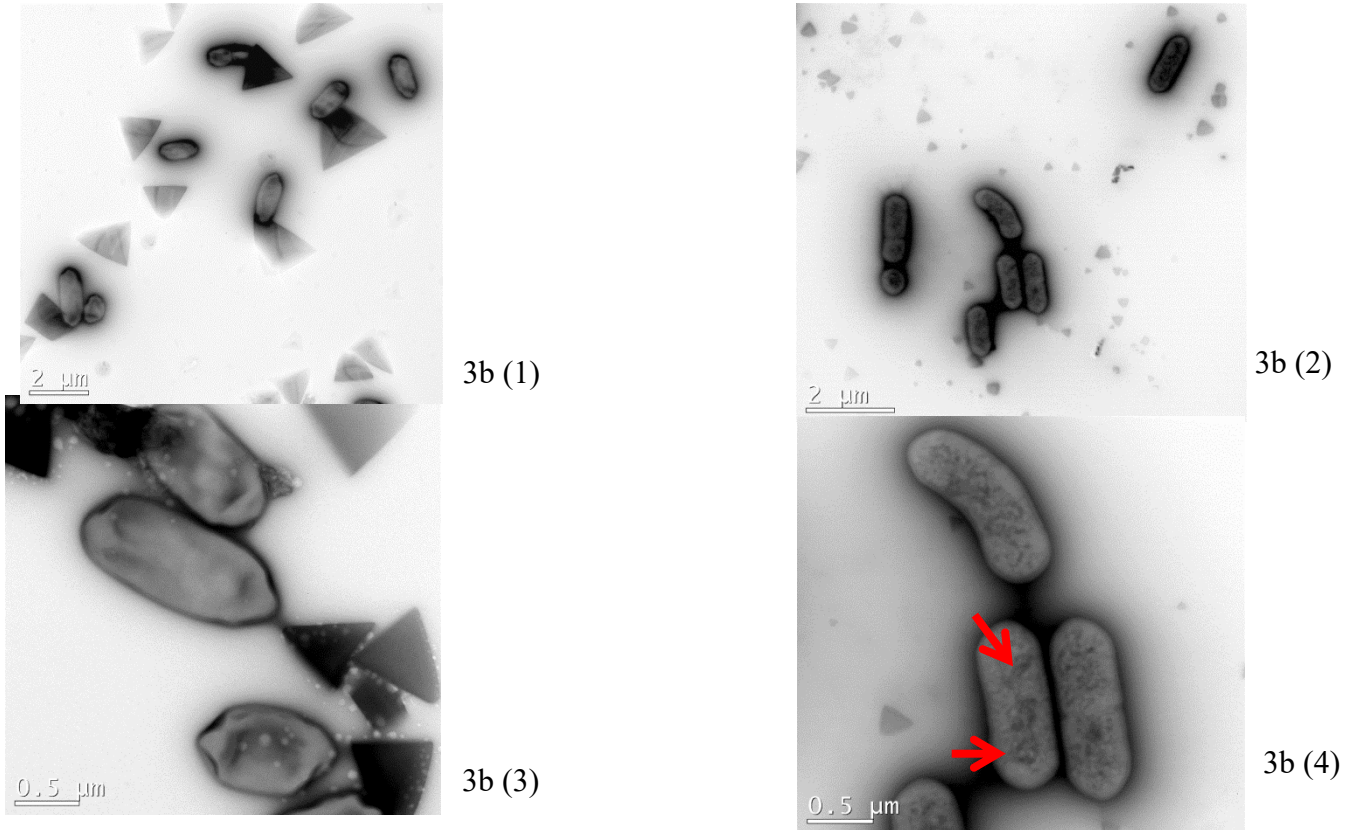


Fig 4.3 (continued) b Transmission electronic microscopy image of negative stained *Listeria monocytogenes*. 2b(1) , 2b(3) were untreated *Listeria monocytogenes*, 2b(2), 2b(4) were *Listeria monocytogenes* treated with 5 mg/mL FA-glu

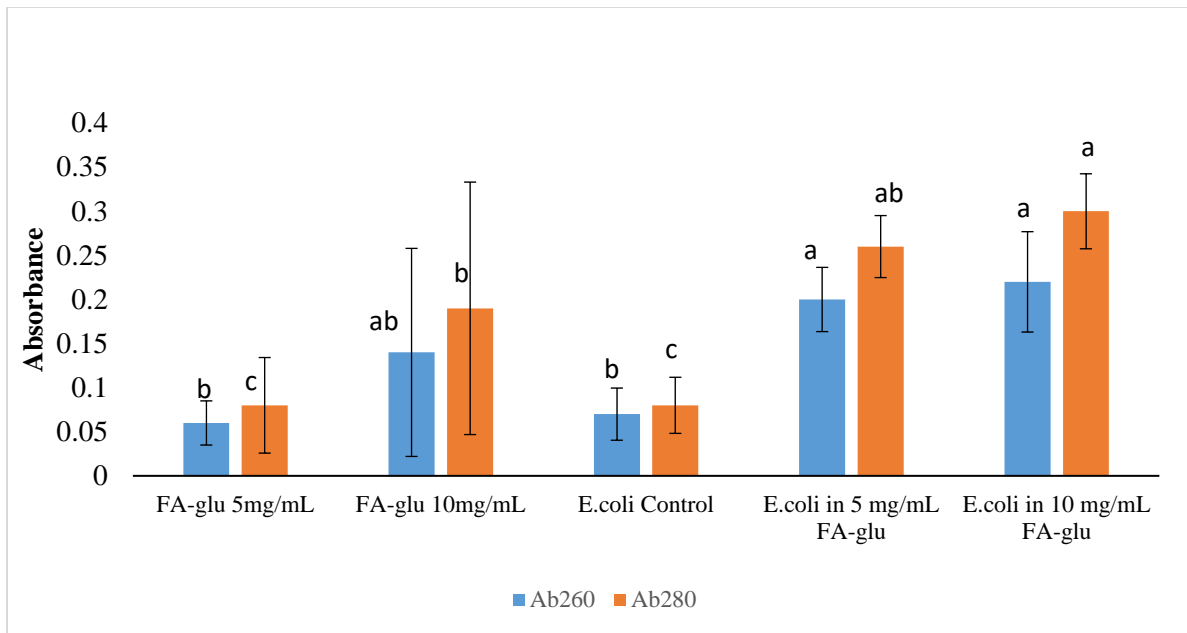


Figure 4.4a Absorbance of leaked content in *E.coli* O157:H. Different letter indicated significant difference among treatments at the same wavelength

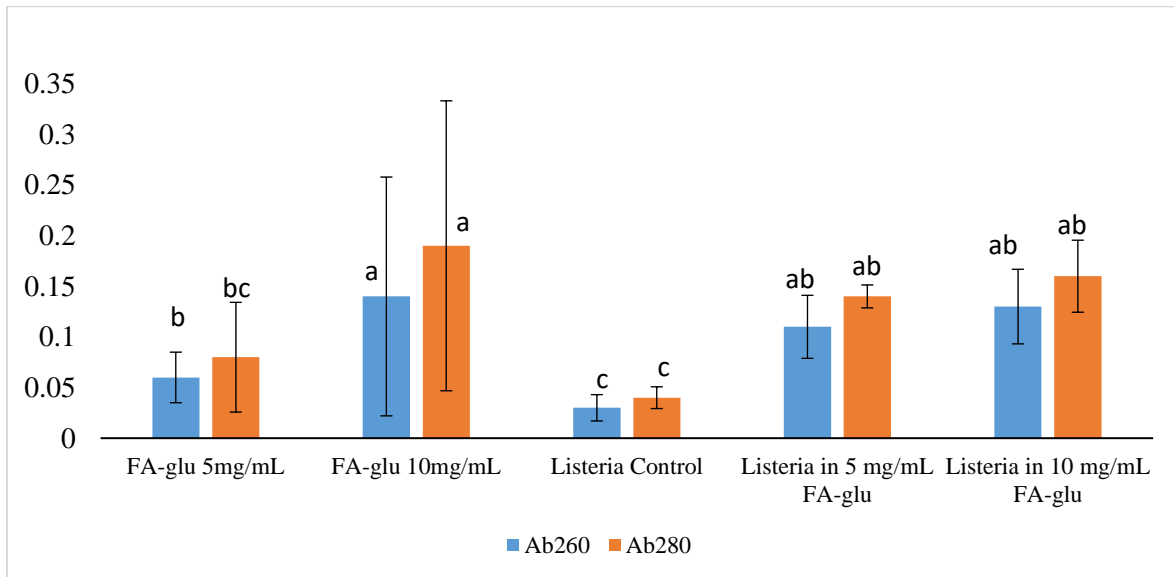


Figure 4.4b Absorbance of leaked content in *Listeria monocytogenes*. Different letter indicated significant difference among treatments at the same wavelength

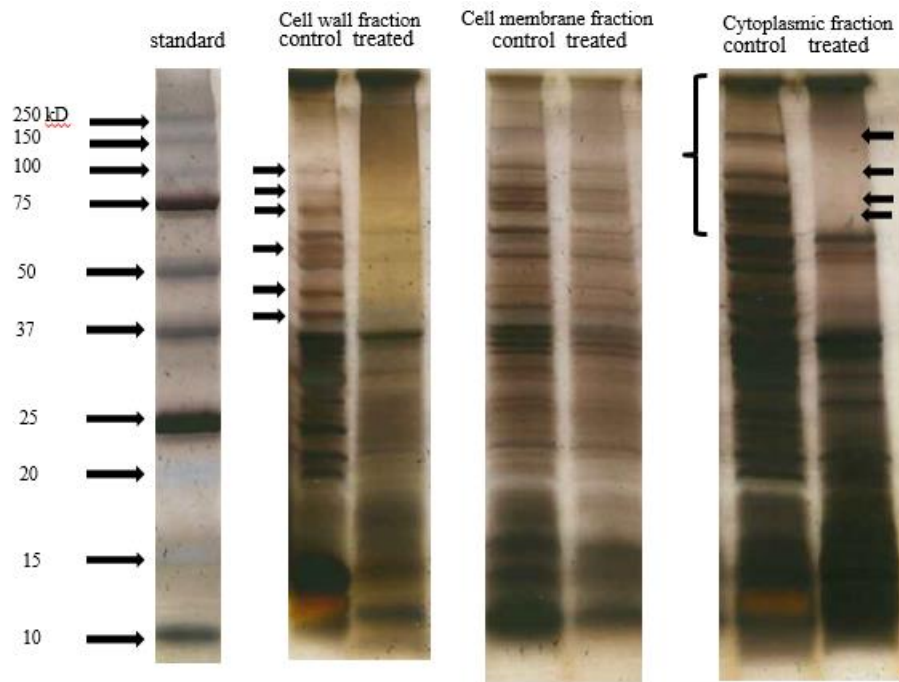


Figure 4.5a Silver staining of protein electrophoresis cell wall, cell membrane and cytoplasmic fractions of *E. coli* O17:H7

