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Characterization of Lactose Fatty Acid Esters for their Microbial Growth Inhibitory Activity and Emulsification Properties

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CHARACTERIZATION OF LACTOSE FATTY ACID ESTERS FOR THEIR MICROBIAL GROWTH INHIBITORY ACTIVITY AND EMULSIFICATION PROPERTIES

by

Seung-Min Lee

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ABSTRACT

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Utah State University, 2018

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Sugar fatty acid esters (also more simply known as sugar esters) have great potential for multiple uses in the food industry. Significant numbers of research studies have identified key roles sugar esters can play in food systems, from emulsification activity to also acting as antimicrobial agents. Sugars can be used to synthesize esters with a wide range of different fatty acids. The most common fatty acids used in synthesis in the literature are lauric, myristic, palmitic, stearic, oleic, behenic and erucic acids. Although it has been shown that sugar esters inhibit bacterial growth and promote emulsion formation and/or stabilization, there is a lack of consensus as to how the length of fatty acid chains in sugar esters influence these properties.

In this study I investigated the fatty acid chain length used to make lactose esters and the subsequent influence on microbiological growth. I synthesized novel lactose

esters including lactose monooctanoate (LMO), lactose monodecanoate (LMD) and lactose monomyristate (LMM). I also looked at previous studies which showed lactose monolaurate (LML) has inhibitory potential against some Gram-positive bacteria. Of these four lactose esters, my testing concluded LML was the most effective antimicrobial, only needing a concentration of between ≤0.05 to ≤5 mg/ml to inhibit the growth of the Gram-positive bacteria I tested (*Bacillus cereus*, *Mycobacterium* KMS, *Streptococcus suis*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Streptococcus mutan*s). LML was then followed in antimicrobial effectiveness by LMD, LMM, and finally LMO. The antimicrobial properties were also affected by the solvents used to dissolve the lactose esters such as ethanol and DMSO (Chapter 3).

Sugar esters are a large class of synthetic emulsifiers used in the food industry, and thus, the second objective of this research was to evaluate the influence of the fatty acid chain length on the emulsification properties of lactose esters in 20% oil-in-water (O/W) emulsions. Results showed that the chain length of the lactose ester influenced the O/W emulsification properties. At a concentration of 0.5%, the best result was LML, LMD showed the second best emulsification activity followed by LMM and LMO. Oil droplet distributions highlighted the same trend, with LML maintaining the smallest droplet sizes and thus the most robust emulsion. These results highlight the importance of the fatty acid chain lengths on emulsion stability (Chapter 4).

Additional research on the microbial inhibitory activity of these esters in milk was investigated for the last objective. Each ester was evaluated for anti-listerial effect at different fat contents (fat free, 2% low fat, whole milk, 7% and whip cream) and temperature variants (5, 24 ℃, and 37 ℃) with a final concentration of 5 mg/ml ester in

each milk sample. The esters that showed the highest anti-listerial activity were LMD and LML. This may be due to the amphipathic nature of lactose esters which allow them to partition to the lipid phase of milk, and thus reducing its antimicrobial activity. My results suggested that the efficacy of both LMD and LML's anti-listerial activity were inversely related the milk fat content of the medium and directly related to the incubation temperature of the medium. In general, listerial log reductions in the LMD milk samples were higher than those of LML. This suggests that LMD may not partition into the milk fat phase as readily as LML and thus remains more available in the water phase of the milk to perform its anti-listerial activity. LMD may have potential in increasing food safety if used as an additive (Chapter 5).

From this research I have found that certain lactose esters do show potential for use in the food industry as both emulsifiers and as antimicrobial agents against Gram-positive bacteria. Specifically, LML was the most effective all around, having the best emulsification properties at all usage levels and also showing antimicrobial qualities. LMD was the only other tested lactose ester that showed usefulness as an emulsifier and antimicrobial. Though not as effective as LML at emulsion stabilizing, LMD was shown to stabilize emulsions if enough was used. LMD did exceed LML, in my testing at least, as an antimicrobial agent in a food system, though both yielded significant log reductions of Gram-positive bacteria when used in a food system and in a growth medium. These lactose esters should be considered for food system usage, as they are emulsifiers, have shown antimicrobial activity and will be relatively inexpensive.

(197 pages)

PUBLIC ABSTRACT

Characterization of Lactose Fatty Acid Esters for
their Microbial Growth Inhibitory Activity
and Emulsification Properties

Seung-Min Lee

Sugar esters, substances made from bonding fatty acid tails to a sugar head, can play a number of key roles in food systems from antimicrobial agents to emulsifiers. These unique and very useful properties result from their water-loving and water-avoiding ends. Lactose, a sugar found in milk, based esters are important, as they are environmentally friendly and inexpensive, however, they are not very well understood. I created four different types of lactose esters: lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM), and then compared them to each other to see which would be the best emulsifier and which would be the best antimicrobial.

My previous studies showed that LML was inhibitory against *Listeria monocytogenes* a common food pathogen. This encouraged us to evaluated the microbial inhibitory (bacterial killing) properties of LMO, LMD, LMM, along with LML, specifically, the influence of the fatty acid chain length in each ester and how that influenced my results. The esters, in order of highest microbial inhibitory properties, were LML, LMD, LMM followed by LMO. LML was inhibitory against all the Gram-positive bacteria tested including *Bacillus cereus*, *Mycobacterium* KMS, *Streptococcus suis*, *L. monocytogenes*, *Enterococcus faecalis*, and *Streptococcus mutans* but not Gram-

negative bacteria (*Escherichia coli* O157:H7).

Sugar esters are a large class of emulsifiers used in the food industry, and so my second research objective was to evaluate the influence of the fatty acid chain length on the emulsification properties of LMO, LMD, LML and LMM and compare them to each other and controls (Tween-20 and Ryoto L-1695) in a standard oil-in-water (O/W) emulsion. I did this by observing how long my emulsions lasted after mixing before they would start to separate. I also looked at the actual size of the oil drops in each of my emulsions, the smaller the oil drops remained, the better they stay in the emulsion and thus the more stable the emulsion. My results showed that the best emulsifier was LML, followed by LMD, LMM, and LMO, respectively. Therefore, my lactose esters contained both microbial inhibitory and emulsification activities.

L. monocytogenes is an infamous food pathogen and one of the largest sources of food-borne illness from dairy foods in the United States. Addition of LMD and LML previously were shown to have microbial inhibitory effects in my lab so I wanted to see how well they would work in a food: milk. In general, bacterial deaths in the LMD milk samples were great and many times greater than the LML samples. However, both were greatly affected by milk fat content and how warm each of the samples were kept. LMD may play a useful role in increasing the safety of some foods.

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Seung-Min Lee

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, NOTATION, AND DEFINITIONS

Abbreviation Key

ANOVA	Analysis of variance
ATCC	American type culture collection
BHI	Brain heart infusion
BS	Backscattering
CDC	Centre for disease control
CFR	Code of federal regulations
CMC	Critical micelle concentration
DMSO	Dimethyl sulfoxide
D (3,2)	Volume-surface mean diameter
ELSD	Evaporative light scattering detector
ETOH	Ethanol
FDA	Food and drug administration
HLB	Hydrophobic lipophilic balance
HPLC	High pressure liquid chromatography
λ	Wavelength
Inc	Incorporation
LB	Luria bertani
LMD	Lactose monodecanoate
LML	Lactose monolaurate
LMM	Lactose monomyristate

LMO	Lactose monooctanoate
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
mM	Millimolar
µm	Micrometer
mm	Millimeter
mm/d	Millimeter/day
MW	Molecular weight
nm	Nanometers
O/W	Oil in water
OD	Optical density
PBS	Phosphate buffered saline
RP-HPLC	Reversed phased high performance liquid-chromatography
RTE	Ready to eat food
STDEV	Standard deviations
STERR	Standard errors
TM3	*Thermomyces lanuginosus*
USA	United states of america
2M2B	Two methyl two butanol
3A	Molecular sieves

CHAPTER 1

INTRODUCTION

Introduction

Sugar fatty acid esters (sugar esters) are non-ionic emulsifiers used in a variety of applications in the food, pharmaceutical, and personal care industries. Sugar esters have been produced for decades by manufacturers such as Dai-ichi Kogyo Seiyaku and Mitsubishi-kagaku Food Inc. in Japan (Kjellin and Johansson 2010). Other producers include Croda (USA), Sisterna and Evonik Goldschmidt (Germany) and Stearinerie Dubois (France) (Szűts and Szabó-Révész 2012). Commercial sugar esters are mixtures with various esterification degrees, manufactured by either chemical or enzymatic methods (Staroń and others 2018). While chemical methods were once preferred, enzymatic synthesis is gaining in popularity because of the greater degree of purity in the product (Staroń and others 2018). Furthermore, enzymatically synthesized sugar esters have other benefits, most notably their lower environmental impact thanks to the renewable resources used in their production (Staroń and others 2018; Rao and McClements 2011; Becerra and others 2008; Holmberg and others 2002; Yang and others 2003). Although various enzymes (including bacterial proteases, esterases and lipases) have proven effective in the manufacture of sugar esters, immobilized lipases have been used predominantly because of their economic advantages (Zhang and others 2014; Neta and others 2012; Walsh and others 2009).

Recent research has shed light on the benefits of sugar esters as antimicrobial agents in foods (Zhang and others 2014; Chen and others 2014; Szűts and Szabó-Révész

2012; Wagh and others 2012; Nobmann and others 2009; Smith and others 2008; Habulin and others 2008; Ferrer and others 2005; Devulapalle and others 2004; Watanabe and others 2000). Below the critical micelle concentration (CMC) of sugar esters, it is believed that interactions on the bacterial cell surface result in increased cellular permeability which enhances inhibitory effects (Blondelle and others 1999). Previous research on esters of laurate attached to sucrose, fructose, galactose, lactose and maltose have been shown by numerous studies to be valuable microbial inhibitory agents, specifically for Gram-positive bacteria (Lee and others 2017; Zhang and others 2014; Chen and others 2014; Wagh and others 2012; Nobmann and others 2009; A. Smith and others 2008; Habulin and others 2008; Ferrer and others 2005; Devulapalle and others 2004; Watanabe and others 2000). Other studies have concluded that sugar esters of decanoic, myristic and palmitic acids exhibit broad antimicrobial activity against Gram-positive bacteria (Lee and others 2017; Zhang and others 2014; Habulin and others 2008; Piao and others 2006). While much of the research on the microbial inhibition of sugar esters has been focused on determining minimum inhibitory concentration (MIC) values (Zhang and others 2014; Chen and others 2014; Wagh and others 2012; Nobmann and others 2009; Piao and others 2006; Ferrer and others 2005; Watanabe and others 2000), few studies have determined minimum bactericidal concentration (MBC) values (Wagh and others 2012). The solvent used to dissolve the esters has also not been thoroughly investigated in terms of its effect on the microbial inhibitory activity against Gram-positive and/or Gram-negative bacteria.

Sugar esters are also used for their emulsification properties. Synthetically produced sorbitan esters and their ethoxylates have been well established as effective

emulsifiers based on their hydrophile-lipophile balance (HLB) and critical micelle concentration (CMC) (McClements 2005). When used at concentrations above the CMC, these sugar esters have been widely shown to emulsify otherwise marginally soluble non-ionic organic compounds (Smith and Burns 2002). Many studies have shown a correlation between fatty acid chain lengths of sugar esters to HLB values in oil-in water (O/W) emulsions (Zhang and others 2014; Becerra eand others 2008; Piao and Adachi 2006; Nakaya and others 2005; Yanke and others 2004; Soultani and others 2003; Garofalakis and others 2000; Hill and Rhode 1999). However, there is very little information about the mechanisms by which lactose esters form emulsions as well as the mechanisms by which they are destabilized (Zhang and others 2014; Neta and others 2012; Garofalakis and others 2000). Most commercial non-ionic emulsifiers are very temperature sensitive (Holmberg and others 2002); however, the emulsification properties of sugar esters are much less influenced by temperature than that of typical commercial emulsifiers. As a result, sugar esters are versatile emulsifiers in a broad array of applications. They are often used in foods at levels around 0.1% but may be used in greater concentrations though they are not to exceed 5% in finished food products as stated in the Code of Federal Regulation (CFR) Title 21 (21CFR172.859).

Though the antimicrobial properties of sugar esters in food systems are not yet fully understood, research performed thus far has yielded promising findings of their usefulness in inhibiting food-borne pathogens (Chen and others 2014; Xiao and others 2011; Yang and others 2003). Two areas of interest that have been studied in previous research but warrant further investigation are the effects of fat content (Chen and others 2014) and incubation temperature (Xiao and others 2011) on the antimicrobial activity of

sugar esters. As lactose esters show promise as emulsifiers in the food industry, and if antimicrobial properties of these esters are better understood both may be used to great synergistic effect in protecting and stabilizing foods. These studies suggest the potential for significant effects of fat content as well as incubation temperature on the microbial inhibition of lactose esters.

Hypothesis

The fatty acid chain length of enzymatically synthesized lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM) has an effect on their microbial growth inhibitory activity and emulsification properties. The fat content of food system and incubation temperature may affect the microbial inhibitory activity of lactose esters.

Objectives

1. Develop methods to synthesize and purify lactose esters. Evaluate the influence of LMO, LMD, LML and LMM in DMSO and/or ethanol against various Gram-positive and Gram-negative bacteria.
    a) Determine the MIC and MBC values of additional lactose esters, LMO, LMD, LML and LMM against seven different Gram-positive bacteria and *Escherichia coli* O157:H7.
    b) Investigate the MIC and MBC values of the esters dissolved in two solvents, DMSO and ethanol.
2. Evaluate the emulsification properties of lactose esters in 20% oil-in-water (O/W) emulsions by observing the thickness of the clarification layer and oil droplet size

distribution. Determine the critical micelle concentration (CMC) and hydrophilic-lipophilic balance (HLB) of these compounds.

a) Record the thickness of the clarification layer and oil droplet size distribution of LMO, LMD and LMM, along with LML compared to Tween-20 and Ryoto L-1695.

b) Determine the CMC value and calculate the HLB value for each ester.

3. Investigate the effect of different levels of milk fat at various temperatures on microbial inhibition, specifically the effectiveness of LMD and LML against *Listeria monocytogenes.*

a) Test the anti-listerial effect of LMD and LML by determining the log reductions in milk at various fat content and temperatures.

References

Becerra N, Toro C, Zanocco AL, Lemp E, Günther G. 2008. Characterization of micelles formed by sucrose 6-O-monoesters. Colloids Surfaces A Physicochem Eng Asp 327:134–139.

Blondelle SE, Lohner K, Aguilar M-I. 1999. Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity. Biochim Biophys Acta - Biomembr 1462:89–108.

Chen Y, Nummer B, Walsh MK. 2014. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Lett Appl Microbiol 58:156–162.

Devulapalle KS, Gómez de Segura A, Ferrer M, Alcalde M, Mooser G, Plou FJ. 2004. Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and

glucosyltransferase activity. Carbohydr Res 339:1029–1034.

Ferrer M, Soliveri J, Plou FJ, Lopez-Cortez NL, Reyes-Duarte DR, Christensen M, Copa-Patino JL, Ballestros A. 2005. Synthesis of sugar esters in solvent mixtures bylipases from *Thermomyces lanuginosus* and *Candida antarctica B*, and their antimicrobial properties. Enzyme Microb Technol 36:391-398.

Garofalakis G, Murray BS, Sarney DB. 2000. Surface activity and critical aggregation concentration of pure sugar esters with different sugar headgroups. J Colloid Interface Sci 229:391–398.

Habulin M, Šabeder S, Knez Ž. 2008. Enzymatic synthesis of sugar fatty acid esters in organic solvent and in supercritical carbon dioxide and their antimicrobial activity. J Supercrit Fluids 45:338–345.

Hill K, Rhode O. 1999. Sugar-based surfactants for consumer products and technical applications. Lipid - Fett 101:25–33.

Holmberg K, Jönsson B, Kronberg B, Lindman B. 2002. Surfactants and polymers in aqueous solution. John Wiley & Sons, Ltd

Kjellin M, Johansson I. 2010. Surfactants from renewable resources. John Wiley & Sons, Ltd

Lee S-M, Sandhu G, Walsh MK. 2017. Growth inhibitory properties of lactose fatty acid esters. Saudi J Biol Sci 24:1483–1488.

McClements DJ. 2005. Food emulsions: principles, practices, and techniques. CRC Press.

Nakaya K, Ushio H, Matsukawa S, Shimizu M, Ohshima T. 2005. Effects of droplet size on the oxidative stability of oil-in-water emulsions. Lipids 40:501–507.

Neta NDAS, Santos JCS, Sancho SDO, Rodrigues S, Gonçalves LRB, Rodrigues LR, Teixeira JA. 2012. Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions. Food Hydrocoll 27:324–331.

Nobmann P, Smith A, Dunne J, Henehan G, Bourke P. 2009. The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. Int J Food Microbiol 128:440–445.

Piao J, Adachi S. 2006. Stability of O/W emulsions prepared using various monoacyl sugar alcohols as an emulsifier. Innov Food Sci Emerg Technol 7:211–216.

Piao J, Kawahara Y, Inoue T, Adachi S. 2006. Bacteriostatic activities of monoacyl sugar alcohols against thermophilic sporeformers. Biosci Biotechnol Biochem 70:263–265.

Rao J, McClements DJ. 2011. Food-grade microemulsions, nanoemulsions and emulsions: fabrication from sucrose monopalmitate & lemon oil. Food Hydrocoll 25:1413–1423.

Smith A, Nobmann P, Henehan G, Bourke P, Dunne J. 2008. Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives. Carbohydr Res 343:2557–2566.

Smith JA, Burns SE. 2002. Physicochemical groundwater remediation. Kluwer Academic.

Soultani S, Ognier S, Engasser J-M, Ghoul M. 2003. Comparative study of some surface active properties of fructose esters and commercial sucrose esters. Colloids Surfaces A Physicochem Eng Asp 227:35–44.

Staroń J, Dąbrowski JM, Cichoń E, Guzik M. 2018. Lactose esters: synthesis and biotechnological applications. Crit Rev Biotechnol 38:245–258.

Szűts A, Szabó-Révész P. 2012. Sucrose esters as natural surfactants in drug delivery systems—A mini-review. Int J Pharm 433:1–9.

U.S. Code of Federal Regulations, Title 21, volume 3. (21CFR172.859). Sucrose fatty acid esters.

Wagh A, Shen S, Shen FA, Miller CD, Walsh MK. 2012. Effect of lactose monolaurate on pathogenic and nonpathogenic bacteria. Appl Environ Microbiol 78:3465–3468.

Walsh MK, Bombyk RA, Wagh A, Bingham A, Berreau LM. 2009. Synthesis of lactose monolaurate as influenced by various lipases and solvents. J Mol Catal B Enzym 60:171–177.

Watanabe T, Katayama S, Matsubara M, Honda Y, Kuwahara M. 2000. Antibacterial carbohydrate monoesters suppressing cell growth of *streptococcus mutans* in the presence of sucrose. Curr Microbiol 41:210–213.

Xiao D, Ye R, Davidson PM, Hayes DG, Golden DA, Zhong Q. 2011. Sucrose monolaurate improves the efficacy of sodium hypochlorite against *Escherichia coli* O157:H7 on spinach. Int J Food Microbiol 145:64–68.

Yang C-M, Luedecke LO, Swanson BG, Davidson PM. 2003. Inhibition of microorganisms in salad dressing by sucrose and methylglucose fatty acid monoesters. J Food Process Preserv 27:285–298.

Yanke L, Shufen Z, Qinghui W, Jinzong Y. 2004. Study on surface activity and critical aggregation concentration of sucrose esters containing different isomers of mono-, di- and polyesters. Tenside Surfactants Deterg 41:26–30.

Zhang X, Song F, Taxipalati M, Wei W, Feng F. 2014. Comparative study of surface-active properties and antimicrobial activities of disaccharide monoesters. PLoS One 9:e114845.

Zhang X, Wei W, Cao X, Feng F. 2014. Characterization of enzymatically prepared sugar medium-chain fatty acid monoesters. J Sci Food Agric 95:1631-1637.