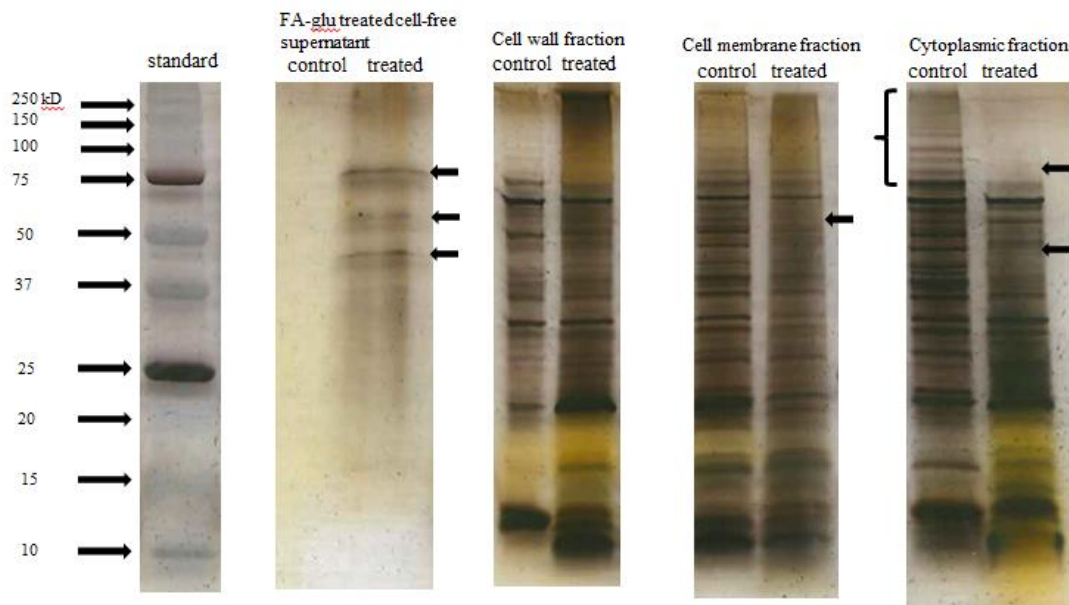


Figure 4.5b Silver staining of protein electrophoresis for cell wall, cell membrane and cytoplasmic fractions of *Listeria monocytogenes*

Table 4.1 Inhibition effect (%) and growth absorbance difference between 0 and 24h (in brackets)

Bio-based surfactant	glucose palmitate					
concentration (mg/mL)	4.15	3	2	1	0.1	0.05
Actual dissolved concentration (mg/mL)	0.56	0.51	0.3	0.24	0.05	0
<i>L. monocytogenes</i>	42 (0.42)	26 (0.74)	28 (0.76)	34 (0.71)	17 (0.9)	12 (0.96)
<i>S. enterica</i>	14 (0.73)	49 (0.53)	25 (0.76)	18 (0.77)	14 (0.93)	10 (1.05)
<i>E.coli O157:H7</i>	41 (0.46)	44 (0.46)	36 (0.53)	20 (0.63)	11 (0.94)	9 (0.97)

Bio-based surfactant	glucose laurate							
concentration (mg/mL)	22.1	14.7	7.36	4.15	3	1	0.1	0.05
Actual dissolved concentration (mg/mL)	6.5	2.8	0.62	0.13	0.085	0.074	0.02	0
<i>L. monocytogenes</i>	91 (-0.03)	87 (0.16)	57 (0.42)	108 (-0.27)	83 (0.39)	30 (0.87)	12 (0.96)	4 (1.05)
<i>S. enterica</i>	85 (-0.05)	89 (0.19)	51 (0.40)	133 (-0.22)	105 (0.05)	35 (0.9)	8 (1.02)	3 (1.08)
<i>E.coli O157:H7</i>	74 (-0.01)	78 (0.31)	38 (0.72)	129.9 (-0.51)	108 (0.01)	40 (0.77)	12 (0.92)	4 (1.02)

Bio-based surfactant	PS750 (sucrose ester)				
concentration (mg/mL)	7.36	4.15	3	2	1
Actual dissolved concentration (mg/mL)	4.72	2.26	1.45	0.89	0.42
<i>L. monocytogenes</i>	45 (0.8)	70 (0.32)	48 (0.59)	13 (0.83)	38 (0.48)
<i>S. enterica</i>	31 (0.93)	52 (0.55)	11 (0.83)	21 (0.68)	14 (0.62)
<i>E.coli O157:H7</i>	26 (1.2)	51 (0.45)	48 (0.48)	38 (0.48)	0 (0.89)

Table 4.1 continued

Bio-based surfactant	surfactin						
concentration (mg/mL)	15	10	5	0.1	0.05	0.01	0.005
<i>L. monocytogenes</i>	6.1 (1.03)	3 (1.05)	22 (0.87)	28 (0.79)	17 (0.93)	13 (0.98)	14 (0.98)
<i>S. enterica</i>	11 (0.87)	9 (0.91)	10 (0.91)	0 (0.97)	0 (1.02)	0 (1.03)	3 (0.94)
<i>E.coli O157:H7</i>	35 (0.78)	35 (0.74)	36 (0.8)	9 (0.93)	3 (1.01)	4 (0.98)	4 (0.98)

Bio-based surfactant	FA-glu (DMSO)						
concentration (mg/mL)	25	20	15	10	5	0.1	0.05
<i>L. monocytogenes</i>	72 (0.29)	83 (0.2)	78 (0.25)	61 (0.43)	57 (0.45)	4 (1.05)	7 (1.02)
<i>S. enterica</i>	65 (0.33)	77 (0.25)	75 (0.26)	62 (0.38)	57 (0.45)	6 (1.05)	2 (1.09)
<i>E.coli O157:H7</i>	80 (0.22)	84 (0.17)	87 (0.14)	81 (0.21)	72 (0.34)	10 (0.96)	7 (0.97)

Bio-based surfactant	FA-glu (Aq)						
concentration (mg/mL)	25	20	15	10	5	3	0.1
<i>L. monocytogenes</i>	100 (0.07)	89 (-0.02)	66 (0.4)	47 (0.59)	47 (0.65)	40 (0.77)	21 (0.97)
<i>S. enterica</i>	96 (0.12)	82 (0.22)	75 (0.34)	42 (0.75)	41 (0.7)	34 (0.85)	13 (1.06)
<i>E.coli O157:H7</i>	100 (0.04)	86 (0.01)	71 (0.26)	65 (0.34)	51 (0.43)	37 (0.63)	0 (1.05)

Note: Inhibition percentage values were calculated according to equation 1.

Table 4.2 Parameters of differential scanning calorimetry

Phospholipid	T _m (Transition Temperature °C)					
	Without FA-glu	1% FA-glu	2.1% Fa-glu	2.6% Fa-glu	5.1% Fa-glu	12.5% Fa-glu
DMPC (Zwitterionic)	23.07a	21.48bc	21.85b	20.63bc	21.02bc	20.29c
DMPG (Anionic)	20.02bc	20.92ab	19.87bc	18.86c	19.01c	-*
DPPE (Zwitterionic)	64.67a	63.44ab	64.11ab	64.20ab	63.02b	62.61b

Phospholipid	$\Delta T_{1/2}$ (1/2 of phase transition temperature range)					
	Without FA-glu	1% FA-glu	2.1% Fa-glu	2.6% Fa-glu	5.1% Fa-glu	12.5% Fa-glu
DMPC (Zwitterionic)	1.78d	2.31c	2.41c	3.41ab	2.93b	3.92a
DMPG (Anionic)	4.26bc	2.80d	3.68c	4.85b	5.10b	7.28a
DPPE (Zwitterionic)	2.97a	2.89a	2.84a	3.0a	3.60a	3.37a

Phospholipid	Enthalpy (J/g)					
	Without FA-glu	1% FA-glu	2.1% Fa-glu	2.6% Fa-glu	5.1% Fa-glu	12.5% Fa-glu
DMPC (Zwitterionic)	23.88ab	25.12a	25.33a	25.47a	23.53ab	21.27b
DMPG (Anionic)	28.48a	28.65a	24.06b	24.62ab	26.86ab	24.23b
DPPE (Zwitterionic)	35.39a	27.62a	29.58a	29.50a	26.73a	21.72a

Note: the different letters indicate significant differences in a row (P<0.05)

Table 4.3 Identification of phospholipids present in the bacterial organic solvent extract and fatty acyl glutamic extract

Molecular weight [M-H] ⁻	Molecular formula	Phospholipid	<i>E.coli</i> O157:H7 choloform and methanol extract	<i>E.coli</i> O157:H7 FA-glu extract	<i>Listeria monocytogenes</i> choloform and methanol extract	<i>Listeria monocytogenes</i> FA-glu extract
579.2724	C ₂₉ H ₅₈ NO ₈ P	PE C12:0/C12:0	√	√		
674.4737	C ₃ H ₆₉ NO ₈ P	PE C14:0/cyC17:0 or PE C15:0/C16:1	√			
688.4918	C ₃₇ H ₇₁ NO ₈ P	PE C15:0/cyC17:0 or PE C16:0/C16:1	√			
691.9761	C ₃₆ H ₆₈ O ₁₀ P	PG C14:0/C16:1	√		√	
693.4711	C ₃₆ H ₇₀ O ₁₀ P	PG C16:0/C14:0	√			
702.5024	C ₃₈ H ₇₃ NO ₈ P	PE C16:0/cyC17:0	√			
719.4864	C ₃₈ H ₇₄ O ₁₀ P	PG C14:0/C18:1 or PG C15:0/cyC17:0 or PG C16:0/C16:1	√		√	
733.5024	C ₃₉ H ₇₄ O ₁₀ P	PG C16:0/cyC17:0	√	√		
747.517	C ₄₀ H ₇₆ O ₁₀ P	PG C16:0/C18:1	√		√	
761.5331	C ₄₁ H ₇₈ O ₁₀ P	PG C16:0/cyC19:0	√			
773.532	C ₄₂ H ₇₇ O ₁₀ P	PG C18:1/C18:1	√	√		√
1307.5316	C ₇₀ H ₁₃₂ O ₁₇ P ₂	bis(phosphatidyl)glyc erol fatty acid combinations 15/15/15/16:1			√	

Note: check mark indicated phospholipid presence in the bacterial extract.

Supplemental material

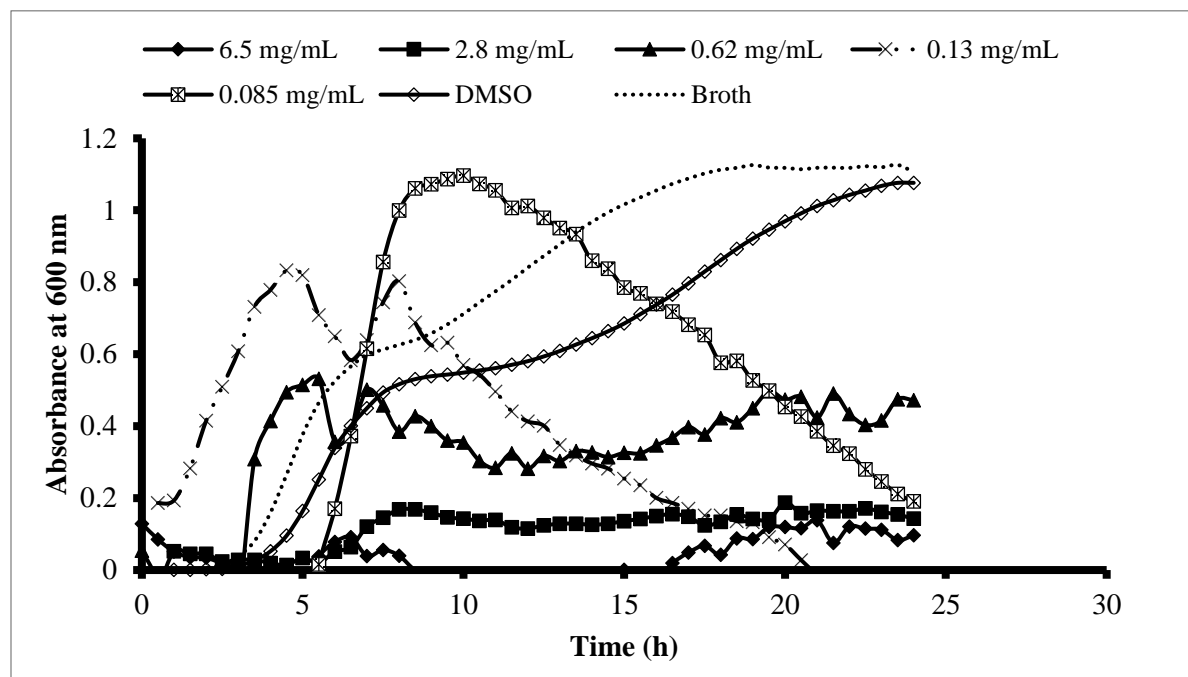


Figure S4.1a Glucose laurate monoesters (LA)'s inhibition effect on *Listeria monocytogenes*. The surfactant had similar effect on *Salmonella* Enteritidis and *E.coli* O157:H7

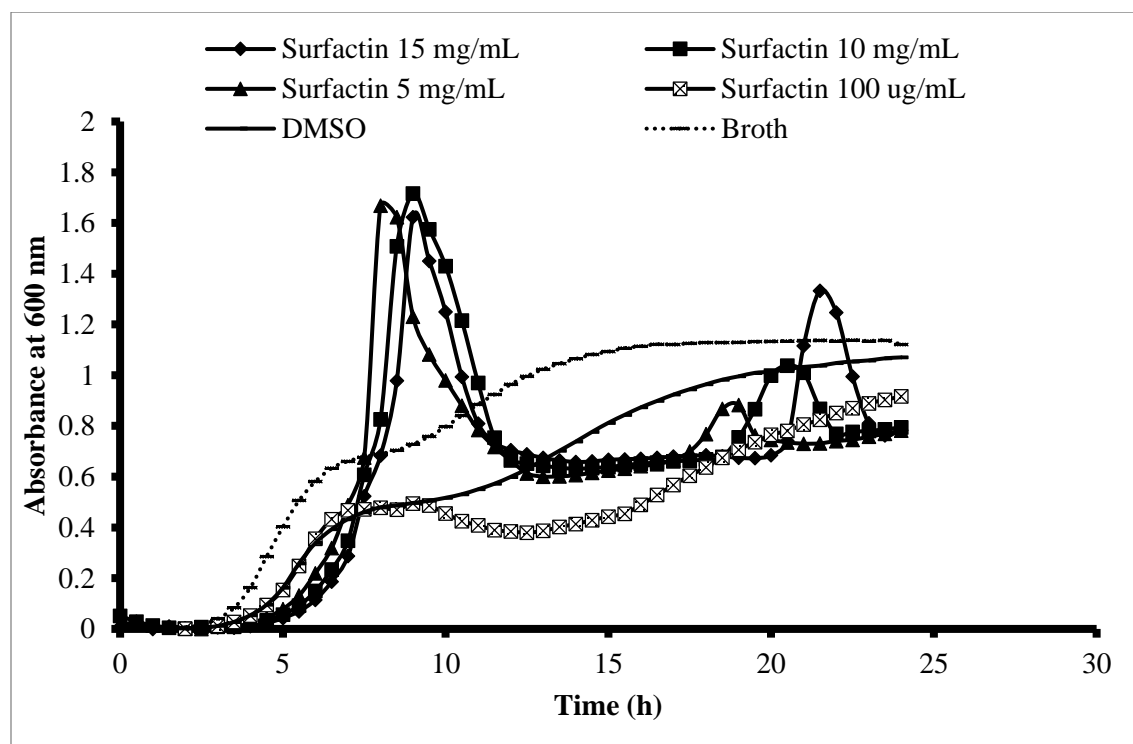


Figure S4.1b Surfactin's inhibition effect on *E.coli* O157:H7 (three bacteria had the same trend)

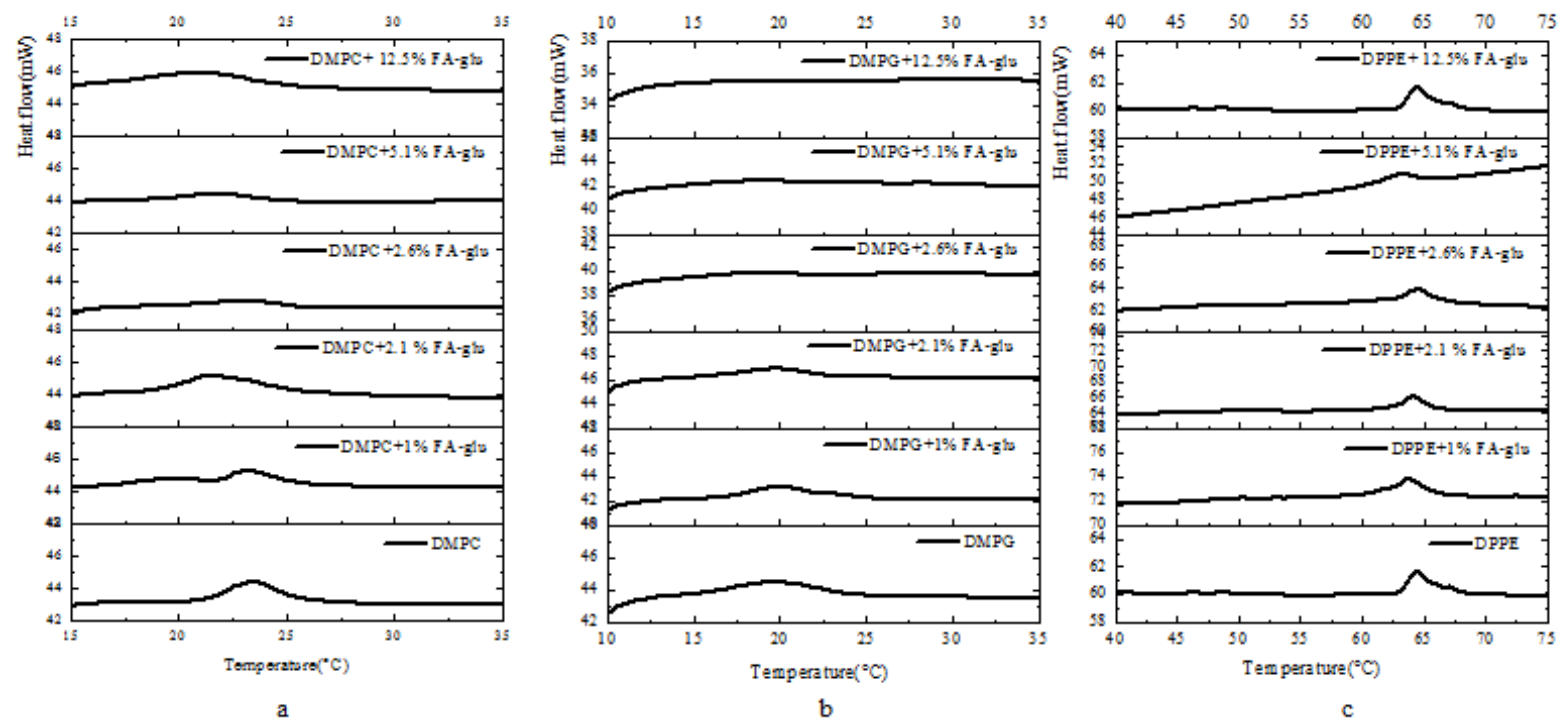


Figure S4.2 DSC heating scan thermograms for phospholipid (4a-DMPC, 4b-DMPC, 4c-DPPE) with different content (mol% of total) of FA-glu

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CHAPTER 5. THE STABILITY OF NANOEMULSIONS AND EMULSIONS CONTAINING CINNAMALDEHYDE AND BIOSURFACTANTS, AND THEIR ANTIMICROBIAL PERFORMANCE AGAINST *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES*

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5.1 Abstract

Two novel biosurfactants - surfactin and its variant fatty acyl glutamic acid (FA-glu) - were compared with commercial emulsifiers - lecithin, and a mixture of Tween 80 and lauric arginate (TLA) - for formation and stability of emulsions and nanoemulsions containing cinnamaldehyde (CM). The nanoemulsions'/emulsions' antimicrobial performance against two common foodborne pathogens *E. coli* O157:H7 and *Listeria. monocytogenes* was also compared. Two emulsifier concentrations (0.5% w/w and 1% w/w) and two homogenizing pressures (9,000 PSI and 18,000 PSI) were compared for droplet stability during storage for 46 days at 4, 25, and 37°C. Surfactin, FA-glu and TLA mixture formed nanoemulsions at both concentrations, but lecithin did not. Droplet sizes did not change significantly during 38 days at temperatures stored for surfactin- and TLA mixture- stabilized nanoemulsions. However, FA-glu and lecithin stabilized emulsions coalesced after 13th day when stored at 37°C; also, FA-glu stabilized

emulsion thickened during elongated storage days at 4°C. The incorporation of CM in nanoemulsions or emulsions did not lower the minimum inhibitory concentration (MIC) for bacteria in broths. However, at the concentrations lower than MIC, nanoemulsions and emulsions containing CM formulated with FA-glu, lecithin, and TLA, showed enhanced effects in inhibiting bacterial growths compared to CM alone, with smaller droplets inhibiting more.