CHAPTER 2

LITERATURE REVIEW

Synthesis of Sugar Esters

Comparison of Ionic and Non-ionic Emulsifiers

The mechanism by which non-ionic emulsifiers normally stabilize emulsions involves steric, hydration, and thermal fluctuation interactions (Li 2012). Still, the non-ionic interfacial membranes are usually unstable enough to rupture when the droplets come into close proximity with each other (McClements 2007). Commercial non-ionic emulsifiers include primarily polyethoxylated products (Tadros 2013). These have a polyethylenglycol chain which acts as the hydrophilic portion (Tadros 2013). Non-ionic emulsifiers can be altered in their properties and applications by the relative amount of hydrophobic and hydrophilic portions included in the emulsifier (Tadros 2013).

These emulsifiers do not have a net charge and frequently are used in tandem with anionic emulsifiers (Gumus 2017). One benefit is their lack of interaction with the calcium and magnesium ions found in hard water (van Os 2010). In cold solutions they tend to be more efficient emulsifiers than their anionic counterparts at similar concentrations (van Os 2010). As a general rule, non-ionic emulsifiers have the least toxic effects among emulsifiers (Staroń and others 2018; Szűts and Szabó-Révész 2012). The two main disadvantages of non-ionic emulsifiers are that some do not produce good foams and that some may produce cloudiness in solutions, potentially leading to phase separation (van Os 2010). Most sugar esters are non-ionic emulsifiers (Szűts and Szabó-Révész 2012).

Even small structural variations of emulsifiers can lead to drastic changes in their properties and effectiveness. The main mechanism by which ionic emulsifiers stabilize emulsions is by electrostatic repulsion (Branen and others 2005). In brief, when added to water they become ionized (McClements 2005). Their ability to behave as effective emulsifiers is thanks primarily to their negative charge (anion) and positive charge (cations) (McClements 2005). The negative charge enables emulsifier molecules to associate with different substances simultaneously, one commercial example being carpet cleaners which interact with carpet fibers and soil particles, suspending soils in micelles (Jesse and Lynn 2009). For this reason, anionic emulsifiers are the most frequently used kind of emulsifier in low moisture carpet cleaners, such as shampoos and encapsulation products (Jesse and Lynn 2009). Cationics are positively charged and work well in formulations like fabric softeners and automobile waxes (Jesse and Lynn 2009). They may also have antimicrobial properties; hence their presence in disinfectants and cleaners (Villapún 2016). One disadvantage of ionic emulsifiers is their tendency to interact with other ions in the solution which may cause precipitates or the formation of a foam (Gumus 2017).

Structures of Lactose Esters, Tween-20 and Ryoto L-1695

Enzymes are very selective catalysts with the ability to distinguish among very similar compounds to act on a single substrate (Staroń and others 2018; Walsh and others 2009). Lipases, esterases, and bacterial proteases are the principal types of enzymes involved in the esterification reactions that form lactose esters (Staroń and others 2018). Much research has gone into studying these enzymes, and it has been elucidated that

enzymes from *Candida antharctica*, *Mucor miehei*, *Pseudomonas cepacia*, *Rhizomucor miehei*, and *Thermomyces lanuginosus* usually act on the C6' OH group which is located at the non-reducing end of lactose (Staroń and others 2018).

In the not too distant past, Walsh and others (2009) were able to synthesize a unique and new sugar ester, lactose monolaurate (LML). A lipase from *Thermomyces lanuginosus* was used in the synthesis of LML (Fig 2.3). The esterification of lactose with fatty acid acyl group (laurate) is most likely to occur at the C6' OH position, and further investigation showed that lactose monooctanoate (LMO) (Fig 2.1), lactose monodecanoate (LMD) (Fig 2.2) and lactose monomyristate (LMM) (Fig 2.4) differ only in the fatty acid acyl group.

Polysorbate 20 (also known as Tween-20) is a non-ionic emulsifier of the polysorbate type which is synthesized by the ethoxylation of sorbitan followed by the introduction of lauric acid (Damodaran and others 2007). Thanks to its relative stability and non-toxicity, it has found many uses as an emulsifier and detergent in domestic, pharmacological and scientific products (Damodaran and others 2007). The process of ethoxylation creates 20 repeated units of polyethylene glycol which are present in 4 different chains, thus creating a wide array of compounds with many commercial applications (Jafari and McClements 2018). As shown below in Figure 2.5, these compounds are polymers of pegylated sorbitan with the total number of poly (ethylene glycol) units being 20 ($w + x + y + z = 20$) with one terminal being capped by a dodecanoyl group (Jafari and McClements 2018).

Ryoto L-1695 is a non-ionic sucrose ester and an emulsifier which contains a hydrophilic group, sucrose, along with a lauric acid side chain (Ye 2007) (Fig 2.6). It has

also found many commercial uses as emulsifier. The esterification of this compound most frequently involves the C6' OH, the same as with lactose esters.

Antimicrobial Properties of Sugar Esters

Application of Sugar Esters as Antimicrobial Agents

While the words "preservative" and "chemical additives" may not elicit a positive response among most consumers, these terms do not necessarily connote unhealthy or unnatural ingredients (Kralova and Sjöblom 2009). Fatty acids and their corresponding esters are one example of natural substances with little to no toxicity which may aid in the shelf-life of products by their antimicrobial activity (Szűts and Szabó-Révész 2012; Kralova and Sjöblom 2009).

In general, research seems to suggest that fatty acid chain length is one factor affecting antimicrobial activity, with medium chain lengths (C10-C12) being more effective against Gram-positive bacteria (Lee and others 2017; Zhang and others 2014; Wagh and others 2012; Nobmann and others 2009; Smith and others 2008). One finding of note is that regardless of the sugar used, the fatty acid seemed to be the main determinant of antimicrobial activity medium chain fatty acids appear to be the most active (Lee and others 2017). Research performed by Davidson and others (2005) has also suggested that among monoglyside derivatives of lengths from C8 to C14, the C12 monoglyceride has the most antimicrobial potency.

The antimicrobial properties of sugar esters are not fully understood in food systems (Wagh and others 2012). The antimicrobial effectiveness of sugar esters may be decreased by interactions with fat, starch and proteins (Kralova and Sjöblom 2009).

Given the complexity of many food matrices, these major components of food may cause a partitioning which may prevent interaction of the sugar ester with the bacteria present in the food system (Chen and others 2014). In order to reduce the presence of food-borne pathogens in a food system, sugar esters are often combined with a number of post-packed decontamination methods, such as thermal pasteurization, application of disinfectant and high-pressure processing (Pesavento and others 2010). The one area where the antimicrobial properties of sucrose esters were employed was in canned milk coffee served from hot temperature vending machines in Japan (Thomas and others 1998). Sucrose monolaurate with sodium hypochlorite exhibited impressive microbial inhibitory properties against *Escherichia coli* O157:H7 in spinach (Xiao and others 2011). Sugar esters exhibited antimicrobial properties when added to weak acid hypochlorous water used on shredded vegetables (Pan and Nakano 2014).

Calculations of MIC and MBC

Minimum inhibitory concentrations (MIC) are the lowest concentration of an antimicrobial compound that still wields an inhibitory effect the growth of a microorganism, monitored by cell counts observed on plated samples over time (Emery PHARMA 2018). Minimum inhibitory concentration is an important value determined in diagnostic laboratories to determine the degree of resistance of microorganisms to an antimicrobial and also to assess the effectiveness of new antimicrobials (Andrews 2001). Minimum bactericidal concentrations (MBC) are the lowest concentration of antimicrobial that will prevent altogether the growth of a microorganism (Andrews 2001). When MIC and MBC values are determined experimentally, a statistical method

known as the student T-test is used to determine statistically significant differences between test compounds and control compounds (Student T-test (paired, two tailed), $p < 0.05$) (Wagh and others 2012).

Structures of Gram-positive and Gram-negative Bacteria

Cell Wall

The cell wall in Gram-positive bacteria consists primarily of peptidoglycan which forms a thick, protective layer around the cell (Fig 2.7) (Karki 2017). The peptidoglycan layer tightly adheres to the cell membrane at its outer surface (Silhavy 2010). In Gram-positive bacteria about 60 to 90% of the cell wall is composed of peptidoglycan (Harisha 2006). The cell wall of most Gram-positive bacteria contains very little protein, and they contain neither an outer membrane, nor a periplasmic space (Hoiczyk and Hansel 2000).

The cell wall of Gram-negative bacteria is generally more complex but thinner in comparison (Fig 2.7) (Silhavy and others 2010). While Gram-negative bacteria still contain peptidoglycan, this layer composes only 10 to 20% of the cell wall (Spellman and Drinan 2012). The remaining portion of the cell wall is made up of polysaccharides, proteins, and lipids (Silhavy and others 2010). To the outside of the cell wall is an outer membrane which interfaces with the surrounding environment around the cell and leaves only a very thin periplasmic layer (Solomon and others 2014). On the inside of the wall there is a greater periplasmic space separating an inner cell membrane from the cell wall (Solomon and others 2014).

Cell Membrane

The cell membrane (also known as plasma membrane or cytoplasmic membrane) is the constant structure that is present in all bacteria, as well as plant and animal cells (Boyle 2008). However, in animal cells, the cell membrane is the only bounding membrane whereas in plants the cell wall serves as an extra layer of protection (Boyle 2008). All protoplasmic structures are included within the outermost membrane of the cell, diagrammed (Fig 2.7) (Boyle 2008).

The cell membrane comes into immediate contact with the surrounding environment around the cell (Kumar 2012). It is dynamic and eclectic in its properties and plays an integral role in numerous functions such as osmosis, selective absorption of mineral nutrients, signal transducing receptors for multiple stimuli (including electrical, light mechanical and chemical) (Solomon and others 2014; Kumar 2012). Proteins embedded in the cell membrane are arranged in a directional fashion (Boyle 2008). In some places, the cell membrane projects inward and may be continuous with the endoplasmic reticulum (Boyle 2008). The cell membrane is also the location where pinocytosis and phagocytosis take place (Boyle 2008). As can be seen, the cell membrane has unique and numerous properties. Its vibrant fluidity prevents it from ever becoming stagnant in shape or function (Solomon and others 2014; Boyle 2008). A more detailed diagram of the cell membrane is shown below (Fig 2.8) (BiologyWise 2018).

Biofilms

Biofilms are populations of bacteria which adhere to each other and/or surfaces and are encapsulated in a biopolymer matrix (Limoli and others 2015; Garrett and others

2008). Bacteria may exist in either planktonic (free) or sessile (attached) forms in their natural environments; biofilms are formed when microorganisms switch to a sessile form of growth (Gu 2014). In order for a biofilm to form, first bacteria must adhere to a surface in a single layer creating a monolayer of cells (Gu 2014; Donlan 2002). Then follows a clustering of cells and microcolony formation (Gu 2014; Donlan 2002). The synthesis of extracellular polymers and subsequent surrounding of cell clusters by a hydrated exopolymer matrix is the next critical step in biofilm maturation (Garrett and others 2008). Nutrients can reach the bacteria through open water channels within the glycocalyx matrix of the biofilm contains open water channels which allow for the transport and delivery of nutrients to all cells within the biofilm (Donlan and Costerton 2002; Donlan 2002).

The regulation of gene expression is achieved by a mechanism known as quorum sensing (Kragh and others 2016). This process is dictated primarily by cell population density fluctuations and involves signaling among cells which facilitates biofilm formation and longevity (Kragh and others 2016). It is believed that quorum sensing is pivotal in determining biofilm thickness (Gu 2014). At a certain point, a maximum biofilm thickness is achieved, at which time cell dispersion begins, involving the release of planktonic cells from the biofilm into the outer environment, colonizing new areas (Montana State University 2017; Gu 2014). The main stages in biofilm formation appear the diagram below (Fig 2.9) (Montana State University 2017).

Food-borne Illnesses of Various Microorganisms

Listeriosis is the term used for a potentially severe infection caused by eating food contaminated with the *Listeria monocytogenes*, a Gram-positive bacterium (Allerberger and Wagner 2010). Older adults, pregnant women, newborns, and adults with weakened immune systems are much more susceptible to Listeriosis (CDC 2011). As Table 2.1 and Table 2.2 below shows, infections caused by *L. monocytogenes* have a relatively high hospitalization rate and death rate (Scallan and others 2011).

Mycobacterium sp. causes an infection which impacts about one-third of the world's population (Moghaddam and others 2016). Due to the emergence of multidrug-resistant strains, *Mycobacterium* sp. is becoming even more of a global concern because of the difficulty in treating with expensive and toxic drug which are also often less effective (Hoagland and others 2016).

Bacillus cereus most often causes illness when 10^5 cfu/g or more are ingested in food. It produces two enterotoxins which have been found to cause food-borne illness (Bennett and others 2013). *B. cereus* is found in soil and is most frequently associated with vegetables, milk, cheese, and a number of other raw and processed foods (Bennett and others 2013). Table 2.3. shows the high frequency of illnesses from *B. cereus* (Scallan and others 2011).

Streptococcus suis is a wide spread pathogen of swine which also at times infects humans (Feng and others 2014). It is considered an emerging pathogen of concern. Of the 1600 or so human cases which have been reported worldwide, most occurred in Southeast Asia (Chatzopoulou 2015).

Streptococcus mutans most commonly causes infections in the mouth, resulting in tooth damage, abnormal speech, problems chewing, and psychological problems which may have detrimental effects on self-esteem, social interactions, concentration, among other things (Forssten and others 2010). Cavities caused by this microorganism are the primary reason for about half of all dental visits in the USA (Brown and Lechtenberg 2006).

Enterococcus faecalis found in the gut microbiota, is linked with colonic lesions and endocarditis (Silva and others 2017). Recently, research about inflammatory intestinal diseases and irritable bowel syndrome have suggested there may be potential mechanisms involving *E. faecalis* which disrupt the epithelial layer of the intestines (Silva and others 2017).

Escherichia coli O157:H7 is a Gram-negative bacterium discovered to be a human pathogen in 1982 (Lim and others 2010). It is one of several serotypes which produces Shiga toxin, known to cause human illness. *E coli* O157:H7 is commonly found in the feces of healthy livestock and is usually passed on to humans through contaminated food, water, or by contact with infected animals or people (Ferens and Hovde 2011). Table 2.1 and Table 2.3 below shows the relatively high hospitalization and frequency of illness associated with the illness (Scallan and others 2011).

Mechanisms of Sugar Esters as Antimicrobial Agents

Sugar esters with various fatty acids and saccharide moieties exhibit differing degrees of antimicrobial activity against the many different strains that have been tested (Zhang and others 2014; Chen and others 2014; Wagh and others 2012; Nobmann and

others 2009; Smith and others 2008; Habulin and others 2008; Piao and others 2006; Ferrer and others 2005; Devulapalle and others 2004; Watanabe and others 2000). The area of inhibition against Gram-positive bacteria is generally larger than that of Gram-negative bacteria, which indicates the stronger antimicrobial effects of sugar esters against Gram-positive bacteria (Lee and others 2017; Wagh and others 2012; Piao and others 2006).

It appears as though in Gram-negative bacteria, diffusion and delivery of sugar esters to the vulnerable cell is greatly inhibited by the outer membrane, thus reducing the effectiveness of sugar esters against Gram-negative bacteria (Moore 1997). Consistent conclusions have also been made in other studies by Jouki and others (2014) and Moore (1997).

The exact mechanism by which autolysis is achieved by these compounds is yet to be fully understood (Nobmann and others 2010). It has been proposed that sugar esters may cause disorganization in the membrane structure and initiate an autolysin control system which may exist in the cell membrane, though this has not been confirmed (Zaika and Fanelli 2003). In their study, they revealed that sugar esters generated significant alterations in the morphology of *L. monocytogenes* cells. Nobmann and others (2010) showed that fatty acids and their derivatives, inactivated *Staphylococcus aureus*, by means of disintegrating the cell membrane.

Another possible mechanism of action involves a disruption in the respiratory activity of cells by the inactivation of enzymes integral to the process of oxygen uptake and/or the absorption of essential amino acids (Moore 1997). Others have claimed that sugar esters at minimum inhibitory concentrations (MIC) decrease production of beta-

lactamases and other exoproteins in *S. aureus* by causing interferences cellular signals (Projan and others 1994).

It is critical to understand biofilm formation, growth and removal as all these strongly affect the susceptibility of microorganisms to antimicrobial compounds. Sucrose esters not only strongly diminished the hydrophobic nature of the cell surface of different *S. aureus* strains, they also disrupted and destroyed the cell membrane (Zhang and others 2015). In this same study, a critical attribute of the bacterial surface for successful adhesion to non-polar surfaces is hydrophobicity. The hydrocarbons test was used to determine the extent of cell surface hydrophobicity after sucrose ester treatments with a correlation being observed between biofilm formation ability and hydrophobicity. In another study, similar effects were observed with *Salmonella enteritidis* with sugar esters (Miyamoto and others 2009).

Emulsifying Properties of Sugar Esters

Forming Emulsions with High-speed Mixers

Ultra-turrax T25 and/or high-speed mixers are generally regarded as the most effective means for the homogenization of oil and water phases in the food industry (Laboratory-Equipment.com 2018; McClements 2005). The oil, water and emulsifiers to be homogenized are all added to an appropriate container (McClements 2005). The mixture is then blended by a spinning head that rotates powerfully, creating a shear force that quickly blends the materials into one homogenous fluid (Laboratory-Equipment.com 2018; McClements 2005). The high-speed mixer generates horizontal as well as vertical flow of the fluids, distributing both throughout the vessel resulting in a homogenous

solution (Fig 2.10) (McClements 2005). This process can be aided by the use of baffles fixed to the inner walls of the container to create a more turbulent flow (McClements 2005).

Ultra-turrax T25 (shown below in Fig 2.11) operates under the rotor-stator principle, generating a very powerful shear force that efficiently homogenizes otherwise incompatible substances into emulsions or suspensions (Laboratory-Equipment.com 2018; Charles Ross & Son Company 2012; McClements 2005). The outer tube, or stator remains stationary while the inner shaft, or rotor, rotates very quickly, creating a strong shear force (Laboratory-Equipment.com 2018). Shear force is created when misaligned forces act on an object from opposing sides. Shear force causes different parts of an object to be pushed in different directions and may result in a rupturing of the object, creating smaller particles and homogenizing substances (Laboratory-Equipment.com 2018).

Forming Emulsions with High-pressure Homogenizers

Microfluidizers or high-pressure homogenizers are another means of efficiently creating emulsions (McClements 2005). Because of their ability to greatly reduce emulsion droplet size, they have commonly been employed in the pharmaceutical industry for preparing pharmaceutical emulsions as well as in the food industry (Szűts and Szabó-Révész 2012). Small quantities of fluids are forced through narrow channels with at least one dimension smaller than 0.1 μm, for dispersion of insoluble fluids (McClements 2005). Mixing occurs in an interaction chamber where the flow of the fluid is directed through microchannels leading to an area called the impingement area (Jafari

and others 2007). The fluid stream is forced through these channels by a pump powered pneumatically that is strong enough to pressurize the system to (80-199 psi) to about 10,000 psi (Yanniotis and others 2013). When this highly pressurized fluid gets to the interaction chamber, extreme shear and impact forces along with cavitation act as the means for a dramatic reduction in droplet size (Anandharamarishnan 2014). Schematic drawing of the homogenization process using the microfluidizer is shown in Fig 2.12 (Wabel 1998).

The extent to which emulsification is achieved is dictated by the microchannel design, the delivering pressure as well as the processing time (McClements 2005). Microfluidizers are effective at creating very small emulsion droplet sizes because the powerful forces applied to the droplets (Anandharamarishnan 2014). The amount of pressure used and the time it is applied for are the major determinants of resulting droplet size (McClements 2005). Table 2.4 shows other types of equipment used in creating emulsions (McClements 2005). These will not be described in detail here, but the table below gives a brief outline of several of the homogenizers. The graph below shows the other types of equipment that can be used in creating emulsions along with the degree of sheer forces which they generate (Microfluidics 2010). As shown, the microfluidizer is by far the most powerful in terms of the sheer force it creates (Fig 2.13) (Microfluidics 2010).