Key words: biosurfactants, surfactin, fatty acyl glutamic acid, nanoemulsion, antimicrobial effect, emulsion droplet size

5.2 Introduction

Biobased products are partially or fully derived from biorenewable agricultural resources (US. Senate Commimittee on Agriculture Nutrition and Forestry, 2006). They have potential use in food, detergent, cosmetic, pharmaceutical, agricultural, and related industries (Banat, Makkar, & Cameotra, 2000). Biobased surfactants can be produced by microbial fermentation and enzymatic synthesis (Ren & Lamsal, 2017; Reznik, Vishwanath, Pynn, Sitnik, Todd, Wu, et al., 2010); since such methods can employ sustainable methods of producing biobased surfactants, they could lower the environmental pollution and reduce usage of petroleum feedstock (Banat, Makkar, & Cameotra, 2000). Various biobased surfactants have been researched for optimization of production rates (Arcos, Bernabe, & Otero, 1998; Degn, Pedersen, & Zimmermann, 1999; Ren & Lamsal, 2017), medical applications (Hayes, 2009), soil bioremediation (Bustamante, Duran, & Diez, 2012), biofilm inhibition (Sriram, Kalishwaralal, Deepak, Gracerosept, Srisakthi, & Gurunathan, 2011), and antimicrobial effects (Y. Chen, Nummer, & Walsh, 2014; Habulin, Šabeder, & Knez, 2008). Surfactin and fatty acid glutamic acid (FA-glu) are microbial biosurfactants which can be produced by *Bacillus subtilis* and have very good surface activity, as they have been shown to reduce the water surface tension to 27 mN/m and 36 mN/m from 72

mN/m, respectively (Reznik, et al., 2010). Their structures are shown in Fig 5.1 (a, b): surfactin is a cyclic peptide with 7 amino acids linked to fatty acid by a peptide bond, whereas, FA-glu is a variant molecule of surfactin with only one hydrophilic amino acid, conferring it increased aqueous solubility. Surfactin was studied for its antimicrobial activity, such as prevention of biofilm formation and anti-fungal activity due to its capability of interacting with or modifying cell membranes (Zhao, Shao, Jiang, Shi, Li, Huang, et al., 2017). It can also enhance biodegradation of diesel-contaminated water and soil (Whang, Liu, Ma, & Cheng, 2008) as it improves the solubility of organic compound or emulsification of liquid pollutant (Volkerling, Breure, & Rulkens, 1997). The FA-glu's emulsion and antimicrobial effects have not been reported in published research, as this is a relatively newer chemical. In many studies for antimicrobial effects of biosurfactants, mostly the minimum inhibition concentrations of biosurfactants as applied in bacterial broth or food medium (eg. milk and beef) were reported (Y. Chen, Nummer, & Walsh, 2014; Hathcox & Beuchat, 1996). However, both their functionality and effectiveness as food emulsifiers, and potential synergistic effect of emulsion droplet size on antimicrobial performance have not been reported. Since surfactin and FA-glu have potential as clean-label food ingredients, studies comparing their functionalities as food emulsifiers and antimicrobial agents are very relevant to food industry.

Antimicrobial effects in food can also be imparted by natural ingredients like essential oils (EO); for example, cinnamaldehyde (CM), is the main constituent of cinnamon bark EO (Ribeiro-Santos, Andrade, Madella, Martinazzo, Moura, de Melo, et al., 2017) and is reported to have antimicrobial effect against a number of bacteria and fungi (Jantan, Karim Moharam, Santhanam, & Jamal, 2008; Shan, Cai, Brooks, & Corke, 2007). However, the use of most EO in food is limited due to their lower solubility in aqueous systems (Chang, McLandsborough, &

McClements, 2015), and strong aroma and flavor characteristics. Emulsions, however, have been studied to incorporate essential oils in food systems for antimicrobial effects. Nanoemulsions, in which the droplet size is usually less than 1 μm (El Kadri, Devanthi, Overton, & Gkatzionis, 2017), were studied for their antimicrobial activity (Chang, McLandsborough, & McClements, 2015; Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012). Various parameters, such as emulsifiers' concentration, droplet size and processing methods were reviewed for the antimicrobial effect, however, no general trends were found (El Kadri, Devanthi, Overton, & Gkatzionis, 2017). For instance, (Wilkinson, 2015) reported improved efficiency of eugenol at intermediate concentration of lecithin (0.01%), not at lower (0.0025%, 0.005%) or higher concentrations (0.015%). Terjung and others (2012) found larger droplets (3 μm) of Tween 80-stabilized emulsions were more effective at microbial inhibition than smaller ones (80 nm), whereas, another study (Topuz, Özvural, Zhao, Huang, Chikindas, & Gölükçü, 2016) found that the nanoemulsions had better bactericidal effect than coarse emulsions. In any case, it is important to study the inhibition mechanism by verifying whether essential oils locate at the oil-water interface or inside the emulsifiers micelles, and how the emulsions' size affected the access of antimicrobial agents to bacteria (Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012). The methods to produce nanoemulsions, such as high pressure homogenization or ultrasonication could also generate heat and shear that can destroy antimicrobial agents (Pestana, Gennari, Monteiro, Lehn, & de Souza, 2015), making processing method also very important. It is, therefore, hard to predict the effect of antimicrobial agents in emulsions without testing in model and real food systems. Understanding such factors affecting stability and antimicrobial properties will help improve processing techniques and functionalities of novel emulsifiers, such as surfactin and FA-glu with antimicrobial agents. The objectives of this study are to 1) evaluate

and compare the stability of some biobased surfactants (emulsifier)-stabilized oil-in-water nanoemulsions/emulsion systems that contained cinnamaldehyde under different processing and storage conditions, and 2) to compare antimicrobial effect of nanoemulsions/emulsions stabilized by biobased emulsifiers on foodborne pathogens *E. coli* O157:H7 and *L monocytogenes* in broth system.

5.3 Materials and Methods

5.3.1 Reagents and bacterial strains

Surfactin and fatty acyl glutamic acid were kindly donated by Modular Genetics, Inc (Woburn, MA). Lecithin and tween 80 were purchased from Fisher Scientific (Fair Lawn, NJ). Cinnamaldehyde was purchased from Sigma-Aldrich (St. Louis, MO). Lauric arginate was donated by A&B ingredients Inc (Fairfield, NJ). Canola oil was purchased from a local grocery store. Brain heart infusion (BHI) broth was purchased from Fisher Scientific (Hampton, NH).

Two common food-borne pathogens, *E. coli* O157:H7 FRIK125 and *L. monocytogenes* Scott A NADC 2045 serotype 4b were used for the antimicrobial tests and they were obtained from Food Research Institute University of Wisconsin-Madison, and USDA/national animal disease center (Ames, IA), respectively.

5.3.2 Nanoemulsions preparation and characterization

Nanoemulsions were prepared with four different emulsifiers: microbial surfactin and FA-glu, and commercial lecithin, and a mixture of Tween 80 and lauric arginate (mass ratio 9:1, denoted as TLA mixture). The emulsifiers were dissolved in phosphate buffer solutions (PBS) at pH 7.2 and then mixed with CM and canola oil. FA-glu, lecithin and TLA mixture easily dissolved in PBS treated with water bath at 80°C. The surfactin was dissolved following a method described in literature (H.-L. Chen, Lee, Wei, & Juang, 2008) with modifications: it was

initially dissolved at 0.1 M NaOH PBS solution, then the pH was adjusted to pH 7.2 by adding proper amount of 0.1 M HCl PBS solution.

Two emulsifier concentrations (0.5% w/w and 1% w/w) were used in emulsion formation at two homogenizing pressures (9,000 PSI and 18,000 PSI). One or 2g of emulsifiers, 2 mL CM, 18 mL canola oil, and 178 mL or 179 mL phosphate buffer solutions (PBS), respectively, for 0.5% or 1% emulsifier levels were initially homogenized using a hand-held lab blender (Bamix Type M 150) for 2 min at 15,000 rpm. The emulsions were then passed three times through a high-pressure homogenizer (EmulsiFlex-D20, Avestin, Inc, Ottawa, Ontario) at either 9,000 or 18,000 PSI. Two steady-state samples (200 mL) for each treatment were then collected for storage stability studies so there were 34 samples in total. After preparation, each emulsion was further sub-divided to store at three different temperatures: 4°C, 25°C and 37°C.

5.3.3 Nanoemulsion/emulsion droplet size determination

The mean droplet size for nanoemulsions (z-average) was measured by a dynamic light scattering instrument (Malvern Zetasizer Nano ZS, Malvern instruments, Worcestershire, UK). On 1st, 13th, 24th, 38th and 46th storage day, 2 mL of the emulsions were placed in a spectrometry cell for droplet size measurement. Each sample had at least 12 measurement runs to obtain a satisfying result determined by the instrument. The emulsion droplet size was measured by Malvern Particle Size Analyzer (Mastersizer Hydro 2000). Emulsions were introduced to the sample cup until an obscuration of 10-20% was obtained. Zeta-potential determination

The emulsion zeta-potential was measured by a particle electrophoresis instrument (Malvern Zetasizer Nano ZS, Malvern instruments, Worcestershire, UK) following a method reported by (Witayaudom & Klinkesorn, 2017): 0.1 mL emulsion was placed in the zeta-cell and

diluted with 2 mL PBS solution, each measurement had enough automatic runs until a satisfying result was obtained.

5.3.4 Minimum inhibitory concentration for nanoemulsions/emulsions containing cinnamonaldehyde against pathogens

Growth curves for two pathogens under study with or without CM or CM-containing nanoemulsions were obtained using a Bioscreen C turbidometer (Growth Curves USA, Piscataway, NJ). Bacterial stock cultures were stored at -80°C and propagated in BHI broth (35°C , 24h). Two consecutive 24-h sub-culturing of the strains in BHI was conducted prior to inoculation. Each bacterial culture was diluted 100-fold in fresh BHI to obtain a cell concentration of 10^7 CFU/mL, and 9.9 mL BHI broth was inoculated with 0.1 mL aliquots of the diluted culture to obtain a concentration of 10^5 CFU/mL. Non-emulsified CM or nanoemulsions/emulsions containing CM were added into the BHI broth to obtain CM concentrations of 0.125 $\mu\text{L/mL}$, 0.25 $\mu\text{L/mL}$, 0.5 $\mu\text{L/mL}$, 0.75 $\mu\text{L/mL}$ and 1 $\mu\text{L/mL}$. The tubes were then vortexed for 10s. Two hundred and fifty μL of each inoculated BHI broth was transferred to 100- well microtiter plate and incubated at 37°C for 48 h. The absorbance at 600 nm of the broth was recorded every 30 min for 48 h. For every treatment, two inoculated broth samples were prepared and two readings from each sample were recorded at every time point. The absorbance differences (ΔA_{624}) between the 0 h and 24 h were calculated to evaluate the growth condition. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of either non-emulsified CM or that contained in the nanoemulsions/emulsions that can prevent bacterial growth throughout 48 h incubation period (Huang, Wei, Zhao, Gao, Yang, & Cui, 2008), as reflected by a flat absorbance curve throughout. The nanoemulsions/emulsions used for droplet size and zeta-potential measurement was from the same batch, while studies on

the nanoemulsions/emulsions for antimicrobial performances were done with another batch. The bacterial broths were inoculated with the nanoemulsions/emulsions containing CM at the second week of storage.

5.3.5 Statistical analyses

Statistical comparisons for the emulsion characterization were conducted using SAS 9.4 software (SAS Institute INC., Cary, NC). Proc mixed test was used to determine how the independent factors (storage days, concentrations and temperatures) or their interactions influenced the response variables (droplet size and zeta potential). Only significant interactions ($P < 0.05$) are presented and discussed.

5.4 Results and Discussion

5.4.1 Stability of Emulsion Droplets

Table 5.1 presents the data on droplet size stability for emulsions stabilized with different emulsifiers, and interaction between relevant parameters. The corresponding visualization of emulsions is presented in Fig 5.2. Only three three-way interactions were significant for the droplet size stability: emulsifier type, storage duration, and storage temperature ($P < 0.05$, Table 5.1a); emulsifier type, concentration and storage temperature ($P < 0.05$, Table 5.1b); and emulsifier type, pressure and storage temperature ($P < 0.05$, Table 5.1c). These are discussed below:

5.4.1.1 Interaction of emulsifier type, storage day and storage temperature

Nanonemulsions stabilized with surfactin and TLA mixture were very stable during storage, with surfactin nanoemulsions size ranging between 400-750 nm, and TLA mixture nanemulsions size ranging between 500-950 nm, and these sizes were not affected by storage temperature (Table 5.1a). For FA-glu, the droplet size increased drastically (1341 nm) only on

the last storage day (38th day) at 37°C. For lecithin-stabilized emulsions at 4°C, the droplet size remained constant, however, at both 25°C and 37°C, smaller sizes were observed. This was due to oil droplet coalescence with a layer of oil on top observed (Fig 5.2b and 5.2c), which was not sampled; instead, samples contained the un-coalesced droplets remaining in the emulsion.

Similar observations were reported by a previous study (Yang, Zhao, Tian, Lu, Zhao, Bao, et al., 2017). The storage temperature played a role in instability of FA-glu and lecithin stabilized emulsions, as it is known that higher storage temperature generated more energy for droplets' Brownian motion resulting in higher chances for droplet to collide and coalesce (Yang, et al., 2017). The storage temperature did not affect the sizes of surfactin and TLA mixture stabilized emulsions, due to effective electrostatic repulsion and steric hindrance.

5.4.1.2 Interaction of emulsifier type, concentration, and temperature

From Table 5.1b, we can see the interaction of surfactant type, concentration, and storage temperature affecting the emulsion droplet size. The concentration and temperature had no effect on the droplet size for surfactin and TLA mixture stabilized emulsions. However, for FA-glu, the higher concentration (1%) resulted in slightly smaller, but not significant, decrease in droplet size at each storage temperature studied. For lecithin, the higher concentration resulted in significantly lower droplet sizes at both 4°C and 25°C, but not at 37°C. Higher concentration of emulsifier in the emulsion generally lead to smaller droplet sizes, as emulsifiers are able to cover more interfacial areas on droplets (McClements, 2004a). However, the sizes are also dependent on how fast the emulsifiers can adsorb to the interface; if the time spent during the homogenization process is too short, or if there is an uneven distribution of disruptive energy that the emulsifiers didn't completely adsorb at the interface, the droplets may not be disrupted and not stabilized (Walstra & Smulder, 1998)). In our study, the three passes during homogenization

might not be sufficient enough for smaller droplet generation and emulsifiers' adsorption, leading to inadequate or incomplete adsorption of emulsifiers at the interface. If the emulsions were passed through the high-pressure homogenizer more number of times, significant reduction in size at higher concentration could be observed.

5.4.1.3 Interaction of emulsifier type, pressure and storage temperature

Table 5.1c shows the three-way interaction of emulsifier type, pressure, and temperature. The pressure and storage temperature had no effect in the droplet size for surfactin and TLA mixture stabilized nanoemulsions. Whereas, for FA-glu stabilized nanoemulsions storage at 4°C resulted in slightly but not significantly smaller droplet sizes (500-600 nm), the nanoemulsions storage at 25°C and 37°C had slightly bigger size droplet (600-800 nm). For lecithin, higher pressure resulted in slightly but not significantly smaller size droplets that were stored at 4°C and 25°C, but larger sizes at 37°C. Although there were some significant increase/decrease in the droplet size, the magnitudes were not very obvious. Higher pressures during homogenization generally produce smaller sized emulsion droplets, due to intense turbulence and shear flow fields (Walstra, 1983). However, although turbulence generally leads to the break-up of the dispersed phase into smaller droplets, it may also generate collision that can result in re-coalescence (Walstra, 1983). The size of the droplets produced during homogenization depends on adsorption time for emulsifiers onto droplet surfaces and collision time duration; if the former is occurring at faster rate, the droplet can be stabilized (McClements, 2004a). Several studies also reported insignificant changes in droplet size generated at higher pressures (Floury, Desrumaux, & Lardieres, 2000; Juttulapa, Piriyaprasarth, Takeuchi, & Sriamornsak, 2017), the reasons being the re-coalescence of newly created fine droplets, insufficient emulsifiers for adequate adsorption, and denaturing of protein or peptides stabilized systems. In our study, the

first two situations might have occurred: we speculate that if our emulsifiers can adsorb at a faster rate, or pressures can be applied for longer times, the higher pressures should generate smaller emulsion sizes. Another reason could be possible pressure fluctuation in the homogenization chamber causing significant variability in applied pressure leading to no effect of applied pressure on emulsion droplet sizes, as the pressure is manually controlled.

5.4.2 Emulsion Stability Beyond 38 Days

On 46th day, the droplet sizes were measured with the Malvern 2000 (Table 5.1d), as FA-glu and lecithin stabilized emulsion droplet sizes were out of the range for the nanosizer. Only a three-way interaction of emulsifier type, concentration, and temperature was observed ($P < 0.05$, Table 5.1d). Again, the concentration and temperature did not affect the droplet size for surfactin and TLA mixture stabilized emulsion on 46th storage day. For FA-glu, 37°C obviously led to the largest droplet size at both concentrations (over 250 μm). Surprisingly, at 4°C, the FA-glu (1%) stabilized emulsion became yogurt-like viscous, leading to a relatively bigger droplet sizes (17 μm); this was not observed at lower concentration (0.5%). This was probably caused by depletion flocculation of the excess amount of un-adsorbed FA-glu, which may have existed as micelles in the emulsion (Bibette, 1991). The attractive force among the micelles is bigger enough to overcome the electrostatic repulsion between the droplets (McClements, 1994) so that the micelles ‘bridge’ among the droplets and form viscous structure. The driving force for this phenomenon is osmotic potential: the micelles concentration is higher in the bulk than in the ‘bridge’ regions so there is a tendency for the micelles to move from bulk to the region between droplets (McClements, 1994). Higher number of such micelles can induce depletion flocculation and form three-dimensional network (McClements, 2004b). It is possible that the lower storage temperature reduced the Brownian movement of both micelles and droplets, so the yogurt-like viscous was

formed. Several other studies also have reported gel-like structure formation due to flocculation (Graça, Raymundo, & de Sousa, 2016; Tang & Liu, 2013), however, their flocculation were caused by different intermolecular forces such as hydrophobic attraction, electrical attraction caused by opposite charge of the surfactant, etc. For lecithin at 0.5%, like FA-glu, 37°C storage led to the largest droplet size (172 μm), and higher concentration (1%) generated smaller sizes. Storage at both 25°C and 4°C led to the smaller droplet sizes on 46th day.