Methods of Measuring Emulsion Stability: Interpretation of Turbiscan Plots

The Turbiscan (a vertical scan macroscopic analyzer) method involves sending photons into a sample (Formulaction 2009). These photons are inevitably scattered

numerous times by objects in suspension, exit the sample and are then measured by the detection device of the Turbiscan (both the degree of transmission and the backscattering of the photons may be reported) (Birk 2015; Bennett and others 2009). The combination of several scans conducted over a period of time enables stability analysis of the sample to proceed anywhere from 20 to 50 times faster than would be possible by mere visual analysis (Fig 2.14) (Turbiscan CLASSIC). This may be used to measure 6 ml samples of emulsions in glass tubes (Garg and others 2010). This method involves sending photons through the length of the tube to detect any changes in backscattering (ΔBS%) (Formulaction 2009). These measurements are collected, and with the help of statistical analysis, conclusions can be made about the absolute thickness of the clarification layer which is located from 0.5-10 mm at the bottom of the tube (Garg and others 2010). This method can also give an overall picture of the homogeneity in the emulsion.

In Fig 2.15 below, the downward peaks at the left of the graph show the lack of backscattering in the clarification layer of the sample, while the upward peaks on the right show the backscattering that occurs in the creaming layer (Formulaction 2009). The gradually increasing depth and height of the peaks represent the decreasing stability of the sample (Bennett and others 2009). The small peaks indicate a greater degree of homogeneity. With time, the peaks gradually increase in size due to the increased creaming and clarification in the solution (Turbiscan CLASSIC). As shown in the examples below, the maximum backscattering value is -30% and the threshold is set at the middle of this at -15% (Turbiscan CLASSIC).

These plots are useful in displaying the degree of stability of an emulsion over time (Turbiscan CLASSIC). They are useful in research about food emulsion stability

because it a relatively simple and inexpensive way of showing to what extent a clarification and creaming layer has formed in a sample (Formulaction 2009). As an emulsion oil migration and destabilizes occurs, a change in thickness of the clarification layer can be shown over time as an indicator of the degree of instability that develops (Fig 2.16) (Turbiscan CLASSIC; Birk 2015). This type of graph is often generated with the help of an excel spreadsheet by importing the data (time and thickness). This type of graph is useful because of the simplicity with which is shows increasing destabilization over time. The slope of the graph indicates the rate of destabilization, often measured in mm of emulsion destabilized per day (Turbiscan CLASSIC).

Droplet Size Measurement with Particle Size Analyzers

The LS Beckman Coulter droplet size analyzer measures the distribution of particle sizes in an emulsion by detecting the pattern of light dispersed by the oil droplets (Beckman Coulter 2011; Garg and others 2010). A scattering pattern is generated by light intensity as determined by the angle at which the light is scattered, and the scattering pattern of each oil droplet is a determinant of its size (Beckman Coulter 2011). Each particle's scattering pattern is characteristic of its size. The pattern produced by the Beckman coulter droplet size analyzer is the total of all patterns scattered by each oil droplet from the sample being measured (Garg and others 2010).

There are numerous other methods of expressing the mean oil droplet size of an emulsion (Garg and others 2010). Three common ways are D (4,3), the mean diameter over volume (also called the DeBroukere mean), D (3,2), the volume/surface mean (also called the Sauter mean) and D (1,0), the arithmetic or number mean (HORIBA 2012). Of

these three methods, Sauter mean diameter (D (3,2)), which is simply an average of particle size, is the most commonly used (HORIBA 2012). It is useful for predicting properties which depend on droplet surface area such as bioavailability, reactivity, dissolution (Malvern 2015; HORIBA 2012). It also accounts for the presence of fine particulates in the size distribution in comparison to the other methods (HORIBA 2012). Another common method of expressing oil droplet size in an emulsion is known as the volume moment mean (D (4,3)) (Malvern 2015). It is most useful when expressing droplet size in many samples since it is a representation of the particle size of the bulk of the oil droplets in a sample emulsion (HORIBA 2012). It is more responsive to larger particulates in the size distribution since these occupy more space and are more heavily accounted for (Malvern 2015). The arithmetic or number mean (D (1,0)) is less commonly used and for that reason will not be discussed here (Malvern 2015).

Oil droplet size distribution is usually a better indicator of emulsion stability compared to D (3,2), which is frequently used as an indicator of stability. D (3,2) (data obtained using a LS Beckman Coulter droplet size analyzer) is often not adequate in estimating the stabilities of the tested emulsifiers due to the wide range and variability of results. Indeed, only oil droplet size distribution adequately captures the variable nature of each tested sugar ester.

Particle Size Result Interpretation: Number vs. Volume Distributions

Interpreting results of a particle size measurement requires an understanding of which technique was used and the basis of the calculation. Generally, most of the instruments called “particle size analyzers” measure particle size distribution based on

the volume standard (Garg and others 2010). Volume distributions display the total volume of the oil droplets at each droplet size, measured by diameter (HORIBA 2012). Thus, in volume distributions, more weight is given to the larger oil droplets since each one occupies more volume. Number distributions give more weight to smaller particles since the volume of each particle is not accounted for in the graph.

Fig 2.17 and Fig 2.18 displayed below are examples of volume and number distributions. As can be seen, the sample size distribution is expressed in terms of either the total volume occupied or the number oil droplets.

HLB Calculation

The HLB (Hydrophobic-lipophilic balance) concept is a widely used method of measuring the ability of an emulsifier to maintain oil droplets suspended in a solution (Zhang and others 2014; Ritthitham 2009**;** Piao and Adachi 2006; McClements 2005; Hait and Moulik 2001). The hydrophile-lipophile balance is expressed by a value indicating the relative affinity of the emulsifier for the oil and aqueous phases (McClements 2005; Hait and Moulik 2001). Each emulsifier is given a HLB value according to its chemical structure (McClements 2005). High HLB values indicate a high proportion of hydrophilic groups to lipophilic groups (McClements 2005; Hait and Moulik 2001). The HLB value of an emulsifier is determined by not only the number but also the type of hydrophilic and lipophilic groups it contains (McClements 2005). A commonly accepted method of calculating the HLB value of an emulsifier is shown below (McClements 2005):

$$HLB = 7 + \sum (\text{hydrophilic values}) - \sum (\text{lipophilic values}) \quad (1)$$

Group numbers used in the HLB calculation above (as hydrophilic or lipophilic values) have been experimentally determined and assigned to many hydrophilic and lipophilic groups (Table 2.5) (McClements 2005). These group numbers can be referenced from this table and then plugged into the above HLB equation to determine the HLB value of the emulsifier (McClements 2005). While using this method to compare the effectiveness of different emulsifiers and the stability of the emulsions they form has its limitations (e.g. the fact that it can't account for the innumerable conformations in which the chemical groups reside in relation to one another), it is non-etheless a useful tool.

The Ritthitham (2009) method to find out the HLB value is based on a scale of 20 and it is given by the formula below:

$$HLB = [(L/T) * 20] \quad (2)$$

In this equation, L is the atomic weight of the hydrophilic portion, while T is the molecular mass of the entire molecule (Ritthitham 2009). An HLB value of 0 would indicate a molecule which is entirely lipophilic in nature (Ritthitham 2009). The HLB value is useful in predicting the phase in which an emulsifier is most likely to remain: oil (HLB <10) or water (HLB >10) (Gündüz 2018; Needs 1976). Thus, this method goes one step further than McClements's (2005) in that it to accounts for the relative strength of hydrophilic groups (Gündüz 2018).

Extreme HLB values (below 3 or above 18) usually mean that an emulsifier will be less effective since they are less likely to stay at the water/oil interface (McClements 2005; Hait and Moulik 2001). In sum, the HLB calculation is useful in making general

predictions about how effective an emulsifier will be at maintaining a stable emulsion. However, there may be exceptions for which it cannot account, and thus experimental work is always recommended in selecting the optimum emulsifier for a given application.

Emulsion Destabilization by Flocculation

Flocculation is a predominant process in the destabilization of food emulsions (Tadros 2013; McClements 2005). It results when droplets begin to associate with each other while retaining integrity of the original droplets (Fig 2.19) (Barnard Health Care 2017). Flocculation may occur because of insufficient emulsifier (McClements 2005). Depending on the food system, it can be either beneficial or detrimental (Guezennec and others 2015; McClements 2005). An example of a disadvantage of flocculation includes the acceleration of the gravitational separation, which is harmful to shelf-life (Tadros 2013; McClements 2005). The average size of particles in emulsions in which flocculation is occurring usually increases over time (McClements 2005). In some cases, only a fraction of the droplets is flocculated while the rest remain separate in the emulsion, resulting in what is termed as bimodal particle size distribution (McClements 2005). If the droplets in an oil-in-water (O/W) emulsion are small enough, gravitational separation can be prevented using an emulsifier that forms a more dense and thick interfacial layer (Chiralt 2005; McClements 2005). This diminishes the difference in density between the liquid and the oil droplets (McClements 2005).

Emulsion Destabilization by Coalescence

Once flocculation occurs, another process may result in further association of oil droplets known as coalescence (Fig 2.19) (Barnard Health Care 2017; Tadros 2013;

Chiralt 2005; McClements 2005). This is the process by which two or more oil droplets combine together forming a one big droplet. In order for coalescence to occur, oil droplets must have some mobility so that they eventually interact with one another and combine together (Pichot 2010; Chiralt 2005; McClements 2005). Gradually the total interfacial area decreases. This causes the average droplet size to increase and may result in complete separation of the oil and water phases (Dreher and others 1999). The main mechanisms by which it occurs are Brownian motion, gravity, applied shear, turbulence, and the nature of the forces between droplet (colloidal and hydrodynamic forces) (McClements 2005). Understanding these mechanisms can aid in preventing undesired changes in emulsions which can result in extending the shelf-life of emulsified products (Tadros 2013).

Phase Separation in Emulsions

In most cases, liquid oil droplets have a lower density than the aqueous solution (McClements 2005; Srinivasan and others 2001). As a result, they may be being to migrate upward, which is called creaming (Fig 2.19) (Barnard Health Care 2017). Oil accumulates at the top while the aqueous portion remains at the bottom. Larger oil droplets tend to move upwards more quickly than smaller oil droplets (Tadros 2013; McClements 2005). When the oil droplets reach the top, they may push together and form a creaming layer (McClements 2005). The process of creaming is not dissimilar to coalescence and flocculation and similarly may eventually lead to a phase separation (Turbiscan CLASSIC; McClements 2005). Flocculations have the effect of increasing creaming velocity, and this is the case largely because of the increase in oil droplet size

which accelerates the degree of migration upwards (McClements 2005). Creaming is a critical process in many food systems since it may greatly affect the sensory acceptability of products, as well as shelf-life and safety (Turbiscan CLASSIC; Chiralt 2005).

Sedimentation, like creaming, occurs in part due to a difference in density of two immiscible liquids (Tadros 2013). Gravitational forces gradually lead to a migration of denser particles downward, and less dense particles upward (Fig 2.19) (Barnard Health Care 2017). This is generally a slow process, but can also occur more rapidly by the application of gravitational centrifugal forces (Turbiscan CLASSIC; McClements 2005). Attributes such as particle size, shape, density are all critical determinants of degree of sedimentation that will occur in a solution (McClements 2005).

References

Allerberger F, Wagner M. 2010. Listeriosis: A resurgent foodborne infection. Clinical Microbiology and Infection 16: 16–23.

Anandharamarishnan C. 2014. Techniques for nanoencapsulation of food ingredients. Springer New York Heidelberg Dordrecht London.

Andrews JM. 2001. Determination of minimum inhibitory concentrations. The Journal of Antimicrobial Chemotherapy 48: 5–16.

Barnard Health Care. 2017. Emulsion science in the food industry. Food Emulsions. https://www.barnardhealth.us/food-emulsions/emulsion-science-in-the-food-industry.html

Beckman Coulter. 2011. LS 13 320 Laser diffraction particle size analyzer instructions for use. Beckman Coulter Inc.

Bennett L, Sudharmarajan S, Silva KJD, Barnett JL, Johnson MA, Stockmann R, Smithers GW. 2009. Use of the Turbiscan for measuring foam stability properties of food ingredients. Application Note TURBISCAN® Classic.

Bennett SD, Walsh KA, Gould LH. 2013. Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*-United States, 1998-2008. Clinical Infectious Diseases 57: 425–433.

BiologyWise. 2018. The fascinating world of cell organelles and their functions. https://biologywise.com/organelles-their-functions

Birk GC. 2015. Formulation development of a highly concentrated suspension of Cilengitide. Villingen-Schwenningen.

Boyle J. 2008. Molecular biology of the cell, 5th edition by B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. Biochemistry and Molecular Biology Education 36: 317-318.

Branen AL, Davidson PM, Salminen S, Thorngate JH. 2005. Food additives. Marcel Dekker, Inc.

Brown JE, Lechtenberg E. 2006. Nutrition through the life cycle. Nelson Education, Ltd.

CDC. 2011. CDC Estimates of food borne illness in the United States. Centers for Disease Control and Prevention.

Charles Ross & Son Company. 2012. Improve emulsion stability through ultra-high shear mixing. Mixing Technology Insight #17. https://www.mixers.com/insights/mti_17.pdf.

Chatzopoulou M, Voulgaridou I, Papalas D, Vasiliou P, Tsiakalou M. 2015. Third case

of *Streptococcus suis* infection in Greece. Hindawi Publishing Corporation.

Chen Y, Nummer B, Walsh MK. 2014. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Letters in Applied Microbiology 58: 156–162.

Chiralt A. 2005. Food emulsions. Food Engineering–Vol. II 381–389. Oxford.

Damodaran S, Parkin KL, Fennema OR. 2007. Fennema's food chemistry. CRC Press.

Davidson PM, Sofos JN, Branen AL. 2005. Antimicrobials in food. CRC Press.

Devulapalle KS, Gómez de Segura A, Ferrer M, Alcalde M, Mooser G, Plou FJ. 2004. Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyltransferase activity. Carbohydrate Research 339: 1029–1034.

Donlan RM. 2002. Biofilms: Microbial life on surfaces. Emerging Infectious Diseases 8: 881–890.

Donlan RM, Costerton JW. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews 15: 167–193.

Dreher TM, Glass J, O'Connor AJ, Stevens GW. 1999. Effect of rheology on coalescence rates and emulsion stability. AIChE Journal 45: 1182–1190.

Emery PHARMA. 2018. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assay. Microbiology and Cell Biology, Medicinal Chemistry–Emery Pharma.

Feng Y, Zhang H, Wu Z, Wang S, Cao M, Hu D, Wang C. 2014. *Streptococcus suis* infection. Virulence 5: 477–497.

Ferens WA, Hovde CJ. 2011. *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. Foodborne Pathogens and Disease 8: 465–487.

Ferrer M, Soliveri J, Plou FJ, Lopez-Cortez NL, Reyes-Duarte DR, Christensen M, Copa-Patino JL, Ballestros A. 2005. Synthesis of sugar esters in solvent mixtures bylipases from *Thermomyces lanuginosus* and *Candida antarctica B*, and their antimicrobial properties. Enzyme and Microbial Technology 36: 391–398.

Formulaction. 2009. Stability of various beverage emulsions. Food Application Paper 1-7. http://www.titanex.com.tw/doc/tecsupport/ANF-Turbiscan-application%20paper%20on%20%20beverage%20emulsions.pdf

Forssten SD, Björklund M, Ouwehand AC. 2010. *Streptococcus mutans*, caries and simulation models. Nutrients 2: 290–298.

Garg N, Martini S, Britt DW, Walsh MK. 2010. Emulsifying properties of lactose-amines in oil-in-water emulsions. Food Research International 43: 1111–1115.

Garrett TR, Bhakoo M, Zhang Z. 2008. Bacterial adhesion and biofilms on surfaces. Progress in Natural Science 18: 1049–1056.

Gu H. 2014. Patterned biofilm formation to investigate bacteria-surface interactions. Syracuse University.

Guezennec A-G, Michel C, Bru K, Touzé S, Desroche N, Mnif I, Motelica-Heino M. 2015. Transfer and degradation of polyacrylamide based flocculants in hydrosystems: A review. Environmental Science and Pollution Research, Springer Verlag. 22: 6390-6406.

Gumus C. 2017. Utilization of natural emulsifiers and their derivatives to formulate emulsion-based delivery systems for hydrophobic nutraceuticals. University of Massachusetts Amherst.

Gündüz G. 2018. Chemistry, materials, and properties of surface coatings: Traditional

and evolving technologies. Middle East Technical University.

Habulin M, Šabeder S, Knez Ž. 2008. Enzymatic synthesis of sugar fatty acid esters in organic solvent and in supercritical carbon dioxide and their antimicrobial activity. The Journal of Supercritical Fluids 45: 338–345.

Hait SK, Moulik SP. 2001. Determination of critical micelle concentration (CMC) of nonionic surfactants by donor-acceptor interaction with iodine and correlation of CMC with hydrophile-lipophile balance and other parameters of the surfactants. Journal of Surfactants and Detergents 4: 303–309.

Harisha S. 2006. Introduction to practical biotechnology: A hand book on practical biotechnology. Laxmi Publication. https://books.google.com/books/about/An_Introduction_to_Practical_Biotechnolo.html?id=YqFMQn5UtGQC.

Hoagland DT, Liu J, Lee RB, Lee RE. 2016. New agents for the treatment of drug-resistant *Mycobacterium tuberculosis*. Advanced Drug Delivery Reviews 102: 55–72.

Hoiczyk E, Hansel A. 2000. Cyanobacterial cell walls: News from an unusual prokaryotic Envelope. Journal of Bacteriology 182: 1191–1199.

HORIBA. 2012. A guidebook to particle size analysis. HORIBA Instruments, Inc

Jafari SM, He Y, Bhandari B. 2007. Production of sub-micron emulsions by ultrasound and microfluidization techniques. Journal of Food Engineering 82: 478–488.

Jafari SM, McClements DJ. 2018. Nanoemulsions: Formulation, applications, and characterization. Elsevier Inc.

Jesse L, Lynn Jr. 2009. Detergency and detergents. Kirk-Othmer Encyclopedia of

Chemical Technology

Jouki M, Mortazavi SA, Yazdi FT, Koocheki A. 2014. Characterization of antioxidant–antibacterial quince seed mucilage films containing thyme essential oil. Carbohydrate Polymers 99: 537–546.

Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AEL, Irie Y, Ø Jensen P, Diggle SP, Allen RJ, Gordon V, Bjarnsholt T. 2016. Role of multicellular aggregates in biofilm formation. mBio 7: e00237.

Kralova I, Sjöblom J. 2009. Surfactants used in food industry: A review. Journal of Dispersion Science and Technology 30: 1363–1383.

Karki G. 2017. Bacterial cell wall: Structure, composition and types. Online Biology Notes.

Kumar S. 2012. Textbook of microbiology. Jaypee Brothers Medical Publishers (P) Ltd.

Laboratory-Equipment.com. 2018. T 25 digital ULTRA-TURRAX® Disperser by IKA. Terra Universal. Inc.

Lee S-M, Sandhu G, Walsh MK. 2017. Growth inhibitory properties of lactose fatty acid esters. Saudi Journal of Biological Sciences 24: 1483–1488.

Li L. 2012. *In vitro* gastrointestinal digestion of oil-in-water emulsions. Massey University.

Lim JY, Yoon J, Hovde CJ. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. Journal of Microbiology and Biotechnology 20: 5–14.

Limoli DH, Jones CJ, Wozniak DJ. 2015. Bacterial extracellular polysaccharides in biofilm formation and function. Microbiology Spectrum 3: MB-0011-2014.

Malvern. 2015. A basic guide to particle characterization. Malvern Instruments Limited.

McClements DJ. 2005. Food emulsions: Principles, practices, and techniques. CRC Press.

McClements DJ. 2007. Critical review of techniques and methodologies for characterization of emulsion stability. Critical Reviews in Food Science and Nutrition 47: 611-649.

Microfluidics. 2010. Webinar series principles of particle principles of particle size reduction and size reduction and characterization. Ian Gunniss Microfluidics Research Engineer.

Miyamoto T, Kawaguchi J, Shimotsu S, Kawagishi J, Honjoh K-I. 2009. Inhibitors of adhesion ability of *Salmonella enteritidis*. Nippon Shokuhin Kagaku Kogaku Kaishi 56: 200–208.