5.4.3 Zeta-potential analysis

Zeta-potential indicates the electric charge distribution in the double layer around the surface-charged particles (droplets). Apparently, surfactin and FA-glu stabilized emulsions had the highest absolute value of zeta-potential at around 50 mV (Table 5.2), followed by lecithin-stabilized emulsion (~30 mV), and TLA mixture stabilized emulsions (~ 2 mV). Three factors- storage day, emulsifier type, and storage temperature- had significant interaction affecting the zeta-potential during storage. For example, the absolute zeta-potential for surfactin-stabilized emulsion was constant around 50- 52 mV, regardless of the temperature till 38 days, indicating the constant droplet repulsion being one of the main reason for better stability. Higher absolute value of zeta potential of a suspension generally means higher stability (Hanaor, Michelazzi, Leonelli, & Sorrell, 2012), but it is not true for every type of emulsions (Bhattacharjee, 2016). For example, although the absolute values of zeta-potential were in the range of 2-5 mV for TLA mixture stabilized emulsions, the droplet size did not change significantly, possibly due to the stearic hindrance (Celus, Salvia-Trujillo, Kyomugasho, Maes, Van Loey, Grauwet, et al., 2018). Whereas the lecithin had much larger absolute value (26-30 mV), it still experienced phase separation at 37°C. It is worth pointing that the zeta potential absolute values were constant at 4°C for both lecithin and TLA mixture stabilized emulsions stored for 38 days; they experienced

only slight increase at 25°C and 37°C storage. This subtle increase in zeta-potential at elevated temperatures can be explained by the increased emulsifiers adsorption density at the interface (Bhattacharjee, 2016): as temperature increased, coalescence occurred resulting in bigger droplets. As a consequence, the surface area of the dispersed phase decreased leading to increase in the number of charges on the droplet surfaces. For FA-glu stabilized emulsions, although the zeta-potential was close to that of surfactin, the stability was lower than those of surfactin and TLA mixture stabilized emulsions. The reason could be lower molecular weight lacking the steric hindrance to form a viscoelastic interface at the oil droplet surface (Celus, et al., 2018). As a result, phase separation was observed on 24th day at temperature 37°C (Fig 5.2 c). The decrease in zeta-potential at 37°C can be attributed to the dissociation of the glutamic acid part of the FA-glu promoted by the elevated temperature, thus the increased level of hydrogen ion reduced the repulsive force (Jackson & Vinogradov, 2015).

From the above discussion, it was very obvious that the surfactin and TLA mixture stabilized emulsion were more stable than those of FA-glu and lecithin stabilized ones. Since the lecithin generated emulsion droplet sizes exceed 1 μm , the lecithin stabilized emulsions cannot be considered as nanoemulsion. Surfactin, although non-ionic, had relatively large magnitude of zeta potential, thus provided electrostatic repulsion leading to higher stability. Tween 80, also provided steric hindrance for stabilizing the droplets from coalescence (Celus, et al., 2018). Lecithin, although provided some electrostatic repulsion, coalescence still occurred at 25°C and 37°C. Another study also reported the use of lecithin promoted coalescence when used at medium-high level (Drapala, Auty, Mulvihill, & O'Mahony, 2015). It is possible that due to the limited hydrophilic property (Colbert, 1998), the adsorption to the interface was affected. The different capabilities to produce different sizes of the emulsion lie in their capability to produce

different interfacial tension between oil and water. According to the theory from previous research (Walstra, 1993), the maximum droplet size that can persist in emulsion is direct proportional to the interfacial tension when continuous phase's viscosity, shear rate and the ratio of viscosities of dispersed and continuous phases are constant.

5.4.4 Antimicrobial inhibition by biobased surfactants-stabilized emulsions and the effect of emulsion droplet size

To study the effect of emulsion droplet size on antimicrobial effect of biobased surfactants-stabilized emulsions containing natural antimicrobial (CM) on common foodborne pathogens, fresh emulsion batches were prepared: after initial homogenization by kitchen blender (denoted as 'coarse emulsion'), and after passing the coarse emulsions through high pressure homogenizer ('fine emulsion'). The emulsion droplet sizes were measured during the 3rd week. There were significant differences in droplet size among emulsions stabilized with different emulsifiers - Surfactin and TLA mixture stabilized emulsion had the smallest sizes (1.2 μm and 7.9 μm for fine and coarse emulsion stabilized by surfactin; 0.4 μm and 8.5 μm for fine and coarse emulsion stabilized by TLA mixture. For lecithin, it was unexpected that fine emulsions had a larger droplet size (mean diameter 122.6 μm) than the coarse one (mean diameter 83.4 μm); it is possible that the lecithin source we purchased did not have good surface activity, which can be reflected by the droplet size data. FA-glu stabilized emulsions had the greater sizes. No significant difference between fine and coarse emulsions for all the emulsifiers.

5.4.4.1 Antimicrobial inhibition by surfactin-stabilized emulsions

The ΔA_{b24} values were calculated to evaluate the inhibition effects of emulsions, and the absorbance changes during the 24 h are presented for each emulsions and bacteria in Table 5.3a.

The ΔA_{b24} values reflect the inhibition strength to some extent, but cannot determine the MIC, as phase separation will decrease the absorbance. Our preliminary result indicated that the MIC for CM against *E. coli* O157:H7 was 0.25 $\mu\text{L/mL}$ when bacterial count was around 10^5 CFU/mL. The surfactin-stabilized coarse and fine emulsions did not lower the MIC, which was also 0.25 $\mu\text{L/mL}$ for both (Table 5.3a). However, emulsified CM was effective in inhibiting growths at a sub-MIC concentration of 0.125 $\mu\text{L/mL}$ compared to non-emulsified CM at the same concentration, indicating improved inhibition effect by the emulsions. On one hand, it is known that surfactin can generally inhibit bacterial growths (Ndlovu, Rautenbach, Vosloo, Khan, & Khan, 2017) and can alter cell membrane resulting in nucleic acid leakage (Gao, Han, Liu, Qu, Lu, & Bie, 2017), on the other hand, surfactin as an emulsifier in the system could limit its own adsorption to the bacteria cell membranes due to its adsorption on the emulsion oil droplets surfaces. It is possible that the synergistic effect of both surfactin and expanded surface area for emulsified CM improved the antimicrobial performance. However, the fine emulsion (average diameter 1.2 μm) did not perform better than the coarse emulsion (average diameter 7.9 μm), indicating the further increasing of surface area did not improve inhibition.

For *L. monocytogenes*, MIC for non-emulsified CM was 0.125 $\mu\text{L/mL}$ when bacteria count was around 10^5 CFU/mL. However, the surfactin-stabilized emulsions did not inhibit these bacteria at 0.125 $\mu\text{L/mL}$, instead, the MICs for the fine and coarse emulsions were 0.5 $\mu\text{L/mL}$ and 0.25 $\mu\text{L/mL}$, respectively. It was unexpected that fine emulsions had higher MIC than coarse emulsions. From these observations, it was seen that the use of surfactin to expand the surface area of emulsified CM for higher antimicrobial behavior did not hold for *L. monocytogenes*, rather, it may depend on competitive/preferential binding of surfactin to bacteria or the oil droplets. The negative droplet charge reflected by the zeta-potential, could also interfere the

contact of CM to negative charged cell membrane. It is important to point out that lower ΔA_{b24} can be associated with stronger inhibition effect of the antimicrobial; for example, at sub-MIC concentration, the ΔA_{b24} was usually higher than 0, whereas, at concentrations higher than MIC, the ΔA_{b24} were close to zero or negative. However, the values were also affected by the phase separation, thus one cannot solely rely on ΔA_{b24} for the determination of MIC. For instance, although ΔA_{b24} was -0.12 for fine surfactin emulsion of *L. monocytogenes*, the bacteria still grew during 48 h (absorbance increased for a period of time), the later decreasing of absorbance was caused by phase separation. Only a flat growth curve of the bacteria can indicate a MIC (Huang, Wei, Zhao, Gao, Yang, & Cui, 2008).

5.4.4.2 Antimicrobial inhibition by FA-glu stabilized emulsions

For FA-glu stabilized emulsions, the MICs against *E. coli* O157:H7 was 0.25 $\mu\text{L/mL}$ for fine emulsion, but was not found for coarse emulsion in the range of 0.125-1 $\mu\text{L/mL}$, thus, indicating the MIC to be beyond this range (Table 5.3b). The fine emulsions had better inhibition performance than coarse emulsions at 0.125 $\mu\text{L/mL}$ concentration, as there was more growth in the coarse emulsion. As the concentration of CM in the coarse emulsion increased, the inhibition effect became stronger. For example, at CM concentration of 0.125 $\mu\text{L/mL}$ in coarse emulsion, the *E. coli* grew from 7.5-31h; at concentration of 0.25 $\mu\text{L/mL}$, the bacteria started to grow very slowly h and absorbance remained almost unchanged for the rest of the incubation period. At higher concentrations (0.5, 0.75 and 1 $\mu\text{L/mL}$ CM concentrations), bacteria started to grow from 3.5-8 h, then continued to grow very slowly to a peak (at 33 h and 25 h respectively), then decreased gradually. The decreased absorbance was caused by the phase separation in emulsion that occurred after 48 h incubation at 37°C (visual observation), and the broth became clearer with a cream layer on top. For *L. monocytogenes*, similar to *E.coli*, the MIC for fine emulsion

was 0.25 $\mu\text{L/mL}$ and the MIC for coarse emulsion was beyond the range of 0.125-1 $\mu\text{L/mL}$. The fine emulsion at 0.125 $\mu\text{L/mL}$ and coarse emulsion at 1 $\mu\text{L/mL}$ had similar effect that the bacteria started to grow at 5-6 h, slowly at first and reaching the peak at around 21 h, and decreasing to a level lower than the initial absorbance. At CM concentration of 0.25 $\mu\text{L/mL}$ in coarse emulsion, although the ΔA_{624} was negative, bacteria grew very slowly with almost no change in absorbance until 23 h then phase separation occurred. At the concentration of 0.5 and 0.75 $\mu\text{L/mL}$ for coarse emulsion, the bacterial lag time reduced to 8-10 h, reaching the highest level around 40-46 h before decreasing. For FA-glu, overall, the fine emulsions had better inhibition effect than coarse emulsions, which indicated that smaller size played a role in enhancing the antimicrobial effect. Compared with surfactin, FA-glu is a smaller molecule, thus, its emulsions may have had better chance to contact bacteria causing slower growth or even inactivation. Although FA-glu had some antimicrobial effect by interacting with cell membrane, the MIC was much higher than the concentration we used in this study (unpublished data), so the role of oil droplet size was more important than the antimicrobial effect of FA-glu itself.

5.4.4.3 Antimicrobial effect by lecithin stabilized emulsions

For lecithin-stabilized emulsions, the MICs for *E. coli* were 0.5 and 0.25 $\mu\text{L/mL}$ for fine and coarse emulsions respectively (Table 5.3c). Other concentrations studied successfully inhibited bacterial growth. For *L. monocytogenes*, the MICs for fine and coarse emulsions were not found. The increase in the CM concentration in both fine and coarse emulsions made the inhibition more effective, but the effect was greater in coarse emulsion. For example, little growth was seen at the concentration range of 0.5-1 $\mu\text{L/mL}$ in coarse emulsion but the range increased to 0.75-1 $\mu\text{L/mL}$ in fine emulsion, indicating the coarse emulsions to be more effective. It should be pointed out that the fine emulsions that underwent the high-pressure

homogenizer had bigger, but not significant, droplets sizes than the coarse emulsions prepared with lab blender, which was unexpected. The results discussed above, however, indicated the smaller droplets had better antibacterial effect than larger droplet size.

5.4.4.4 Antimicrobial inhibition by TLA mixture stabilized emulsions

For TLA mixture stabilized emulsions, the MIC for *E. coli* (Fig 4g) was 0.25 $\mu\text{L/mL}$ for both fine and coarse of emulsions (Table 5.3d). However, the fine emulsion inhibited bacterial growth better than the non-emulsified CM and coarse emulsion at the CM concentration of 0.125 $\mu\text{L/mL}$: The bacterial growth in fine emulsion was much weaker than in coarse emulsion, as the overall absorbance was much lower throughout the entire incubation period. This growth phenomenon was also shown by *L. monocytogenes* and the MIC was 0.25 $\mu\text{L/mL}$ for both coarse and fine emulsions. It was very obvious that the fine emulsion had much better effect than coarse emulsion, because the bacteria only grew to a small extent from 6.5 to 20 h and was inhibited in the fine emulsions, whereas they grew starting at 13.5 h and continued until 48 h in the coarse emulsions. The smaller droplet size significantly improved the antibacterial effect in TLA mixture stabilized emulsions. The lauric arginate is a food grade cationic surfactant known to have antimicrobial activity against a wide range of bacteria (Chang, McLandsborough, & McClements, 2015); unlike surfactin whose effect was not influenced by droplet size, the decreased droplet size in the TLA mixture stabilized emulsion increased the inhibition effect.

From the discussion above on antimicrobial activity of emulsion droplets and EO itself, our results revealed that the incorporation of CM as emulsion did not lower MIC of CM against *E. coli* and resulted in increased MIC against *L. monocytogenes*. Similar findings were reported in some other studies that creating emulsions did not lower the MIC (Burt & Reinders, 2003; Xue, Davidson, & Zhong, 2017). However, at the EO concentrations that were lower than MIC,

the emulsion suppressed the growth of *E. coli* better compared to the non-emulsified EO, which was also found in other studies (S. Li, 2011; Xue, Davidson, & Zhong, 2017). This indicated that the emulsification assisted the essential oils to disperse in the bacterial broth and improve their efficacy at some concentrations. We also found that for the biosurfactant that possess antimicrobial property (surfactin), the emulsion droplet size did not affect the biosurfactant's antibacterial property. It is possible that surfactin has caused damage to the bacteria before CM can reach the bacteria, as surfactin molecules exist on the surface of the droplets that may access bacteria easier than the oil phase where CM is located in. For other three emulsifiers, regardless of their own antimicrobial property, the decrease of the droplet sizes increased the antibacterial property; this can be explained by the fact that CM in smaller droplets emulsions have larger surface areas that it had more access to bacteria to achieve the inhibition effect.

5.5 Conclusions

This study reports the stabilizing functions of four emulsifiers and antimicrobial effect of their emulsions against two common foodborne pathogens. Surfactin and TLA mixture had the best stabilizing functions under all temperatures, due to their relatively bigger molecules that provide effective steric hindrance; the surfactin-coated droplets also have electric repulsion that kept the droplet from coalescence. For the emulsions stabilized with FA-glu and lecithin, they were not stable at 25 and 37°C, and experienced substantial coalescence. The bacterial growth curves revealed that encapsulation of CM into emulsion did not lower the MIC, but emulsion improved the efficacy of CM when the concentrations were lower than MIC. Except for surfactin, the size of emulsion droplet was an important factor influencing the inhibition effect. Due to the good antibacterial property of surfactin itself, the decrease in the droplet sizes did not further improve the inhibition effect of its emulsions.

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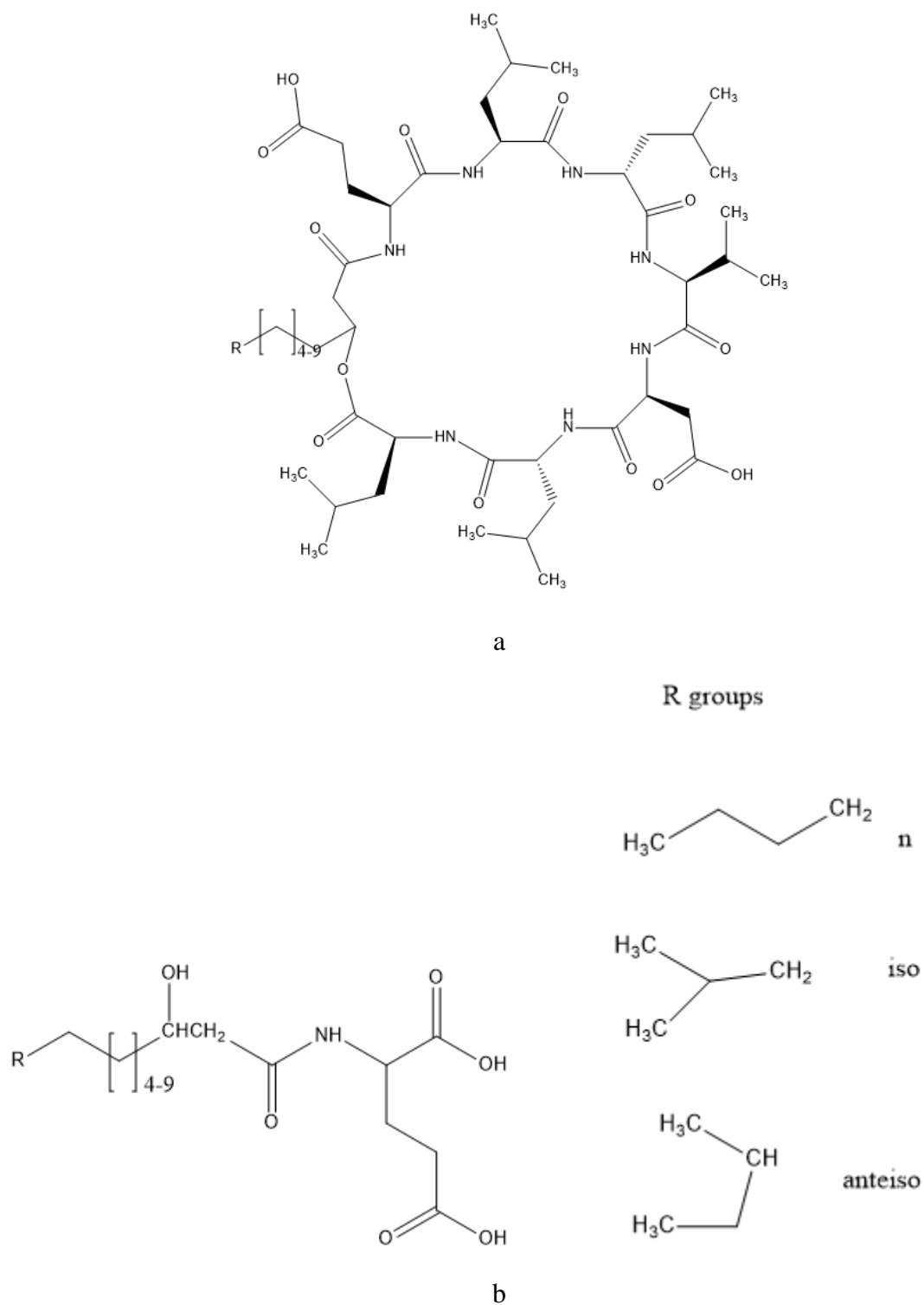


Fig 5.1 Surfactin (a) and fatty acyl glutamic acid (b) produced by *Bacillus subtilis* (Reznik 2010)



Figure 5.2 Emulsions stabilized by surfactin (1), FA-glu (2), lecithin (3), Tween&lauric arginate (4), and control without any emulsifiers (5) at 4°C (a), room temperature (b) and 37 °C (c) on 24th day

Table 5.1a Type × Day × Temperature interaction for Z-average values (nm)

	Surfactin			FA-glu			Lecithin			TLA mixture		
	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)
D1	469A	733A	621A	607B	780B	736B	2252ABC	2438AB	2013BC	712A	815.65A	925A
D13	627A	588A	528A	561B	720B	677B	2423AB	2525A	1958BC	619A	808A	580A
D24	559A	605A	631A	566B	555B	560B	2359ABC	1917C	1228D	918A	620A	839A
D38	551A	559A	597A	520B	528B	1341A				697A	913A	571A

Note: the upper case indicated significant difference (P<0.05) for combinations of Day and Temperature for one single emulsifier

Table 5.1b Type × Concentration × Temperature interaction for Z-average values (nm)

	Surfactin			FA-glu			Lecithin			TLA mixture		
	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)
C1 (0.5%)	548A	759A	588A	666AB	650AB	903A	2689A	2638A	1810B	711A	844A	807A
C2 (1%)	554A	484A	600A	461B	641AB	754AB	1959B	1906B	1614B	762A	734A	651A

Note: the upper case indicated significant difference (P<0.05) for combinations of Concentration and Temperature for one single emulsifier

Table 5.1c Type × Pressure × Temperature interaction for Z-average values (nm)

	Surfactin			FA-glu			Lecithin			TLA mixture		
	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)	T1 (4 °C)	T2 (25°C)	T3 (37°C)
P1 (9000PSI)	572A	641A	690A	589AB	638AB	769AB	2359A	2382A	1466C	790A	870A	728A
P2 (18000PSI)	530A	601A	499A	537B	654AB	888A	2289AB	2163AB	1958B	684A	709A	730A

Note: the upper case indicated significance difference (P<0.05) for combinations of Pressure and Temperature for one single emulsifier

Table 5.1 (continued) d Day 46 Emulsion droplet size (D[4,3], μm)

	Surfactin			FA-glu			Lecithin			TLA mixture		
	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)
C1 (0.5%)	0.5A	0.5A	0.5A	0.58C	0.58C	395A	4B	13B	172A	0.57A	0.46A	0.44A
C2 (1%)	0.7A	0.4A	0.4A	17C	0.64C	286B	1B	3B	66B	1.1A	0.69A	0.55A

Note: the upper case indicated significance difference ($P < 0.05$) for combinations of Concentration and Temperature for one single emulsifier

Table 5.2 Type \times Day \times Temperature interaction for zeta potential

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	Surfactin			FA-glu			Lecithin			Tween		
	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)	T1 (4°C)	T2 (25°C)	T3 (37°C)
D1	-51.4A	-50.8A	-52.5A	-54.4D	-52.8CD	-54.4D	-26.9A	-26.7A	-27.5AB	-2.2AB	-1.9A	-1.7A
D13	-51.2A	-51.2A	-51.4A	-52.2BCD	-54.3D	-48.2A	-27.9ABC	-28.2ABC	-29.1ABCD	-2.5AB	-2.7AB	-3.1AB
D24	-51.6A	-51.9A	-53.5A	-54.0D	-52.8CD	-50.5ABC	-29.7BCDE	-30.5CDE	-31.9E	-2.7AB	-3.6AB	-4.3AB
D38	-51.7A	-52.2A	-51.9A	-50.8ABC	-49.7AB	-50.1ABC	-29.0ABCD	-30.5CDE	-31.6DE	-2.8AB	-3.5AB	-4.9AB

Note: the different upper case letters indicated significant difference ($P < 0.05$) of temperature and day combinations for a single emulsifier.