Moghaddam T, Moghadam HZE, Khademi G, Bahreini A, Saeidi M. 2016. Tuberculosis: Past, present and future. Mashhad University of Medical Sciences 4: 1243–1254.

Montana State University. 2017. Biofilm basics: Section 1. Montana State University Center for Biofilm Engineering.

Moore SL. 1997. The mechanisms of antibacterial action of some nonionic surfactants. The University of Brighton in collaboration with Unilever Research, Port Sunlight.

Needs A. 1976. The HLB system a time-saving guide to emulsifier selection. ICI Americas Inc.

Nobmann P, Smith A, Dunne J, Henehan G, Bourke P. 2009. The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. International Journal of

Food Microbiology 128: 440–445.

Nobmann P, Bourke P, Dunne J, Henehan G. 2010. *In vitro* antimicrobial activity and mechanism of action of novel carbohydrate fatty acid derivatives against *Staphylococcus aureus* and MRSA. Journal of Applied Microbiology 108: 2152-2161.

Pan X, Nakano H. 2014. Effects of chlorine-based antimicrobial treatments on the microbiological qualities of selected leafy vegetables and wash water. Food Science and Technology Research 20: 765–774.

Pesavento G, Ducci B, Nieri D, Comodo N, Lo Nostro A. 2010. Prevalence and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. Food Control 21: 708–713.

Piao J, Adachi S .2006. Stability of O/W emulsions prepared using various monoacyl sugar alcohols as an emulsifier. Innovative Food Science & Emerging Technologies 7: 211–216.

Piao J, Kawahara-Aoyama Y, Inoue T, Adachi S. 2006. Bacteriostatic activities of monoacyl sugar alcohols against *Thermophilic sporeformers*. Bioscience, Biotechnology, and Biochemistry 70: 263-265.

Pichot R. 2010. Stability and characterisation of emulsions in the presence of colloidal particles and surfactants. University of Birmingham

Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP. 1994. Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other *Staphylococcal* exoproteins by interfering with signal transduction. Journal of Bacteriology 176: 4204–4209.

Ritthitham S. 2009. Synthesis of sucrose fatty acid esters as catalyzed by alkaline protease AL 89 and *Candida antarctica* lipase B in hydrophilic solvents. Aalborg University.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States–Major pathogens. Emerging Infectious Diseases 17: 7–15.

Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology 2: a000414.

Silva ECDF, Montalvão CR, Bonafé S. 2017. Infectious endocarditis from *Enterococcus faecalis* associated with tubular adenoma of the sigmoid colon. Case Reports in Infectious Diseases. Hindawi: 1–4.

Smith A, Nobmann P, Henehan G, Bourke P, Dunne J. 2008. Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives. Carbohydrate Research 343: 2557–2566.

Solomon E, Martin C, Martin DW, Berg LR. 2014. Biology. Cengage Learning.

Spellman FR, Drinan JE. 2012. The drinking water handbook. CRC Press.

Srinivasan M, Singh H, Munro PA. 2001. Creaming stability of oil-in-water emulsions formed with sodium and calcium caseinates. Journal of Food Science 66: 441–446.

Staroń J, Dabrowski JM, Cichoń E, Guzik M. 2018. Lactose esters: Synthesis and biotechnological applications. Critical Reviews Biotechnology 38: 245–258.

Szűts A, Szabó-Révész P. 2012. Sucrose esters as natural surfactants in drug delivery systems—A mini-review. International Journal of Pharmaceutics 433: 1–9.

Tadros TF. 2013. Emulsion formation and stability. Wiley-VCH Verlag GmbH & Co.

KGaA.

Thomas LV, Davies EA, Delves-Broughton J, Wimpenny JWT. 1998. Synergist effect of sucrose fatty acid esters on nisin inhibition of Gram-positive bacteria. Journal of Applied Microbiology 85: 1013–1022.

Turbiscan CLASSIC. User Guide ©Formulaction. Formulaction Smart Scientific Analysis.

van Os NM. 2010. Nonionic surfactants: Organic chemistry–Surfactant science series Volume 72. CRC Press.

Villapún VM, Dover LG, Cross A, González S. 2016. Antibacterial metallic touch surfaces. Materials 9: 736.

Wabel CT. 1998. Influence of lecithin on structure and stability of parenteral fat emulsions. Erlangen.

Wagh A, Shen S, Shen FA, Miller CD, Walsh MK. 2012. Effect of lactose monolaurate on pathogenic and nonpathogenic bacteria. Applied and Environmental Microbiology. 78: 3465–3468.

Walsh MK, Bombyk RA, Wagh A, Bingham A, Berreau LM. 2009. Synthesis of lactose monolaurate as influenced by various lipases and solvents. Journal of Molecular Catalysis B: Enzymatic 60: 171–177.

Watanabe T, Katayama S, Matsubara M, Honda Y, Kuwahara M. 2000. Antibacterial carbohydrate monoesters suppressing cell growth of S*treptococcus mutans* in the presence of sucrose. Current Microbiology 41: 210–213.

Xiao D, Ye R, Davidson PM, Hayes DG, Golden DA, Zhong Q. 2011. Sucrose monolaurate improves the efficacy of sodium hypochlorite against *Escherichia coli*

O157:H7 on spinach. International Journal of Food Microbiology 145: 64–68.

Yanniotis S, Taoukis P, Stoforos NG, Karathanos VT. 2013. Advances in food process engineering research and applications. Springer-Verlag New York Inc.

Ye F. 2007. Porous polymeric materials derived from bicontinuous microemulsions for drug delivery. University of Akron.

Zaika LL, Fanelli JS. 2003. Growth kinetics and cell morphology of *Listeria monocytogenes* scott a as affected by temperature, NaCl, and EDTA. Journal of Food Protection 66: 1208–1215.

Zhang H, Liu Y, Wu C, Wang D, Deng W, Song J, Zhao G, Jiang Y. 2015. Inhibitory effects of sucrose fatty acid esters on biofilm formation by food-borne *Staphylococcus aureus*. Research & Reviews: Journal of Microbiology and Biotechnology 4: 57–61.

Zhang X, Song F, Taxipalati M, Wei W, Feng F. 2014. Comparative study of surface-active properties and antimicrobial activities of disaccharide monoesters. PLoS ONE 9: e114845.

Zhang X, Wei W, Cao X, Feng F. 2014. Characterization of enzymatically prepared sugar medium-chain fatty acid monoesters. Journal of the Science of Food and Agriculture 95: 1631-1637.

Table 2.1. Ranking of rates of hospitalization for representative food-borne pathogens identified in the hazard identification (Scallan and others 2011)

Agent	Hospitalization Rate (Percent)
L. monocytogenes	94
C. botulinum	82.6
E. coli O157:H7	46.2
Hepatitis A virus	31.5
Salmonella (non-typhoidal)	27.2
Cryptosporidium	25
S. aureus	6.4
B. cereus	0.4
Norovirus	0.03

Table 2.2. Ranking of rates of death for representative food-borne pathogens identified in the hazard identification (Scallan and others 2011)

Agent	Death Rate (Percent)
C. botulinum	17.3
L. monocytogenes	15.9
Hepatitis A virus	2.4
E. coli O157:H7	0.5
Salmonella (non-typhoidal)	0.5
Cryptosporidium	0.3
S. aureus	<0.1
Norovirus	<0.1
B. cereus	0

Table 2.3. Ranking of illness for representative food-borne pathogens identified in the hazard identification (Scallan and others 2011)

Agent	Frequency of Illness (Mean Number of Annual Episodes)
Norovirus	5,461,731
Salmonella (non-typhoidal)	1,027,561
S. aureus	241,148
B. cereus	63,400
E. coli O157:H7	63,153
Cryptosporidium	57,616
L. monocytogenes	1,591
Hepatitis A virus	1,566
C. botulinum	55

Table 2.4. Comparison of the attributes of different types of homogenizers (McClements 2005)

Homogenizer Type	Throughput	Relative Energy Efficiency	Minimum Droplet Size	Simple Viscosity
High-speed mixer	Batch or continuous	Low	2 μm	Low to medium
Colloid mill	Continuous	Intermediate	1 μm	Medium to high
High-pressure homogenizer	Continuous	High	0.1 μm	Low to medium
Ultrasonic probe	Batch or continuous	Low	0.1 μm	Low to medium
Ultrasonic jet homogenizer	Continuous	High	1 μm	Low to medium
Microfluidization	Continuous	High	<0.1 μm	Low to medium
Membrane processing	Batch or continuous	Very high	0.3 μm	Low to medium

Table 2.5. Selected HLB group numbers (McClements 2005)

Hydrophilic Group	Group number	Lipophilic Group	Group Number
$-SO_4^- Na^+$	38.7	-CH-	0.475
$-COO^- H^+$	21.2	$-CH_2-$	0.475
Tertiary amine	9.4	$-CH_3$	0.475
Sorbitan ester	6.8	-CH=	0.475
Glyceryl ester	5.25		
-COOH	2.1		
-OH	1.9		
$-O^-$	1.3		
$-(CH_2-CH_2-O)-$	0.33		

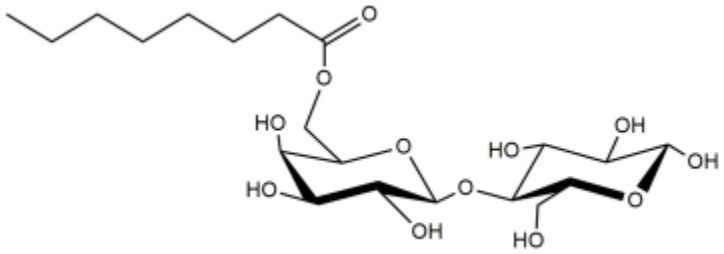

Figure 2.1. Representative structure of lactose monooctanoate (LMO)

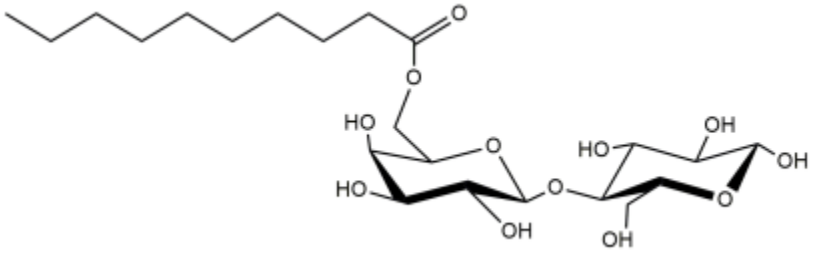

Figure 2.2. Representative structure of lactose monodecanoate (LMD)

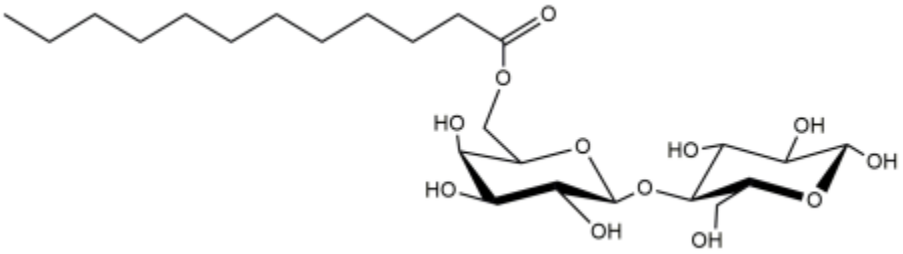

Figure 2.3. Representative structure of lactose monolaurate (LML)

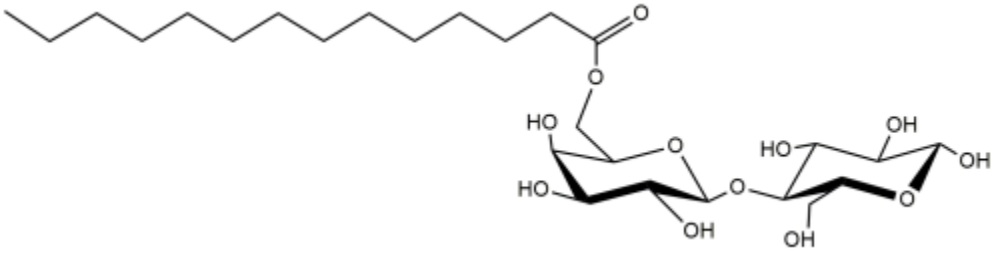

Figure 2.4. Representative structure of lactose monomyristate (LMM)

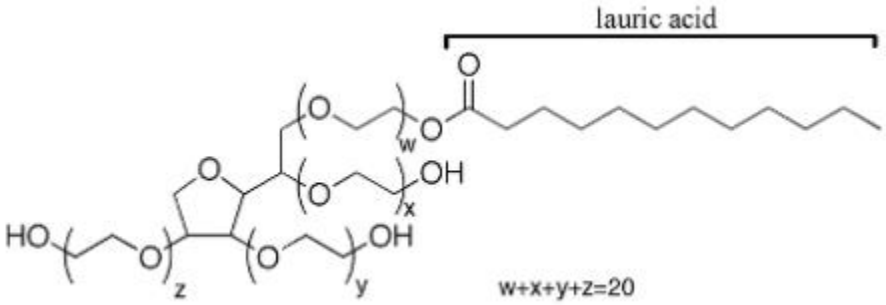

Figure 2.5. Representative structure of Tween-20 (Jafari and McClements 2018)

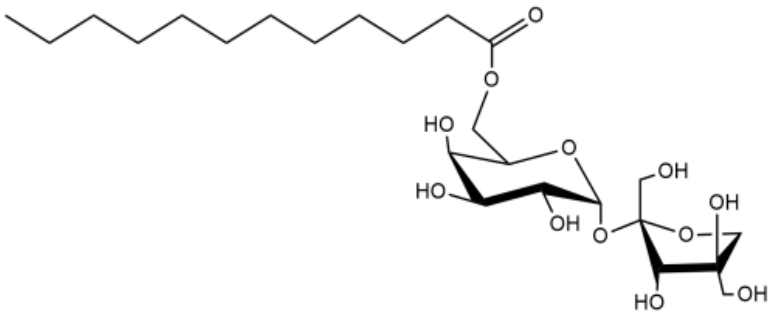

Figure 2.6. Representative structure of Ryoto L-1695

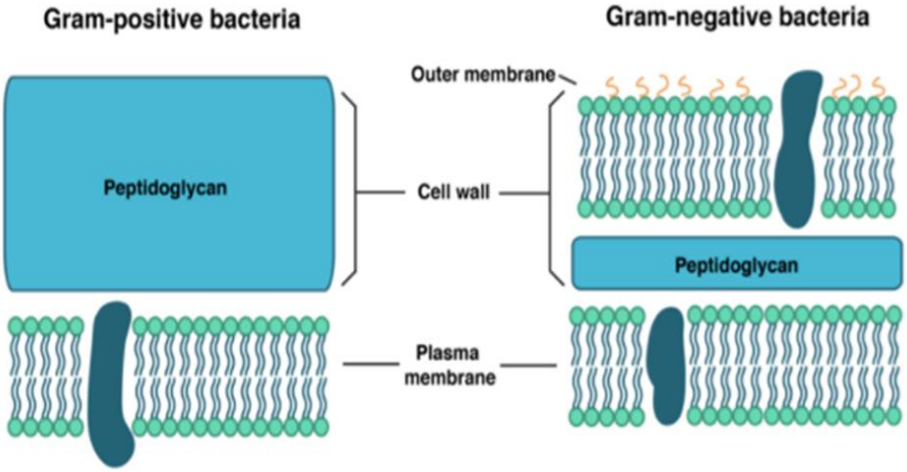

Figure 2.7. Comparison of bacterial cell wall structure (Karki 2017)

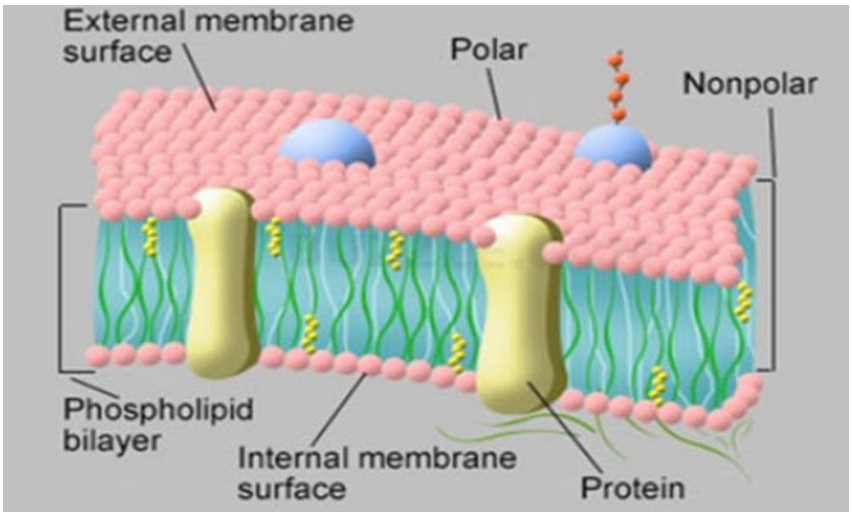

Figure 2.8. Structure of the cell membrane (BiologyWise 2018)

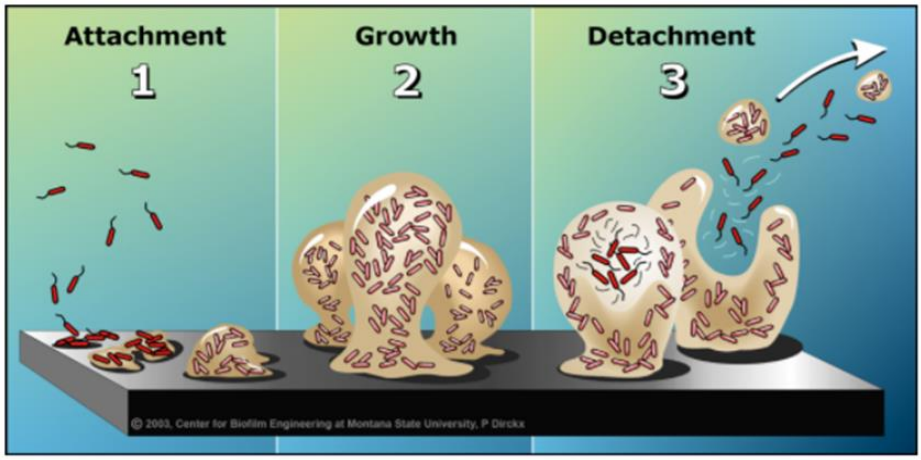

Figure 2.9. Introduction to the biofilm life cycle (Montana State University 2017)

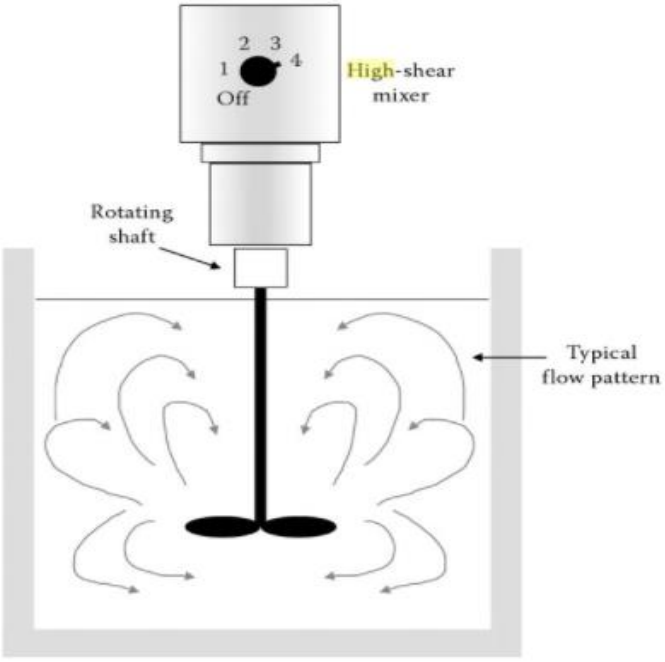

Figure 2.10. General diagram of a high-speed mixer used to create a homogenous emulsion out of initially separate oil and water phases (McClements 2005).

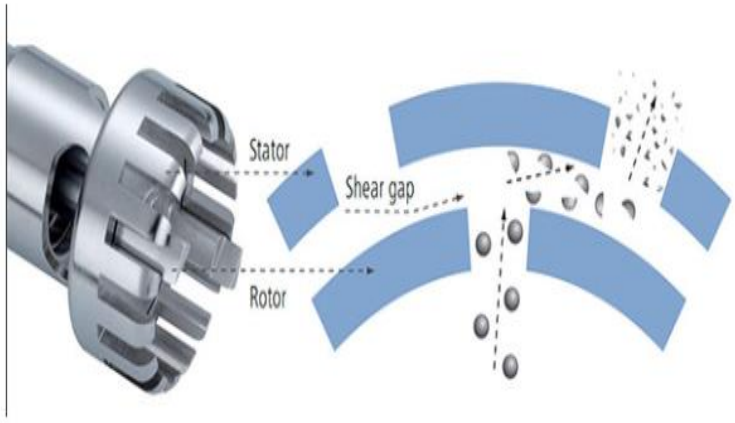

Figure 2.11. Diagram of high-speed mixer head operating under rotor-stator principle (Laboratory-Equipment.com 2010)

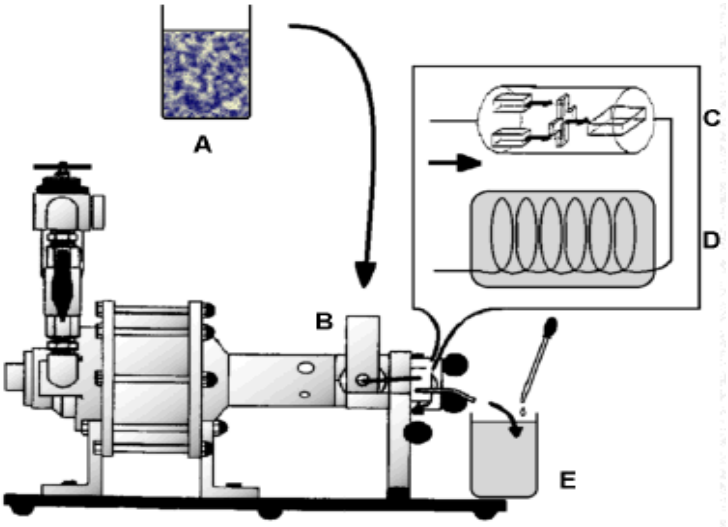

Figure 2.12. Schematic drawing of the homogenization process using the microfluidizer. The crude dispersions (A) were filled into the reservoir (B) and cycled through the dissipation zone (C), cooled or heated, respectively, by passing the heat exchange coil (D) and then collected at the outlet (E) to be refilled in the reservoir and recycled (Wabel 1998)

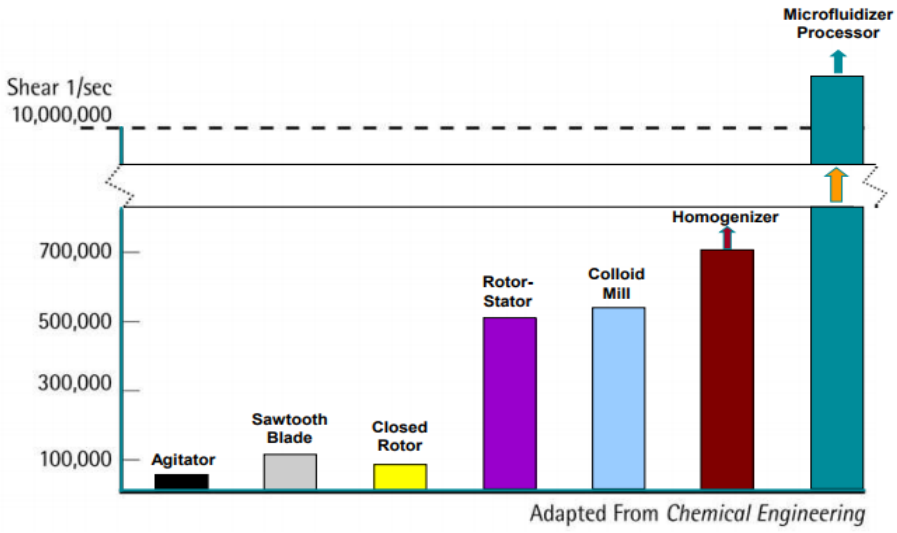

Figure 2.13. Shear rates for various technologies (Microfluidics 2010)

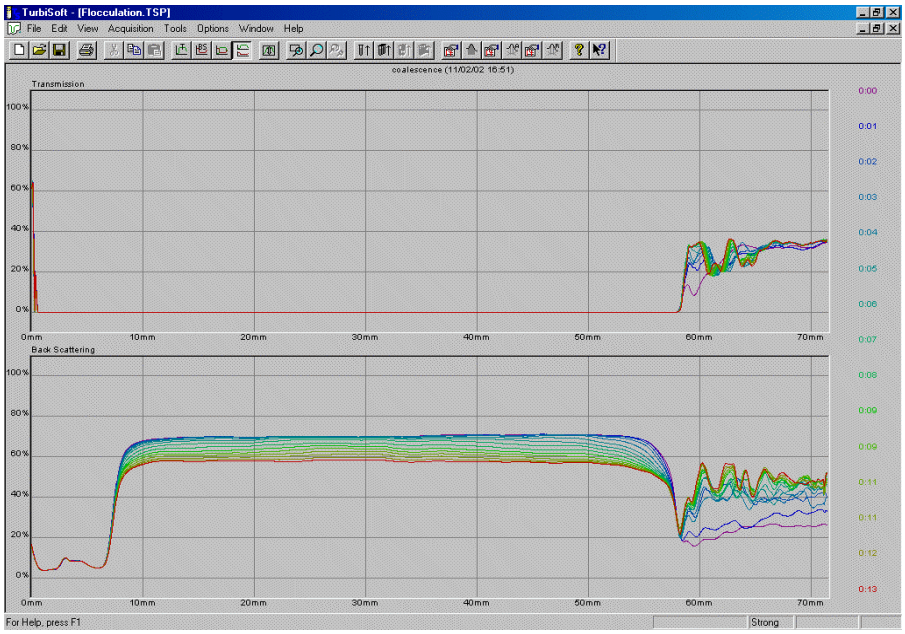

Figure 2.14. Display of backscattering and transmission of photons. The top graph shows transmission of photons while the bottom one shows backscattering over the length of the tube as a function of time in the emulsion including lactose monolaurate (Turbiscan CLASSIC)

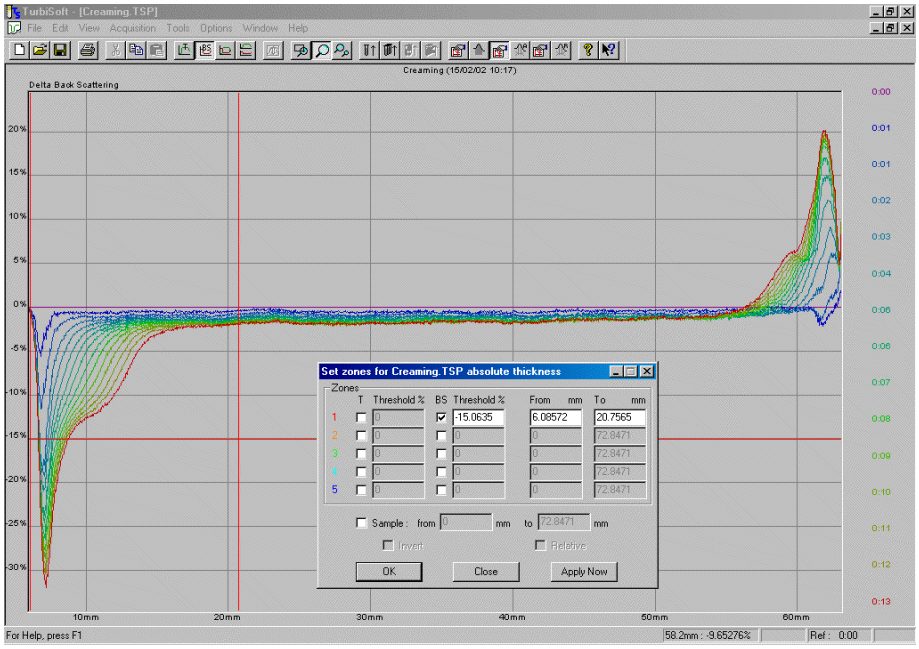

Figure 2.15. Turbiscan reading showing ever increasing depth and height of peaks, representing destabilization of an emulsion and the formation of a clarification layer, shown by the downward peaks, and a creaming layer, shown by the upward peaks (Turbiscan CLASSIC)

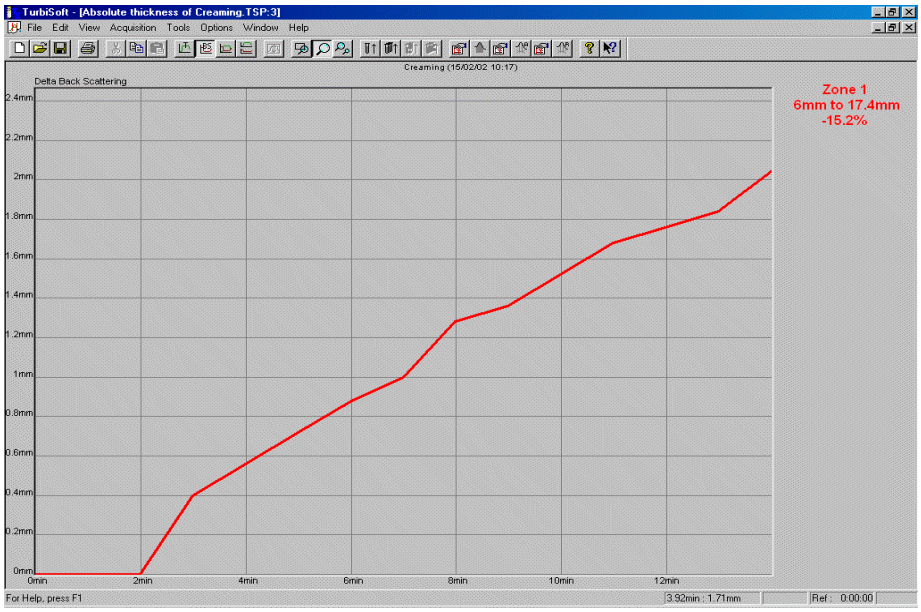

Figure 2.16. Migration velocity of oil droplets in an emulsion as shown by increase in clarification layer thickness over time (Turbiscan CLASSIC)

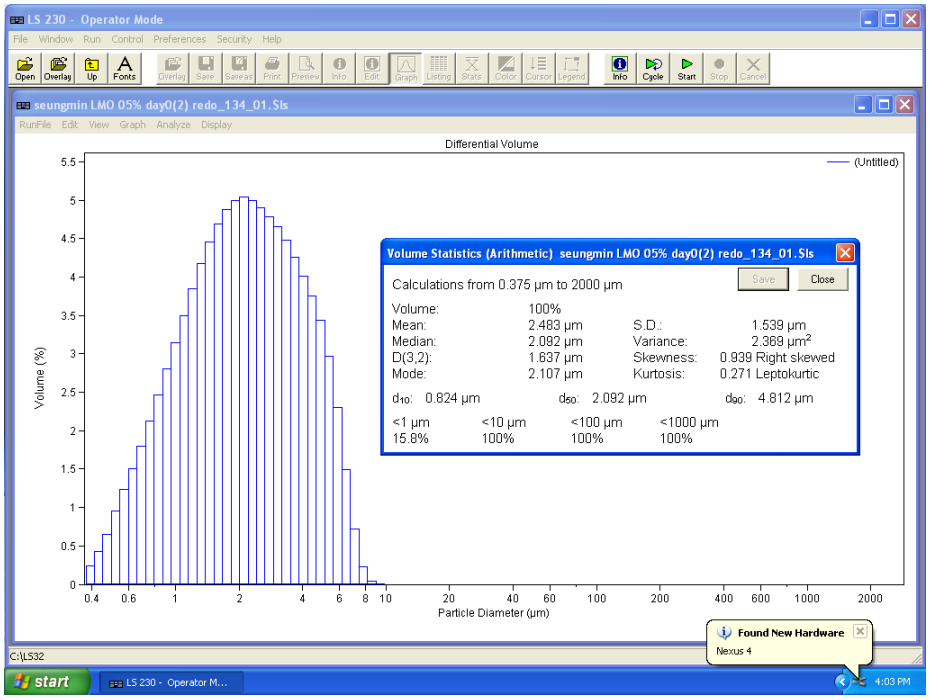

Figure 2.17. Example of volume (%) distribution against oil droplet diameter (µm) of an emulsion stabilized by lactose monooctanoate (LMO)

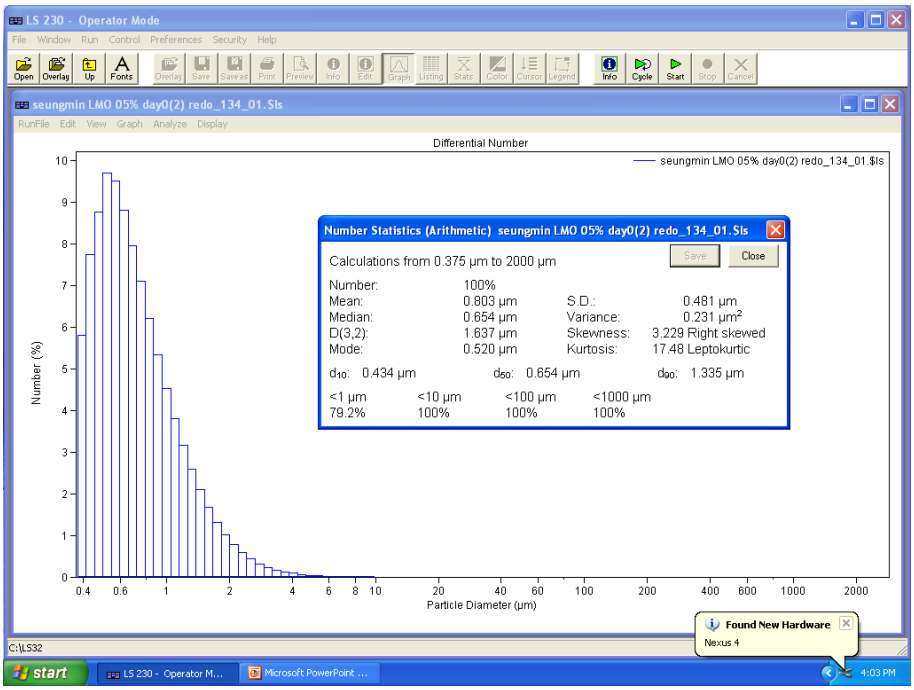

Figure 2.18. Example of number (%) distribution against oil droplet diameter (µm) of an emulsion stabilized by lactose monooctanoate (LMO)

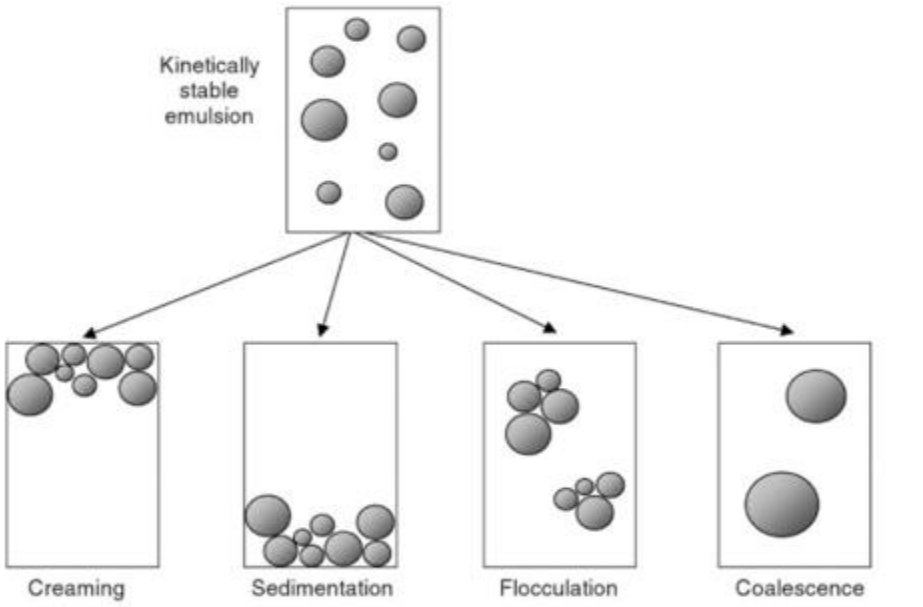

Figure 2.19. Food emulsions may become unstable through a variety of physical mechanisms, including creaming, sedimentation, flocculation, coalescence, and phase inversion (Barnard Health Care 2017).

CHAPTER 3[1]

GROWTH INHIBITORY PROPERTIES OF LACTOSE FATTY ACID ESTERS

Abstract

Sugar esters are biodegradable, non-ionic emulsifiers which have microbial inhibitory properties. The influence of the fatty acid chain length on the microbial inhibitory properties of lactose esters was investigated in this study. Specifically, lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM) were synthesized and dissolved in both dimethyl sulfoxide (DMSO) and ethanol. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined in growth media. LML was the most effective ester, exhibiting MIC values of ≤0.05 to ≤5 mg/ml for each Gram-positive bacteria tested (*Bacillus cereus*, *Mycobacterium* KMS, *Streptococcus suis*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Streptococcus mutans*) and MBC values of ≤3 to ≤5 mg/ml for *B. cereus*, M. KMS, *S. suis*, and *L. monocytogenes*. LMD showed MIC and MBC values of ≤1 to ≤5 mg/ml for *B. cereus*, M. KMS, *S. suis*, *L. monocytogenes*, and *E. faecalis*, with greater inhibition when dissolved in ethanol. LMM showed MIC and MBC values of ≤1 to ≤5 mg/ml for *B. cereus*, M. KMS, and *S. suis*.

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[1] Saudi Journal of Biological Science. Growth Inhibitory Properties of Lactose Fatty Acid Esters. Volume 24, Issue 7, November 2017, Pages 1483-1488. Seung-Min Lee, Guneev Sandhu, Marie K. Walsh. (original copyright notice as given in the publication in which the material was originally published) “With permission of Elsevier”.

LMO was the least effective showing a MBC value of ≤5 mg/ml for only *B. cereus*, though MIC values for *S. suis* and *L. monocytogenes* was observed when dissolved in DMSO. *B. cereus* and *S. suis* were the most susceptible to the lactose esters tested, while *S. mutans* and *E. faecalis* were the most resilient and no esters were effective on *Escherichia coli* O157:H7. This research showed that lactose esters esterified with decanoic and lauric acids exhibited greater microbial inhibitory properties than lactose esters of octanoate and myristate against Gram-positive bacteria.

Introduction

Sugar esters are non-ionic emulsifiers used in a variety of applications in the food, pharmaceutical, and personal care industries. The microbial inhibitory activity of sugar esters has been studied. Although it has been shown that sugar esters inhibit bacterial growth, there is a lack of consensus as to which bacteria are most susceptible. While some studies have shown inhibitory effects of Gram-negative bacteria (Zhang and others 2014; Smith and others 2008; Habulin and others 2008; Ferrer and others 2005), others have shown inhibition of only Gram-positive bacteria (Wagh and others 2012; Piao and others 2006). Studies have shown that esters containing laurate were inhibitory against both Gram-positive and Gram-negative bacteria (Zhang and others 2014; Nobmann and others 2009; Smith and others 2008). A study on the microbial inhibitory activity of lactose monolaurate showed low minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for *Listeria monocytogenes* and *Mycobacterium* sp. strain KMS, and no inhibitory activity against *Escherichia coli* or *Salmonella* (Wagh and others 2012).

The antimicrobial activity of sugar esters is related to the fatty acid chain length. Medium chain fatty acids appear to exhibit the strongest antimicrobial properties. Previous research showed that fatty acid derivatives such as monolaurin are highly inhibitory and more inhibitory than lauric acid (Nobmann and others 2009; Smith and others 2008). Others have reported that sugar monoesters of decanoic, myristic and palmitic acids were microbial inhibitory (Zhang and others 2014; Habulin and others 2008; Piao and others 2006). There was one study investigating the microbial inhibition of sugar octanoate esters which showed no inhibitory effects (Zhang and others 2014).