Table 5.3a ΔA_{b24} and description of growth situation for bacteria in surfactin stabilized emulsions						
			<i>E.coli</i> O157: H7		<i>Listeria monocytogenes</i>	
Emulsifier	Emulsion type	Concentration	ΔA_{b24}	Growth condition description	ΔA_{b24}	Growth condition description
Surfactin	Fine emulsion	0.125	0.59cd	Started to grow at 4hr and kept growing slowly until 48hr	0.31a	Started to grow at 11hr and growly slowly until 28, after which the absorbance remained unchanged
		0.25	-0.37op	-	-0.12hij	Started to grow at 33 hr and kept growing slowly until 48hr
		0.5	-0.32nop	-	-0.046fghi	-
		0.75	-0.13hijk	-	-0.027fghi	-
		1	-0.082hi	-	-0.05fghi	-
	Coarse emulsion	0.125	0.51d	Started to grow at 4hr and kept growing slowly until 48hr	0.25abc	Started to grow after 14 hr and growing slowly until 36hr, after which the absorbance remained unchanged
		0.25	-0.43p	-	-0.38klm	-
		0.5	-0.46p	-	-0.39lm	-
		0.75	- 0.26lmno	-	-0.22jk	-
		1	- 0.25kl,mo	-	-0.25jkl	-

Table 5.3 (continued) b ΔA_{b24} and description of growth situation for bacteria in FA-glu stabilized emulsions

			<i>E.coli</i> O157: H7		<i>Listeria monocytogenes</i>	
Emulsifier	Emulsion type	Concentration	ΔA_{b24}	Growth condition description	ΔA_{b24}	Growth condition description
FA-glu	Fine emulsion	0.125	0.10fg	Started to grow at 4.5hr until 38hr and absorbance decreased	-0.37klm	Started to grow at 5hr and reached at peak at 21hr after which absorbance decreased and remained unchanged for the rest of incubation period
		0.25	-0.27mno	-	-0.12hij	-
		0.5	-0.23jklmn	-	-0.12hij	-
		0.75	-	-	-0.11hij	-
		FA-glu 1	-0.15ijklm	-	-0.12hij	-
	Coarse emulsion	0.125	0.29e	Started to grow at 4hr and absorbance started to decrease at 31 hr	0.14bcde	Started to grow at 6.5hr and reached at peak at 21hr after which absorbance decreased and remained unchanged for the rest of incubation period
		0.25	-0.10hij	Started to grow at 7.5 and the absorbance remained for the rest of the incubation period	-0.10hij	Started to grow at 28.5hr and grew very slowly until the end of incubation period
		0.5	-	Started to grow at 4 hr and continued until 37 hr, then absorbance decreased	0.08defg	Started to grow at 8 hr and growing slowly until 46hr and then absorbance decreased
		0.75	-0.35nop	Started to grow at 3.5 hr and continued until 27hr, then absorbance decreased	0.07degf	Started to grow at 10hr and growing slowly until 40hr and then became flat
		1	0.19ef	Started to grow at 11 hr and kept increasing until 48hr	-0.47m	Started to grow at 3.5 hr growing very slowly until 21hr, then absorbance decreased

Table 5.3 (continued) c ΔA_{b24} and description of growth situation for bacteria in lecithin stabilized emulsions

			<i>E.coli</i> O157: H7		<i>Listeria monocytogenes</i>	
Emulsifier	Emulsion type	Concentration	ΔA_{b24}	Growth condition description	ΔA_{b24}	Growth condition description
Lecithin	Fine emulsion	0.125	1.08a	Started to grow since 2hr and kept growing	0.27abc	Started to grow at 3hr and absorbance increased until 20hr then became flat after that
		0.25	0.76b	Started to grow since 6hr and kept growing	0.28a	Started to grow at 5hr and kept growing until 23hr, after which the absorbance kept unchanged
		0.5	-0.03ghi	-	0.17abcd	Started to grow at 8hr and kept growth until 48hr
		0.75	- 0.013ghi	-	0.013defghi	Grew extremely slowly during the entire period
		1	- 0.032ghi	-	0.0082efghi	Grew extremely slowly during the entire period
	Coarse emulsion	0.125	0.71bc	Started to grow since 4.5hr and kept growing. The absorbance was lower than these of NE lecithin emulsion at 0.125 uL/mL level	0.15abcde	Started to grow at 6.5hr, then increase until 28hr and became flat afterwards. The absorbance was lower than these of NE lecithin emulsion at 0.125 uL/mL level
		0.25	- 0.028ghi	-	0.0012efghi	-
		0.5	- 0.019ghi	-	0.01defghi	Grew extremely slowly during the entire period
		0.75	- 0.006hg	-	0.019defghi	Grew extremely slowly during the entire period
		1	- 0.014ghi	-	0.02defgh	Grew extremely slowly during the entire period

Table 5.3 (continued) d ΔA_{b24} and description of growth situation for bacteria in Tween&lauric arginate stabilized emulsions

			<i>E.coli</i> O157: H7		<i>Listeria monocytogenes</i>	
Emulsifier	Emulsion type	Concentration	ΔA_{b24}	Growth condition description	ΔA_{b24}	Growth condition description
Tween&lauric arginate	Fine emulsion	0.125	-0.077hi	Started to grow at 15hr and grew very slowly until 48hr	-0.033fghi	Started to grow at 6.5hr and the grew was very slow, reached the highest level at 20 hr and kept flat then decreased
		0.25	-0.081	-	-0.025fghi	-
		0.5	-0.095hij	-	-0.029fghi	-
		0.75	-0.092hij	-	-0.032fghi	-
		1	-0.11hij	-	-0.058ghi	-
	Coarse emulsion	0.125	0.17ef	Started to grow at 5hr and continued to reach a peak level at 9hr and decreased a little then remained flat.	0.11cdef	Started to grow since 13.5hr and kept grwoing slowly
		0.25	-0.23jklmn	-	-0.22jk	-
		0.5	-0.14hijklm	-	-0.14ij	-
		0.75	-0.13hijkl	-	-0.11hij	-
		1	-0.14hijkl	-	-0.11hij	-

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CHAPTER 6. GENERAL CONCLUSION

6.1 Summary

The overall objectives of the study were to optimize the synthesis condition of some sugar-fatty acid esters and characterize functionalities and antimicrobial properties of these esters' and some microbial fermentation based biosurfactants. The first study (Chapter 3) used lipase to catalyze esterification reaction between three types of fatty acids and glucose, purified the esters, and studied their emulsification properties. It was found that the highest conversion rate was achieved when the fatty acid and glucose ratio was 3:1. The in-house glucose fatty acid esters demonstrated emulsion-stabilizing effect, with longer-chain fatty acid esters having better stabilizing effect. For example, glucose palmitate had better emulsion stability and prevented coalescence better than glucose laurate. However, the stabilizing effect of the glucose esters were weaker than commercial sucrose esters, due to their lower solubility in water. The second study (Chapter 4) compared the antimicrobial performance of various biobased surfactants, including in-house glucose-fatty acid esters and lipopeptides (surfactin and fatty acyl glutamic acid (FA-glu)) produced via microbial fermentation. All of them showed antimicrobial properties against common food borne pathogens *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Enteritidis. However, minimum inhibitory concentration (MIC) was not found for all of them. Among sugar-fatty acid esters, glucose laurate monoester had the best antimicrobial effect with the MIC at 6.5 mg/mL. In-house and commercial surfactin did not show good antimicrobial effect in this study. The minimum inhibitory concentration (MIC) for FA-glu was found to be 25 mg/mL. At sub-MIC concentration of 5 mg/mL, it was able to disrupt bacterial cell membrane and cause cellular leakage. The third study (Chapter 5) investigated the stabilities and antimicrobial properties of nanoemulsions/emulsions containing cinnamaldehyde (CM)

stabilized with various emulsifiers, including surfactin and FA-glu. The surfactin and FA-glu stabilized nanoemulsions and emulsions containing CM did not lower the MIC of the pure CM against foodborne pathogens studied. However, at the sub-MIC concentrations, the nanoemulsions and coarse emulsions had better inhibition effect than pure CM. The droplet sizes had influence on the antimicrobial performance for some nanoemulsions/emulsions: smaller droplets had better inhibition effect than bigger droplets for FA-glu, lecithin, and Tween 80 and lauric ariginate mixture stabilized nanoemulsions/emulsions. However, the droplet size of emulsion did not affect antimicrobial performance of emulsions stabilized with surfactin.

6.2 Recommendations for Future Work

This research explored optimization of synthesis conditions for some sugar-fatty acid esters in terms of substrate ratios, and functionalities of these and other biobased surfactants. However, there still are knowledge gaps in synthesis and application of biobased surfactants that could be addressed in future research. For example, lipase catalyzed esterification reaction still faces challenges in terms of substrates and solvent selection to improve their miscibility and product yields. To expand the usage of microbial biosurfactants, safety studies of the biosurfactants in human food consumption and the producing microorganisms are needed. Also, improving the yield of the biosurfactants is one of the most important future study directions, which may be accomplished by genetic engineering and media manipulation. For antimicrobial usage of biosurfactants in foods, besides the interaction with artificial cell membrane studies, the influence of biosurfactant on physiological metabolism of microbes could be studied. Moreover, the possible mechanisms for antimicrobial activity of biosurfactant-stabilized emulsions and access of antimicrobial agents the mechanisms need to be investigated to see if emulsions prevent or promote the bacteria to access the antimicrobial agents.