Of the carbohydrate fatty acid esters previously investigated, sucrose esters have been the most thoroughly studied (Nobmann and others 2009). Other oligosaccharide esters of laurate, including maltose, fructose and galactose have been synthesized and have generally been shown to be very effective microbial inhibitory agents (Nobmann and others 2009; Habulin and others 2008; Devulapalle and others 2004; Watanabe and others 2000), whereas hexose laurate did not suppress microbial growth significantly (Watanabe and others 2000).

While many studies examine the microbial inhibition of sugar esters in terms of MIC values, few studies have determined the MBC values of sugar esters. In this study the microbial inhibitory properties of lactose esters (MIC and MBC) in microbial growth media were determined against Gram-positive (*Bacillus cereus, Mycobacterium* KMS, *Streptococcus suis, L. monocytogenes, Enterococcus faecalis* and *Streptococcus mutans*) and the Gram-negative bacteria, *Escherichia coli* O157:H7. Furthermore, we also determined MIC and MBC values of the esters dissolved in two solvents, DMSO and

ethanol. This allowed us to ascertain the role of the solvents in the microbial inhibitory activity.

Materials and Methods

Bacterial Strains

Bacterial strains used are listed in Table 3.1. *Enterococcus faecalis* V538 and *Listeria monocytogenes* EGDe were received from Dr. Andy Benson of the University of Nebraska, Lincoln. Different clinical isolates of *Listeria* (FSL J1-177, FSL N3-013, FSL R2-499 and FSL N1-227) were obtained from Dr. Martin Wiedmann, director of the international Life Sciences Institute North American Database at Cornell University. *Streptococcus suis* 89/1591 was received from Dr. Richard Higgins of University of Montreal, Qubec, Canada. M. KMS was isolated by Utah State University from treatment soils in Champion International Superfund Site, Libby, Montana. *Bacillus cereus* ATCC 13061, *Streptococcus mutans* ATCC 25175 and *Escherichia coli* O157:H7 EDL 931 stains were obtained from ATCC (Manassas, VA).

Materials and Equipments

A high-performance liquid chromatography (HPLC) (Beckman System Gold 125 Solvent Module, Ontario, Canada) equipped with Luna 5 lm C18 100 A ° (250 mm X 4.6 mm, Phenomenex, Torrance, CA, USA), an evaporative light scattering detector (Agilent Technologies, Santa Clara, CA, USA), incubator shaker (Beckman, USA), spectrophotometer (Beckman, Portland, OR, USA) and Ultra-turrax T25 (Janke and Kunkel, Staufen, Germany) were provided by Utah State University. Lactose (Proliant, IA, USA), 48 microtiter well plates (Becton Dickinson, NJ, USA) and acetonitrile (HPLC

grade, Thermo Fisher, PA, USA) were also prepared. Brain heart infusion (BHI) media, Luria–Bertani (LB) media, lipase TM3 (immobilized from *Thermomyces lanuginosus*), Whatman glass microfiber filters, molecular sieves (3A), 2-methyl-2-butanol (2M2B) (dried using 10% 3A molecular sieves), dimethyl sulfoxide (DMSO), Tween-80 and 1, 5 ml macro cuvettes were supplied by Sigma (Aldrich, MO, USA). Vinyl octanoate, vinyl decanoate, vinyl laurate and vinyl myristate were from TCI (Portland, OR, USA).

Lactose Esters Synthesis and Purification

Enzymatic synthesis of LML was performed according to Walsh and others (2009). Synthesis of LMO was conducted using lactose, vinyl octanoate, molecular sieves and immobilized lipase enzyme TM3. For a 60 ml reaction in 2M2B, 3 g of lactose, 6 g of dried molecular sieves, 1.7 ml of vinyl octanoate (lactose to fatty acid ratio of 1:2.1) and 1.8 g TM3 were combined. The reactions were assembled in a 100 ml glass bottle and incubated at 60 ℃ and 90 rpm for 2 days. The amount of LMO synthesized was determined using HPLC with the evaporative light scattering detector set at 60 ℃ with a nitrogen gas pressure of 3.55 bar. There was a gradient from 10% acetonitrile–water (40:60, v/v) to 100% acetonitrile–water (95:5, v/v) as the mobile phase. Synthesis of LMM and LMD was done as described above for LMO using the different molar ratios of lactose to fatty acid; vinyl myristate (lactose to fatty acid ratio of 1:2.14) for LMM and vinyl decanoate (lactose to fatty acid ratio of 1:1) for LMD.

For ester purification, the 2M2B reaction was filtered through a Whatman glass microfiber filter then dried in a hood for 48 hrs. The dry solids of LML and LMM were suspended in a 50% hexane–water while the dry solids of LMO and LMD were

suspended in a 50% ethanol–water and placed in a separatory funnel. This was done due to the differences in hydrophobic/hydrophilic properties of LML and LMM versus LMO and LMD. Hexane, being a non-polar solvent, was used with the products with the greatest hydrophobic potentials; and ethanol, being polar, was used with those possessing shorter fatty acid chains, thus with less hydrophobicity. The lower aqueous layer was drained into a beaker and dried in a hood for 48 hrs. After completely drying, the product powder was suspended in hexane, and then centrifuged for 15 min at room temperature at 2000 x g and the supernatant analyzed via HPLC for the presence of di- tri- or higher saccharides (see appendix A). The hexane or ethanol extraction was repeated until only the monoester was present in the pellet. All purified lactose esters were dry powder types (see appendix A).

Microbial Inhibitory Studies

Stock solutions of LMO (60 mg/ml) and LMD (25 mg/ml) were prepared in 30% ethanol–water. Stock solutions of LML (60 mg/ml) were prepared in 50% ethanol–water and 100% DMSO. Stock solutions of LMO and LMD (60 mg/ml) were prepared in 100% DMSO. LMM was not soluble in 60% ethanol–water hence a stock (60 mg/ml) was prepared in 100% DMSO. Controls were 30% ethanol–water, 50% ethanol–water and 100% DMSO. The activity of esters was tested in nutrient agar plates in terms of the growth of the microorganism. Ester stock solutions were added into growth media to give final ethanol concentrations ranging from 0.5% to 10% and final DMSO concentrations ranging from 2% to 8%. All seven stocks of esters and controls were tested on the bacteria listed in Table 3.2.

Analysis of microbial inhibitory activities of LMO was performed by making a 5-strain cocktail of *L. monocytogenes* including C1-056, J1-177, N1-277, N3-013, and R2-499. The individual 5 stocks were stored at -80 ℃, and each individual freezer stock (20 μl) was added to 15 ml of BHI media. The *Listeria* strains were grown at 37 ℃ and 200 rpm for 24 hrs. Aliquots (2 ml) from each strain were combined in a test tube to develop the 5-strain stock cocktail. Aliquots, 315 ll, of the stock cocktail were grown in BHI media (12 ml) and incubated with shaking at 37 ℃ for 4 hrs. Aliquots of the 5-strain stock cocktail were kept at -80 ℃. Stock solutions of the other bacteria were maintained at -80 ℃. Aliquots of bacterial stock solutions (300 μl) were grown in 15 ml media at 37 ℃, 200 rpm for 24 hrs. Aliquots of the overnight growths (300 μl) were added to 12 ml media and grown again at 37 ℃, 200 rpm for 4 hrs before use. The growing cultures were monitored by optical density measurements at 660 nm (OD600) and diluted with fresh media to reach an OD600 of 0.2 which was approximately 1 x 10^8 cfu/ml. An aliquot of the culture, 100 μl, was mixed with 10 ml fresh media containing 0.1% Tween-80.

The ester stock ester solutions were added to each well for final concentrations of 0.05, 0.1, 0.5, 1, 3, and/or 5 mg/ml and each well contained a total of 0.5 ml. Controls contained the same concentration of ethanol or DMSO as the treatments. Each treatment and control were performed in triplicate and replicated three times. A paired T-test was used to compare the treatments with the controls at each concentration to determine if the treatments were significantly different from the controls. All controls and treatments were plated on appropriate agar and incubated at 37 ℃ for 24 hrs to obtain plate counts. The MIC of each compound was determined as the lowest concentration which showed a

significant difference in the number of cells in treatments as compared to those in controls as determined by plate counts. Similarly, the MBC of each compound for each organism was reported as the minimum concentration of ester at which there was no cell growth as determined by plate counts.

Results

Minimum Inhibitory Concentrations (MIC) of Lactose Esters

In our earlier work, we showed that the novel lactose ester, LML (in 50% ethanol–water) was antimicrobial towards *L. monocytogenes* and M. KMS, but had no activity against Gram-negative bacteria (Wagh and others 2012). In this study, additional lactose esters, LMO, LMD, and LMM were synthesized, and along with LML, were dissolved in both ethanol and DMSO, and tested for microbial inhibitory activity against Gram-positive bacteria and *E. coli* O157:H7. The control samples contained the same concentration of solvent as the treatments. MIC values of the lactose esters against various Gram-positive bacteria are listed in Table 3.3. LML was found to be the most effective microbial inhibitory ester since it showed MIC values (≤0.05 to ≤5 mg/ml) for each Gram-positive bacteria tested in each solvent. On average, there were lower MIC values with LML/ETOH for M. KMS, *L. monocytogenes* and *E. faecalis*. The MIC for LML/DMSO with *E. faecalis* was 5 mg/ml, which was the highest MIC value for LML among the bacteria tested. MIC values of LMD/DMSO ranged from ≤1 to ≤3 mg/ml for *B. cereus*, M. KMS and *S. suis*. The MIC for LMD/DMSO for *E. faecalis* and *S. mutans* was above 5 mg/ml. MIC values for LMD/ETOH ranged from ≤3 to ≤5 mg/ml with no MIC values for *S. mutans*. Ethanol itself was inhibitory, specifically with M. KMS which

showed no cells in the control or treatment with 5 mg/ml LMD/ETOH (corresponding to 10% ethanol), therefore, no MIC could be determined. LMD/ETOH inhibited the growth of *E. faecalis* while LMD/DMSO showed no inhibitory effects on the bacteria. LMM in DMSO showed inhibitory activity against *B. cereus*, M. KMS and *S. suis* with MIC values between ≤1 mg/ml and ≤5 mg/ml. However, MIC values for LMM with *L. monocytogenes*, *E. faecalis* and *S. mutans* were ≥5 mg/ml. LMO/ETOH showed no inhibitory effect at concentrations up to 5 mg/ml but LMO/DMSO was inhibitory to *B. cereus, S. suis* and *L. monocytogenes*. *S. suis* and *L. monocytogenes* were more sensitive with MIC values ≤3 mg/ml than *B. cereus* with an MIC value ≤5 mg/ml. No ester dissolved in either DMSO or ethanol showed microbial inhibitory activity against the Gram-negative bacteria tested (*E. coli* O157:H7).

Minimum Bactericidal Concentrations (MBC) of Lactose Esters

MBC of the lactose esters are reported in Table 3.4 as well as the log reductions in the treatments as compared to the controls. No esters showed bactericidal activity against *S. mutans*. Out of the 4 compounds tested, LML was the only lactose ester to exert a bactericidal effect against *B. cereus*, M. KMS, *S. suis* and *L. monocytogenes* in both solvents used. MBC values of LML/DMSO were ≤1 mg/ml for *B. cereus*, M. KMS, and *S. suis*. MBC concentrations of LML were lower in DMSO compared to ethanol for *B. cereus* and *S. suis*.

In tests against the Gram-positive bacteria, LMD/ETOH showed broad antimicrobial activity against *B. cereus*, *S. suis*, *L. monocytogenens* and *E. faecalis* with MBC values between ≤3 mg/ml and ≤5 mg/ml. However, LMD/DMSO was not shown to

be bactericidal to *L. monocytogenes* or *E. faecalis* at concentrations up to 5 mg/ml. Furthermore, bactericidal activity of ethanol was shown against M. KMS, with no cells growing in the control or treatment at 10% ethanol as stated earlier for the MIC values. LMM/DMSO was effective against *B. cereus*, M. KMS and *S. suis* with MBC values between ≤3 and ≤5 mg/ml.

LMO/ETOH showed no bactericidal effects up to concentrations of 5 mg/ml whereas LMO/DMSO was only shown to have bactericidal activity against *B. cereus* at ≤5 mg/ml. DMSO was itself inhibitory towards *S. suis* with no growth in the treatment of controls with LMO/DMSO containing 8% DMSO, therefore no MBC could be determined. *S. mutans* and *E. faecalis* were observed to be the most resilient among the bacteria tested and *B. cereus* was the most susceptible. Only LMD/ETOH was observed to be bactericidal against *E. faecalis*. Average log cfu/ml of lactose esters dissolved in either DMSO or ethanol showed against the Gram-positive bacteria tested (see appendix A).

Discussion

Carbohydrate fatty acid derivatives are biodegradable, non-toxic and non-skin irritant emulsifiers with microbial inhibitory activity (Szűts and Szabó-Révész 2012). The microbial inhibitory properties of these derivatives are increasingly of interest and many of these compounds have been shown to inhibit Gram-positive rather than Gram-negative bacteria (Wagh and others 2012; Piao and others 2006).

This study evaluated both microbial inhibitory and bactericidal properties of lactose esters. LML/ETOH was shown to be the most effective lactose ester in preventing

microbial growth, yielding the lowest MIC values in the range of ≤0.05 mg/ml to ≤5 mg/ml (0.095 mM to ≤9.53 mM) against each Gram-positive bacteria tested. Moreover *B. cereus* and *S. suis* appeared to be the most susceptible with MIC values obtained for each ester tested, and the lowest MIC value was obtained with LML/ETOH and M. KMS (≤0.05 mg/ml or ≤0.095 mM). With regards to previous studies of bacterial inhibition with lactose esters, LML/ETOH showed inhibitory activity against *L. monocytogenes* at concentrations of 0.1 mg/ml (0.19 mM) (Wagh and others 2012). Similar microbial inhibitory effects of LML were observed in another study in which LML/ETOH inhibited the growth of *L. monocytogenes* in milk, low fat yogurt and cheese at ≤5 mg/ml (Chen and others 2014).

Once it passes through the cell wall, the primary target of LML, as well as other lactose esters, is the cell membrane in Gram-positive bacteria. Some molecules are polar, and thus cannot pass through membranes very easily. However, LML is non-ionic and because of its amphiphilic nature, it would pass through membranes quite easily. Moreover, LML, along with most other sugar esters, is small enough to pass through peptidoglycan followed by the hydrophobic lipid cell membrane. LML would continuously permeate the cell membrane by passive transport since it is generally moving from a higher concentration outside of the cell to a lower concentration inside until a concentration equilibrium is achieved. Once LML enters the cell, there are several possible mechanisms by which it damages Gram-positive bacteria. LML can alter the fluidity of the cell membrane by entering into the membrane, with the polar head remaining near the outer surface while the fatty acid chain inserts itself into the inner portion of the membrane. This insertion and penetration creates wedges, holes and spaces

in the membrane, resulting in an alteration in cell membrane fluidity. This leads to leakage of cellular constituents such as carbohydrates, proteins and nucleic acids. As the number of holes formed increases, there would be increased membrane movement which severely compromises the membrane's functionality, resulting in cell death.

It is known that the identity of the sugar group attached to the ester plays a role in modulating the antimicrobial activity (Smith and others 2008; Ferrer and others 2005). The antimicrobial effect of sugar esters has traditionally been measured and reported as MIC values, with no MBC values given. Nobmann and others (2009) and Smith and others (2008) reported MIC values in the range of 0.04 mM to 0.31 mM for lauric methyl D-glucopyranoside and lauric ester of methyl α-D-mannopyranoside with *S. aureus* and *Listeria* strains. Watanabe and others (2000) also showed inactivation of *S. mutans* by both galactose laurate and fructose laurate, with MIC values of 0.05 mg/ml and 0.2 mg/ml respectively, whereas hexose laurate did not suppress microbial growth. In a similar study, inhibitory effects of the sugar esters 6'-O-lauroylmaltose, 6'-O-lauroylsucrose, and 6''-O-lauroylmaltotriose were observed against *Streptococcus obrinus*, with MIC values of 0.1 mg/ml (Devulapalle and others 2004). Therefore, laurate sugar esters have previously been shown to be microbial inhibitory against Gram-positive bacteria.

The importance of the fatty acid was investigated in this study using octanoic, decanoic, lauric, and myristic acids esterified to lactose. LMM and LMD were effective in controlling the growth of *B. cereus*, M. KMS and *S. suis*. Previous research showed that erythritol and xylitol monomyristoyl suppressed *Bacillus* growth with MIC values between 6.3 μg/ml and 12.5 μg/ml (Piao and others 2006), which are lower than reported

here. As for short chain esters, Zhang and others (2014) reported that sucrose and glucose octanoate had no inhibitory effect against *S. aureus* and *E. coli* H7:O157. In contrast, we showed LMO/DMSO to have microbial inhibitory activity against *B. cereus, S. suis* and *L. monocytogenes* with MIC values ranging from 3 mg/ml to 5 mg/ml respectively.

Zhang and others (2014) reported that sucrose and glucose monodecanoate showed inhibitory effects against *S. aureus* at 4 mg/ml and 3 mg/ml, respectively. In a similar study, Nobmann and others (2009) and Smith and others (2008) reported that a glucose fatty acid ether containing decanoic acid showed the greatest activity against *S. aureus* and *Listeria* at concentrations of 0.04 mM but was effective against *E. coli* at 20 mM. In this study, we showed that LMD had MIC values for all bacteria tested except *S. mutans*, although the MIC values were solvent dependent for M. KMS, *L. monocytogenes* and *E. faecalis*.

Our previous research (Wagh and others 2012) showed that LML was not inhibitory to the Gram-negative bacteria, *E. coli* O157:H7, *Salmonella enterica* or *Klebsiella pneumonia* and this study showed that the other esters (LMO, LMD and LMM) were not inhibitory to *E. coli* O157:H7 (data not shown). On the other hand, there are a limited number of studies showing microbial inhibitory properties of sugar esters against Gram-negative bacteria. Habulin and others (2008) and Ferrer and others (2005) both reported limited inhibition of *E. coli* by sucrose monolaurate with MIC values of 4 mg/ml and 6.25 mg/ml, respectively. Zhang and others (2014) showed that methyl α-D-glucopyranoside monolaurate was effective in inhibiting the growth of both *S. aureus* and *E. coli* O157:H7 at a concentration of 0.188 mg/ml. The antimicrobial method of action of lactose esters is not well described. However, it is almost certainly related to the

interaction of these esters to bacteria cell walls. Gram-positive bacteria, it seems, are very susceptible showing great reductions in populations when treated with these lactose esters. On the other hand, it is easy to understand why Gram-negative bacteria would be more resistant to this sort of interaction due to their cell walls having additional protection and different formation from those of Gram-positive bacteria making them resistant to lactose ester's antimicrobial effects.

Compared to the amount of literature on the microbial inhibitory properties of sugar esters, there is very little information about the effects of the solvent used. Previous studies on microbial inhibitory activities of sugar esters involved dissolving sugar esters into an ethanol solution (Chen and others 2014; Wagh and others 2012; Nobmann and others 2009; Smith and others 2008) or DMSO (Ferrer and others 2005) before diluting into growth media. Others have added esters directly into growth media (Piao and others 2006; Devulapalle and others 2004). All of the esters used in the current study were soluble in a 50% ethanol solution except LMM; therefore, we only tested LMM in DMSO. Previous studies with LML showed that final ethanol concentrations greater than 7.5% were microbial inhibitory towards *L. monocytogenes* (Chen and others 2014). In this study we found that 10% ethanol was antimicrobial to M. KMS and 8% DMSO was antimicrobial/inhibitory to *S. suis.* Ethanol and DMSO are lipid solvents that can disrupt the lipid bilayer in the cell membrane and damage the cell wall. Both also act as dehydrating agents, damaging cells by causing them to lose water. The effect of the solvent on the cell growth can be observed by the log reductions in Table 3.4, specifically for *S. suis* with LMM/DMSO and LMO/DMSO.

In general, the MIC values of the LML/ETOH treatments were lower than the LML/DMSO treatments suggesting compounding stress of both LML and ethanol lead to growth inhibition as suggested by Chen and others (2014). Similar results are seen with LMD/ETOH, where MIC values were obtained for *E. faecalis*, but not with LMD/DMSO. Conversely, the MBC values of LML/DMSO were lower or equal to the LML/ETOH values. Therefore, the effect of ethanol on the MBC values is not understood.

Conclusions

The results suggest that the chain length of the fatty acid ester significantly influences the microbial inhibitory and bactericidal activity of lactose esters towards Gram-positive bacteria. Lactose esters containing decanoate and laurate were more microbial inhibitory than esters containing octanoate and myristate. No esters inhibited the growth of the Gram-negative bacteria *E. coli* O157:H7. The solvent used to dissolve the esters influenced the microbial inhibitory activity for some bacteria. Ethanol (>7.5%) and DMSO (≤8%) inhibited the growth of *L. monocytogenes* and *S. suis* respectively. Additional research on the microbial inhibitory activity of these esters in food systems without the need to prior dissolve in either ethanol or DMSO is needed.

References

Chen, Y., Nummer, B.A., Walsh, M.K. 2014. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Ltt. Appl. Microbiol. 58, 156-162. http://dx.doi.org/10.1111/lam.12169.

Devulapalle, K.S., de Segura, A.G., Ferrer, A., Alcalde, M.G., Mooser, G., Plou, F.J. 2004. Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyl transferase activity. Carbohydrate Res. 339, 1029-1034.

Ferrer, M., Soliveri, J., Plou F.J., Lopez-Cortez, N.L., Reyes-Duarte, D.R., Christensen, M., Copa-Patino, J.L., Ballestros, A. 2005. Synthesis of sugar esters in solvent mixtures bylipases from *Thermomyces lanuginosus* and *Candida antarctica B*, and their antimicrobial properties. Enzyme Microb. Technol. 36, 391-398. http://dx.doi.org/10.1016/j.enzmictec.2004.02.009.

Habulin, M., Sabeder, S., Knez, Z. 2008. Enzymatic synthesis of sugar fatty acid esters in organic solvent and in super critical carbon dioxide and their antimicrobial activity. J. Supercrit. Fluids 45, 338-345. http://dx.doi.org/10.1016/j.supflu.2008.01.002.

Nobmann, P., Smith, A., Dunne, J., Henehan, G., Bourke, P. 2009. The anitmicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. Int. J. Food Microbiol. 128 (3), 440-445. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.10.008.

Piao, J., Kawahara-Aoyama, Y., Inoue, T., Adachi, S. 2006. Bacteriostatic activities of monoacyl sugar alcohols against thermophilic sporeformers. Biosci. Biotechnol. Biochem. 70, 263-265

Smith, A., Nobmann, P., Henehan, G., Bourke, P., Dunne, J. 2008. Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives. Carbohydrate Res. 343, 2557-2566. http://dx.doi.org/ 10.1016/j.carres.2008.07.012.

Szűts, A., Szabó-Révész, P. 2012. Sucrose esters as natural emulsifiers in drug delivery systems. Int. J. Pharm. 433, 1-9. http://dx.doi.org/ 10.1016/j.ijpharm.2012.04.076.

Wagh, A., Shen, S., Shen, F.A., Miller, C.D., Walsh, M.K. 2012. Effect of lactose monolaurate on pathogenic and nonpathogenic bacteria. Appl Environ. Microbiol. 78 (9), 3465-3668. http://dx. doi.org/10.1128/AEM.07701-11.

Walsh, M.K., Bombyk, R.A., Wagh, A., Bingham, A., Berreau, L. 2009. Synthesis of lactose monolaurate as influenced by various lipases and solvents. J. Mol. Catal. B Enzym. 60 (3-4), 171-177. http://dx.doi.org/10.1016/j.molcatb.2009.05.003.

Watanabe, T., Katayama, S., Matsubara, M., Honda, Y., Kuwahara, M. 2000. Antibacterial carbohydrate monoesters suppressing cell growth of *Streptococcus mutans* in the presence of sucrose. Curr. Microb. 41, 210-213.

Zhang, X., Wei, W., Cao, X., Feng, F. 2014. Characterization of enzymatically prepared sugar medium-chain fatty acid monoesters. J. Sci. Food Agric. 95 (8), 1631-1637. http://dx.doi.org/10.1002/ jsfa.6863.

Table 3.1. Series of microorganisms involved in the study

No.	Microorganisms	ATCC no./serovar	Gram reaction[a]	Growth medium
1	*Bacillus cereus*	13061	+	BHI
2	*Mycobacterium* sp. strain KMS	NA	+	LB
3	*Streptococcus suis*	89/1591	+	BHI
4	*Listeria monocytogenes*	FSL C1-056	+	BHI
5	*Listeria monocytogenes*	FSL J1-177	+	BHI
6	*Listeria monocytogenes*	FSL N3-013	+	BHI
7	*Listeria monocytogenes*	FSL R2-499	+	BHI
8	*Listeria monocytogenes*	FSL N1-227	+	BHI
9	*Enterococcus faecalis*	V538	+	BHI
10	*Streptococcus mutans*	25175	+	BHI
11	*Escherichia coli* H7:O157	35150	_	LB

[a] +, positive; -, negative
NA = not available

Table 3.2. Final concentrations of ethanol and DMSO used in the study

Stock solutions	Concentrations		
	1 mg/ml	3 mg/ml	5 mg/ml
LMO 30% ethanol 50 mg/ml	0.5%	1.5%	2.5%
LMD 30% ethanol 25 mg/ml	1.2%	3.6%	10%
LML 50% ethanol 60 mg/ml	0.8%	2.5%	4%
LML 50% ethanol 18 mg/ml	2.8%	8.3%	13.9%
LML 50% ethanol 20 mg/ml	2.5%	7.5%	12.5%
60% ethanol 60 mg/ml	1%	3%	5%
100% DMSO 60 mg/ml	1%	3.2%	8%

Table 3.3. Minimum inhibitory concentrations of lactose esters as both mg/ml and mM concentrations. Esters were tested at concentrations up to 5 mg/ml

	LMO DMSO	LMD DMSO	LMD ETOH	LML DMSO	LML ETOH	LMM DMSO
B. cereus	≤5 mg/ml ≤10.7 mM	≤3 mg/ml ≤6 mM	≤3 mg/ml ≤6 mM	≤1 mg/ml ≤1.9 mM	≤1 mg/ml ≤1.9 mM	≤1 mg/ml ≤1.8 mM
M. KMS	No	≤1 mg/ml ≤2 mM	X[1]	≤1 mg/ml ≤1.9 mM	≤0.05 mg/ml[2] ≤0.095 mM	≤5 mg/ml ≤9 mM
S. suis	≤3 mg/ml ≤6.4 mM	≤3 mg/ml ≤6 mM	≤5 mg/ml ≤10.1 mM	≤1 mg/ml ≤1.9 mM	≤1 mg/ml ≤1.9 mM	≤3 mg/ml ≤5.4 mM
L. monocyto genes	≤3 mg/ml ≤6.4 mM	≤3 mg/ml ≤6 mM	≤3 mg/ml ≤6 mM	≤3 mg/ml ≤5.7 mM	≤0.1 mg/ml[2] ≤0.19 mM	No
E. faecalis	No	No	≤5 mg/ml ≤10.1 mM	≤5 mg/ml ≤9.5 mM	≤1 mg/ml ≤1.9 mM	No
S. mutans	No	No	No	≤1 mg/ml ≤1.9 mM	≤3 mg/ml ≤5.7 mM	No

X[1]= no growth in treatment or control at 5 mg/ml
[2]Data obtained from Wagh and others (2012)
No= no growth inhibition value obtained

Table 3.4. Minimum bactericidal concentrations of lactose esters as both mg/ml and mM concentrations. Esters were tested at concentrations up to 5 mg/ml. The log reductions of the treatment samples compared to the controls are given as log values

	LMO DMSO	LMD DMSO	LMD ETOH	LML DMSO	LML ETOH	LMM DMSO
B. cereus	≤5 mg/ml ≤10.7 mM 7 log	≤3 mg/ml ≤6 mM 9 log	≤5 mg/ml ≤10.1 mM 7 log	≤1 mg/ml ≤1.9 mM 7 log	≤5 mg/ml >9.5 mM 8 log	≤3 mg/ml ≤5.4 mM 8 log
M. KMS	No	≤1 mg/ml ≤2 mM 8 log	X[1]	≤1 mg/ml ≤1.9 mM 7 log	≤1 mg/ml[2] ≤1.9 mM 4 log	≤5 mg/ml ≤9 mM 8 log
S. suis	X[1]	≤3 mg/ml ≤6 mM 7 log	≤5 mg/ml ≤10.1 mM 5 log	≤1 mg/ml ≤1.9 mM 7 log	≤5 mg/ml ≤9.5 mM 8 log	≤5 mg/ml ≤9 mM 2 log
L. monocyto genes	No	≤5 mg/ml ≤9.5 mM 9 log	≤3 mg/ml ≤6 mM 6 log	≤5 mg/ml ≤9.5 mM 8 log	≤5 mg/ml[2] ≤1.9 mM 5 log	No
E. faecalis	No	No	≤5 mg/ml ≤10.1 mM 4 log	No	No	No
S. mutans	No	No	No	No	No	No

X[1]= no growth in treatment or control at 5 mg/ml
[2]Data obtained from Wagh and others (2012)
No= no minimum bactericidal value obtained

CHAPTER 4

EMULSIFICATION PROPERTIES OF LACTOSE FATTY ACID ESTERS

Abstract

Sugar esters are a class of synthetic emulsifiers used in the food, pharmaceutical, and personal care industries. The influence of the fatty acid chain length on the emulsification properties of lactose esters such lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM) was investigated in this study. The change in emulsion thickness (mm) (as an increase in the clarification layer express as mm/d) and oil droplet size distribution in 20% soybean oil-in-water emulsions were measured at 0.1%, 0.25% and 0.5% of lactose ester usage. At a concentration of 0.5% emulsifier, LML showed the most stable emulsion (0.5 mm/d). LMD (0.72 mm/d) showed the second best emulsion destabilization rate followed by LMM (1.11 mm/d) and LMO (7.19 mm/d). Oil droplet distributions highlighted the same trend, with LML and LMD maintaining the smallest droplet sizes and thus the most robust emulsion. An observed increase in hydrophilic-lipophilic balance (HLB) value was seen along with an increased critical micelle concentration (CMC) value for each lactose esters, showing the strength of the linear relationship between these two measured values. The CMC values of LMO, LMD, LML and LMM by the dye micellization method was determined to be 0.96 mM, 0.89 mM, 0.72 mM, and 0.56 mM, respectively. This research showed that LML and LMD formed more stabilized emulsions, even with HLB and CMC values higher than those of LMM, perhaps due to its HLB value regions

compared to the other lactose esters tested suggesting HLB and CMC values alone do not predict emulsifier effectiveness.

Introduction

The most common sugar esters consist of glucose, fructose, xylose or sucrose as the hydrophilic head group. Previous studies have investigated these esters in different colloidal systems (Zhang and others 2014; Becerra and others 2008; Yanke and others 2004; Piao and Adachi 2006; Soultani and others 2003; Ferrer and others 2002; Garofalakis and others 2000). As non-ionic emulsifiers, the use of lactose esters has been recently studied in food, cosmetics and pharmaceuticals (Zhang and others 2014; Neta and others 2012; Walsh and others 2009).

Studying the mechanisms by which emulsions destabilize is an important approach in understanding the emulsification properties of sugar esters. An oil-in-water (O/W) emulsion is an aqueous dispersion of oil droplets in colloidal suspension in water. These emulsions can exhibit aggregation due to the density differences between the two phases over an elapsed period of time (Chiralt 2005; McClements 2005). Gravitational forces play a critical role in the destabilization of emulsions (McClements 2005). The rate at which this takes place can be described mathematically using Stokes' law with considerations of Brownian movement to yield a velocity of the migration of oil droplets out of suspension and into aggregation (McClements 2005):

$$v = \frac{-2\mathrm{g}r^2(\rho^2 - \rho^1)}{2\eta^1}$$

Where v is the velocity of the migrating oil (creaming), r is the radius of the particle, g is the acceleration due to gravity, ρ is the density and η is the shear velocity.

The subscripts 1 and 2 refer to the continuous and dispersed phase. According to Stokes' law, O/W emulsions are normally stable if the oil droplet size remains constantly at or under 1.0 μm (McClements 2005).

Flocculation is of practical importance as it causes the droplets to clot, leading to a growth in the mean oil droplet diameter causing droplets to coalescence and the emulsion to destabilize (Formulaction 2009; McClements 2005). Differences in comparative densities cause the two destabilizing phases to move toward different locations: water accumulates at the bottom, oil floats to the top, and any remaining emulsion sits in between (Walsh and others 2009). Bands are thus formed leading to the nomenclature we use to describe these destabilized emulsions: clarification or the formation of visually clear bands due to oil and water separating and creaming or the movement of fat and oil to the top of a solution. Thus, the clarification and creaming processes indicate unstable emulsions. Emulsions are often stabilized in the long term via homogenization processes which dramatically decrease oil droplet sizes combating gravitational forces with increasing shear forces (Bai and McClements 2016; Trujillo-Cayado and others 2015; McClements 2005).

Clarification and creaming can be easily measured using a Turbiscan (vertical scan macroscopic analyzer) (Kaombe and others 2013; Huck-Iriart and others 2011; Garg and others 2010). Light scattering detection can show the amount of suspension in an emulsion thus highlighting its respective stability (Formulaction 2009). Alternatively, many studies concluded that oil droplet size distribution within the emulsion is a better indicator of emulsion stability which can be measured with a LS Beckman Coulter droplet size analyzer (BeckmanCoulter 2011; Garg and others 2010; McClements 2005).

Noted characteristics of sugar esters feature a high tendency to remain at the O/W interface in an emulsion measurable by the ratio of hydrophile to lipophile balance (HLB) (Zhang and others 2014; Szűts and Szabó-Révész 2012; Piao and Adachi 2006; Soultani and others 2003). This implies that the HLB value of an emulsion is an effective measurement of emulsification stability and other properties such as water solubility and critical micelle concentration (CMC). It has been investigated that the type of headgroup of sugar esters (Zhang and others 2014; Neta and others 2012; Piao and Adachi 2006; Soultani and others 2003; Ferrer and others 2002; Garofalakis and others 2000; Patist and others 2000) and/or degree of esterification (Ferrer and others 2002) can explain the variations observed in HLB and CMC values in emulsions made with these emulsifiers. The importance of these values, as it relates to the fatty acid chain used to generate an emulsifying ester, has been well established (Zhang and others 2014; Szűts and Szabó-Révész 2012; Becerra and others 2008; Piao and Adachi 2006; Suradkar and Bhagwat 2006; Yanke and others 2004; Soultani and others 2003; Ferrer and others 2002; Hait and Moulik 2001; Garofalakis and others 2000; Patist and others 2000), however, there has been little attention given to sugar esters made with lactose attached to fatty acid chains and the resulting emulsifier properties of such compounds.

The primary objective of this research was to evaluate the emulsification ability of lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM) synthesized using an immobilized lipase in 20% soybean O/W emulsions compared to Tween-20 and Ryoto L-1695, commercially available emulsifiers (for example, sucrose esters). The influence of various concentrations (0.1%, 0.25% and 0.5%) over time on emulsion stability was also

evaluated. Evaluating the possible correlations between HLB and CMC in this way will provide a better understanding of the emulsification properties of these lactose-based sugar esters and their stability.

Materials and Methods

Materials and Equipments

Tween-20 (Acros Organics, Thermo Fisher Scientific, Fairlawn, NJ, USA) and Ryoto L-1695 (Mitsubishi-Kagaku, Tokyo, Japan) were prepared to act as standards for comparison. Soybean oil used was obtained from a local grocery store and was of the brand Western Family Inc., Madison, WI, USA. Lactose (Proliant, Iowa, USA), 3A molecular sieves, vinyl octanoate, vinyl decanoate, vinyl laurate, vinyl myristate, HPLC grade solvents (acetonitrile and water), immobilized lipases from *Thermomyces lanuginosus* lipase enzyme (TM3), Whatman glass microfiber filters, 1, 5 ml macro cuvettes and EosinY were all obtained from Sigma Aldrich, St. Louis, MO, USA. Additional supplies of 2-methyl-2-butanol (2M2B) (dried using 10% 3A molecular sieves) was purchased from Thermo Fisher, MA, USA. A reversed-phased high-performance liquid chromatography (RP-HPLC) (Beckman System Gold 125 Solvent Module, Ontario, Canada) equipped with Luna 5 lm C18 100 A ° (250 mm X 4.6 mm, Phenomenex, Torrance, CA, USA), an evaporative light scattering detector (ELSD) (Agilent Technologies, Santa Clara, CA, USA), incubator shaker (Beckman, USA), Ultra-turrax T25 (Janke and Kunkel, Staufen, Germany), microfluidizer (Microfluidics Corporation, Newton, MA, USA), LS Beckman Coulter droplet size analyzer (LS 230, Coulter Corporation, Miami, FL, USA), Turbiscan (MA2000, Toulouse, France) and

spectrophotometer (Beckman, Portland, OR, USA) were provided by Utah State University.

Synthesis of Lactose Esters

Enzymatic synthesis of LML was performed using the method as described by Walsh and others (2009). Synthesis of LMO was conducted using lactose, vinyl octanoate, molecular sieves and TM3. For a 60 ml reaction in 2M2B, 3 g of lactose, 6 g of dried molecular sieves, 1.7 ml of vinyl octanoate (lactose to fatty acid ratio of 1:2.1) and 1.8 g TM3 were combined. The resulting mixture was placed in 100 ml glass bottles and then placed in the incubator/shaker and was allowed to react at a temperature of 60 °C while agitated by incubator/shaker at the 90 rpm setting for 2 days. The other lactose esters, LMD and LMM, were also synthesized enzymatically, using the methods described above, just using different molar ratios of lactose to fatty acid and the respective substrates of vinyl myristate (lactose to fatty acid ratio of 1:2.14) for LMM and vinyl decanoate (lactose to fatty acid ratio of 1:1) for LMD.

The amount of lactose esters synthesized was determined using RP-HPLC with ELSD set at 60 °C with a nitrogen gas pressure of 3.55 bar. Once a sable baseline was obtained, the sample mixture in 2M2B is manually injected to a C18-based sorbent (stationary phase) with flow rate of 1 ml/min. A linear gradient from 10% acetonitrile–water (40:60, v/v) to 95% acetonitrile–water (95:5, v/v) over 18 min to elute the sample was also used as the mobile phase. The final eluents of target components were converted to a fine spray via a nebulizer of ELSD with nitrogen gas. Light was then focused on the remaining components and scattered light was detected (El Rassi 1995).

Purification of Lactose Esters

For ester purification, the 2M2B reaction was filtered through a Whatman glass microfiber filter then dried in a hood for 48 hrs. The dry solids of LMM and LML were suspended in a 50% hexane–water while the dry solids of LMO and LMD were suspended in a 50% ethanol–water. These were then placed in a separatory funnel. The lower aqueous layer was drained into a beaker and dried in a hood for 48 hrs. After completely drying, the product powder was suspended in hexane and/or ethanol, and then centrifuged for 15 min at room temperature at 2000 x g and the supernatant analyzed via HPLC for the presence of di- tri- or higher saccharides. The hexane and/or ethanol extraction was repeated until only the monoester was present in the pellet. The purities of the lactose esters were confirmed to be greater than 85% by HPLC analysis.

HLB Calculation

The calculated HLB^a values of lactose esters, Tween-20 and Ryoto L-1695 were determined using the formula HLB= 7 + ∑ (hydrophilic values) - ∑ (lipophilic values) (McClements 2005). An alternative equation for HLB^b calculation is the following formula: HLB= [(L/T) * 20] (L is the molecular weight of the hydrophilic part of the molecule, and T is the total molecular weight) (Ritthitham 2009). Superscripts were used in the data to differentiate which calculation method was used for comparative analysis.

CMC Determination

The CMC values of lactose esters, Tween-20 and Ryoto L-1695 were measured by the dye micellization method using eosin Y (Patist and others 2000). A 10 X stock solution of the dye (0.19 mM) was also prepared which was diluted to a working standard

(2 X solution). Identical stock solution concentrations (0.001 g/ml) of each of LMO, LMD, LML and LMM were prepared in distilled water. The stock solutions were then added to each well for a final concentration 0.002 μM and each well contained a total of 1 ml in 1, 5 ml macro cuvettes. Light absorbance was measured at 538 nm and 518 nm on a Shimadzu Biospec 1601 (Portland, OR, USA) spectrophotometer at emulsifier concentrations between 1 μM to 1 mM, and the CMC was determined as described by Patist and others (2000). The concentrations of lactose ester were calculated using their molecular weights disregarding the presence of any probable impurities.

Emulsion Preparation

Emulsion samples of 20% soybean oil and water were prepared by combining 40 ml of water and 10 ml of soybean oil in a beaker; and each sample received one treatment of emulsifier at one concentration rate. Concentrations of 0%, 0.1%, 0.25%, and 0.5% emulsifier were prepared for each of the dry solid compounds: LMO, LMD, LML and LMM. Positive controls, using Tween-20 and Ryoto L-1695, were also prepared at these same usage rates: 0%, 0.1%, 0.25%, and 0.5%. Each emulsifier was first stirred in the 40 ml of water for 15 min before the addition of the 10 ml of oil. The water and oil phases were mixed with a high-speed blender (Ultra-turrax T25) at 18,000 rpm for 5 min and then passed immediately through a microfluidizer three times at 17.4 ± 1.6 MPa (~15,000 psi). All emulsion samples were prepared in triplicate. Emulsion destabilization, and oil droplet size measurements started on day 0 (the day the emulsions were prepared) and continued daily until day 4 at room temperature.

Destabilization Measurement

The stability of each 20% oil-in-water (O/W) sample was determined using a Turbiscan, a vertical scan macroscopic analyzer, with Turbiscan MA2000 software. Testing samples were prepared by placing 5 ml of sample into 11 cm glass tubes and then left let to sit for a period of 5 days. The Turbiscan and software where used twice daily to measure the thickness (in mm) of the clarification layer at the bottom of the tubes over the course of the 5 days (day 0 through day 4) as described by Garg and others (2010). All emulsion samples were individually evaluated and pooled in replicate. The destabilization thickness in mm for each sample was plotted against the number of days. The resulting slope of the scatter plot was used to determine instability of emulsions in mm/d. A two-way analysis of variance (ANOVA) SAS for the different rates of destabilization values (mm/d) was conducted between the type, days and concentrations of emulsifiers (appendix B).

Droplet Size Distribution Measurement

The oil droplet diameter distribution of the emulsion samples was measured using a LS Beckman Coulter droplet size analyzer (LS 230) with the polarization intensity differential set for scanning small fluid modules. Emulsions containing LMO, LMD, LML and LMM, along with Tween-20 and Ryoto L-1695, were analyzed from day 0 to day 4 for change in droplet diameter over time. The oil droplet measurements were taken by the angular dependence of the intensity of the laser light (λ= 623.8 nm) scattered by emulsions as described by Garg and others (2010). Droplet diameter curves were constructed as a function of the total volume of oil droplets as a percentage (y-axis)

versus droplet diameters (x-axis). The curve expressed what percent of the total oil droplet volume (y-axis) was to be found at the corresponding oil droplet diameter (x-axis).

Results

Emulsion Destabilization

Destabilization of emulsions can be observed through changes in light backscattering properties of the emulsions. In destabilized emulsions multiple layers can develop from an oil layer on top, a creaming layer directly underneath, to a thick and clearing layer (clarification layer) at the bottom. Using a vertical scan macroscopic analyzer 6 ml samples of emulsions in glass tubes were measured along the length of the tube for changes in backscattering (ΔBS%). Measurements were collected, and statistical analysis was used to determine the change thickness of the clarification layer (0-10 mm) from the bottom of each tube. Fig 4.1 shows how, after 5 days, the emulsions' clarification layers changes in emulsions with and without emulsifier. In Fig 4.1 (A), a control sample of 20% O/W emulsion was monitored and a destabilized result was observed with in a thin oil layer on top and a clarification layer on the very bottom after 5 days. Conversely, Fig 4.1 (B) is a 20% O/W emulsion containing 0.5% LML after 5 days and has no visible clarification layer.

Fig 4.2 shows the change in destabilization (mm) (as an increase in the clarification layer) over the 5 days tested for 20% O/W emulsions containing the four synthesized lactose esters (LMO, LMD, LML, LMM) and two commercial non-ionic emulsifiers (Tween-20 and Ryoto L-1695). By measuring the slope of the increasing size

of the clarification layer, we can determine the rate of destabilization; which, by definition for a stable emulsion, is less than 1.0 mm/d (McClements 2005). Three different levels (0.1%, 0.25% and 0.5%) of each emulsifier were compared to a negative control, the oil and water mixture without an emulsifier had a destabilization value of 3.91 mm/d. Using two controls, a positive and negative, gives us contrast to compare my potential emulsifiers emulsion stabilization ability. In this study, the average destabilization rates of emulsion stabilized with 0.5%, 0.25% and 0.1% of Tween-20 were 0.5, 0.83 and 1.7 mm/d, respectively (Fig 4.2 (E)). This shows that the destabilization rates of Tween-20 emulsions are proportionate to the Tween-20 concentration. Emulsification ability of other lactose esters can be measured relative to Tween-20.

The emulsions containing Ryoto L-1695 at 0.5% and 0.25% concentrations produced a destabilization rate of 0.42 and 0.5 mm/d respectively, implying this emulsion was stable over the time frame (Fig 4.2 (F)). However, with a decreased concentration of 0.1%, Ryoto L-1695 there yielded a destabilization rate of 1.6 mm/d, implying this emulsion was unstable over 5 days.

LMO stabilized emulsions were highly unstable at 0.5%, 0.25% and 0.1% tested with a fairly consistent destabilization rate of 7.19, 7.65 and 7.66 mm/d, respectively (Fig 4.2 (A)). In order to get the observed initial destabilization slop, additional testing was preformed every 12 hrs to quantify the rapid destabilization of these emulsions. LMO treated samples were the only ones to be tested this often, and were only done so out of necessity. While much better than the negative control with no emulsifier, it fails the

desired level of less than 1.0 mm/d, which we have defined as an emulsifier with effective stabilization ability.

The emulsions containing LMD at 0.5% concentration produced the destabilization rate of 0.72 mm/d, implying this emulsion was stable over the time frame (Fig 4.2 (B)). However, with decreased concentrations, such as 0.25% and 0.1%, LMD stabilized emulsions became less stable with destabilization rates of 1.14 and 2.16 mm/d, respectively, showing less than the 1.0 mm/d destabilization required to be an effective emulsifier.

In Fig 4.2 (C), the average destabilization rates for the LML at concentrations of 0.5%, 0.25% and 0.1% were 0.51, 0.84 and 1.79, respectively. LML at concentrations of 0.5% and 0.25% were successful at stabilizing emulsions, showing less than the 1.0 mm/d destabilization, and thus formed effective emulsions although the emulsions formed using 0.1% LML were not stable.

LMM produced less stable emulsions which decreased in stability even more as the concentration decreased (Fig 4.2 (D)). Destabilization rates of 1.11, 1.64 and 2.14 mm/d at 0.5%, 0.25% and 0.1% LMM concentrations, respectively were obtained. Therefore, it is not a suitable O/W emulsifier, based on the destabilization rates.

The "*type 3 fixed effects*" were observed with each main effect and were found to be statistically significant (appendix B). Testing for concentration amount, lactose ester with Tween-20 and Ryoto L-1695 used, and time (as measured in days) yielded *p*-values of <0.0001, 0.0129 and <0.0001, respectively. The interaction effect is negligible ($p > 0.05$), giving greater confidence in focusing the interpretation in main effects population marginal means comparisons. Moreover, this means that the effect of lactose esters, after

controlling for the effect of concentration and the day of measurement, was statistically significant across all groups leading to confidence in declarations of differences between each. "*Post hoc"* analyzes yielded an interesting result in comparisons of thickness of the clarification layers with only LMO showing significant difference from the controls of Tween-20 and Ryoto L-1695. No other statistically significant difference was found comparing each lactose ester to each other or to the positive controls.

The "*post hoc"* analysis of clarification layer thickness yielded results that are contradictory with only one LMO showing significant difference from controls. These controls were not found to be significant in difference from each other, nor from any of the other lactose esters. These other lactose esters were found to not be significant in difference from each other including no significant difference from LMO. This confusing and self-contradictory result required other means of analysis form other tests and graphical result interpretations.

Oil Droplet Size Distribution and Droplet Size Measurement

The stability of the emulsion for each lactose ester was also studied by measuring droplet size distribution of the emulsion after each treatment compared with that of Tween-20 and Ryoto L-1695 over the 5 days. Fig 4.3 shows the droplet size distribution, as a percent, of droplets at a certain volume of the emulsions at 0.5% concentration of emulsifier over time. The droplet size distribution of the 20% O/W emulsions stabilized by 0.25% and 0.1% lactose esters, Tween-20 and Ryoto L-1695 are shown in appendix B. The stability of an emulsion can be influenced by preventing flocculation and/or coalescence (Tadros 2013; McClements 2005). Thus, stable emulsions generally have

small oil droplets. Using a high-speed blender, oil droplets sizes ranging between 2 and 10 μm can be produced (McClements 2005). Even smaller oil droplets, less than 0.1 μm range, can be generated by microfluidization (McClements 2005). In this study, high-speed blending followed by microfluidization was used to test emulsion stability as influenced by oil droplet size with the emulsifiers.

In Fig 4.3 (E), the 20% O/W emulsions stabilized by 0.5% Tween-20, the positive control, on day 0 shows a monomodal size distribution, with a peak at 15% of the oil droplets in the range of 0.8-2 μm. After 2 days, a peak at 13% of the oil droplets remained in this range, and this droplet size distribution stayed relatively constant over 5 days. Additionally, these peaks stayed narrow, with a small range of distribution, without bimodality developing over time. This exemplifies an effective emulsifier.

At day 0, the 0.5% Ryoto L-1695 stabilized emulsion had a significant population of droplets in the 0.5-3 μm range peaking at 13%, and this droplet size distribution stayed relatively constant over 5 days (Fig 4.3 (F)).

Emulsions containing 0.5% LMO on day 0 show a bimodal drop size distribution, which peaked at 10% of oil droplets in the range of 0.5-1 μm and peaked again at 7% of the droplets in the range of 1-4 μm (Fig 4.3 (A)). This oil droplet size distribution on day 2 remained in the same range. After 4 days, the droplet diameters increased to 0.5-10 μm, and the droplet size distribution became wider and skewed to the right, meaning the population of large oil droplets sizes became the greater percentage of total oil droplets in the emulsion.

In Fig 4.3 (B), the emulsion containing 0.5% LMD on day 0 has a peak at 9% of oil droplets in the range of 1-3 μm and another peak at 0.5% in the range of 6-10 μm. On

day 2, the smaller oil droplet sizes kept a similar distribution, but the 6-10 μm range of peaks increased to 3%. At day 4, this trend continued with a bimodal skew to larger oil droplets.

At day 0, the 0.5% LML stabilized emulsion had a significant population of droplets in the 0.5-5 μm range peaking at 7% with a second right skewed distribution peak at 1% in the range of 8-10 μm (Fig 4.3 (C)). On day 2, the 0.5-5 μm ranged peak increased to 10% of droplets and the second peak increased to 2% while widening its range to 5-10 μm. This same droplet size distribution remained on day 4.

Emulsions with 0.5% LMM exhibited noticeable destabilization during the 5 days of storage (Fig 4.3 (D)). LMM emulsions were characterized by broad droplet size distributions with large bimodal peaks. On day 0 and day 2, there was significant number of smaller droplets ranging from 0.1-1 μm. By day 4, this 0.1-1 μm range disappeared and droplet size was shifted to higher values, though bimodality remained. A peak of 9% of droplets was in the range of 0.5-3 μm and a peak of 6% of the droplets was in the range of 3-10 μm.

Statistical data of the mean D (3,2) values of each sample with standard deviations are given in appendix B. It was observed that time had no significant difference on the mean droplet size diameter of the emulsions made using either lactose ester, Tween-20 and Ryoto L-1695. Emulsions with either emulsifier had lower droplet diameter value at 0.5% concentration of emulsifier than at 0.1%. In contrast, 0.5% and 0.25% are at times very close in values to each other complicating interpretation. Additionally, day 0 to day 5 values showed great variability in data for Ryoto L-1695 0.5% and 0.25%; Tween-20 0.5%; LMM 0.5%, 0.25% and 0.1%; LML 0.5%; and LMD

0.5% and 0.25%. These complications in the data required us to look more deeply with droplet size distribution to see difference between lactose esters compared to controls.

Surface-active Properties

The values of MW, HLB, CMC (mM), and CMC (mM reported from other sources) for LMO, LMD, LML, LMM, Tween-20 and Ryoto L-1695 as emulsifiers are presented in Table 4.1. The MW of Tween-20 with an ethoxylated sorbitan was greater than Ryoto L-1695 and the lactose esters (which where themselves comparable, only differentiated by their different bound fatty acid chains). The HLB scale is a basic indicator of an emulsifier's solubility and is the primary criteria for selecting an emulsifier in a food system (Hait and Moulik 2001). Typically, the maximum HLB value of non-ionic emulsifiers is 20 (Ritthitham 2009; McClements 2005), with emulsifiers with HLB values ranging from 8 to 18 are generally used in O/W emulsions (McClements 2005; Whitehurst 2004). The results in Table 4.1 show that Tween-20, Ryoto L-1695, and all tested lactose esters are greater than 8 but less than 18, thus all show potential at stabilizing 20% O/W emulsions. All the HLB^a values of the tested emulsifiers are between 14.1 and 16.9 as determined by the McClements (2005) equation. Using an alternative equation for HLB^b calculation (Ritthitham 2009), we still find a very similar HLB value range of 13.4 to 15.8.

Like HLB values, CMC values are parameters used to characterize the potential of emulsifiers (Zhang and others 2014; Hait and Moulik 2001). As such, the CMC values of the commercial emulsifiers and the lactose esters, as measured by the dye micellization method, were obtained (Table 4.1). Of the agents tested, Tween-20 and Ryoto L-1695

had lower CMC values, 0.07 mM and 0.44 mM, respectively than the lactose esters. LMO, LMD, LML, and LMM had CMC values of 0.96 mM, 0.89 mM, 0.72 mM and 0.56 mM, respectively (see appendix B). These results would normally suggest that these lactose esters would be expected to underperform as emulsifiers when compared to the positive controls Tween-20 and Ryoto L-1695. These reported results on CMC values closely agree with other reported CMC values. Patist and others (2000) used the dye micellization method and Tween-20 had similarly lower CMC value of 0.042 mM. Using a surface tension method, Zhang and others (2014) determined CMC values of Ryoto L-1695, LMO, LMD and LML at only slightly below the CMC values found in this research. It is worth noting that the surface tension method, if used on impure samples, would yield a lower CMC than the dye micellization method. As the surface tension method, is very sensitive to the presence of impurities (Patist and others 2000).

An observed increase in CMC value was seen along with an increased HLB value showing the strength of the relationship between these two measured values in Fig 4.4 (A). There is also an increase in both HLB and CMC values with a decrease in fatty acid chain lengths in Fig 4.4 (B) and Fig 4.4 (C), respectively. This shows a linear relationship between HLB and CMC values (R^2= 0.9756). Further, HLB values/fatty acid chain lengths' (R^2= 0.9995) high correlation and CMC values/fatty acid chain lengths' (R^2= 0.9727) high correlation show even more how close of a linear relationship these values have to each other and how similarly they are influenced by the same factor: fatty acid chain length.

Discussion

Destabilization of emulsions was observed through the change in thickness of the clarification layer of the 20% O/W emulsions for lactose esters compared to the commercial emulsifiers Tween-20 and Ryoto L-1695. Tween-20, a sorbitan monolaurate, is a non-ionic detergent approved by the U.S. FDA for food use (Smith 1991). Due to its wide current usage in industry and its' relative similarity to the agents tested in this research (the lactose esters), Tween-20 was selected as a model control for comparative testing. Ryoto L-1695, a sucrose ester, also has several physical similarities to lactose esters and so was selected as another control for some comparative analysis. Out of the 4 compounds tested, LML at a concentration of 0.5% was the only lactose ester to exert an emulsifying activity comparable to Tween-20 and Ryoto L-1695 over 5 days. Even though LML produced less stable emulsions as the concentration rate decreased, Tween-20 and Ryoto L-1695 showed a similar behavior at lower concentrations.

While there are a large variety of properties we can use to characterize the emulsification stabilities of sugar esters, the HLB values can give an insight into emulsion stabilization effectiveness. A well-balanced ratio of hydrophilic head to hydrophobic tail allows for oil droplets to be well stabilized by the emulsifier resulting in low destabilization rates. The results are consistent with the limited number of studies which have investigated the HLB values of sugar esters which concluded that sugar esters made with laurate were better emulsifiers with greater emulsion stability than alternative length fatty acid chains. Piao and Adachi (2006) reported that erythritol with lauric acid (HLB; 16) resulted in greater stabilized emulsions than emulsions made with glycerol, arabitol, ribitol and xylitol at 0.05% O/W emulsions. Zhang and others (2014) prepared

sugar monoesters including sucrose, maltose, lactose with fatty acid chain lengths from 8 to 12 and showed that lauryl (HLB; 13.1) esters were significantly better at stabilizing emulsions compared to octanoyl (HLB; 14.5) and decanoyl (HLB; 13.8) esters in 20% O/W emulsions. These demonstrated that the HLB values of emulsifiers influenced the stable emulsion.

The importance of the HLB values as it relates to the fatty acid chain used to generate an emulsifying ester was investigated in this study. It was evident that as LML was the most effective emulsifier tested. While the best result was still LML, LMD showed the second lowest emulsion destabilization rates followed by LMM and LMO, respectively. This does bring up an interesting point, all of the tested lactose esters had comparable HLB values and yet very different emulsification properties. This does suggest that HLB is not the only property influencing emulsification potential.

The 0.5% LML stabilized emulsion had a significant population of droplets in the 0.5-5 μm range. Correspondingly, Tween-20 and Ryoto L-1695 showed emulsification properties at concentrations of 0.5% with an oil droplet distribution range of 0.8-2 μm and 1.9-2 μm, respectively. Emulsifying activity effects of Tween-20 were observed in another study in which oil droplet sizes decreases from 25 μm to 150 nm as the concentration of the emulsifier increases from 1% to 4%; oil droplet sizes then remained constant on further Tween-20 addition up to 5% (Pawlik and others 2016). Emulsions containing 0.5% LML produced narrow distributions in ranges comparable to Tween-20, though bimodality was present and range distributions widened over time, while Ryoto L-1695 and Tween-20 were consistent over time. However, these widening distributions shifted left, suggesting oil droplets were decreasing in size as time progressed. Moreover,

the bimodality was quite minor; with the right peak remaining small and showing the same left shifting change observed in the primary peak. Destabilization of emulsion shows LML is comparable with Tween-20 and Ryoto L-1695, even with the differences in oil droplet diameters and LML's bimodality. It is the end result, however, of LML usage that strengthen the assertion of comparability with Tween-20 and Ryoto L-1695, LML produces emulsions that are as stable as the positive controls. The differences in oil droplet sizes and distributions are quite minor with the ranges of distributions matching up quite well with the controls. It is also interesting to consider that LML at the 0.5% concentration seems to be decreasing oil droplet sizes with only a factor of advancing time, a trend that may increase emulsion stability and delay phase separation. This result may be an artifact of testing but if not could be part of the explanation of LML's aforementioned comparable emulsification stability abilities with Tween-20 and Ryoto L-1695.

While Tween-20 and Ryoto L-1695 are excellent at forming and stabilizing initial emulsions, LML may actually be actively working to increase emulsion stability over time in addition to its initial stabilization. It is feasible that the HLB values of LML indicate favorable absorption during emulsification which lead to positive interacts with the oil droplets and the water phase. LMD, with such a similar HLB value as well as other physical properties, may also benefit in a similar manner to LML in its stabilization properties in spite of its observed bimodal distribution of oil droplet sizes.

Another explanation of the bimodal oil size distribution in the observed results for LML (along with other lactose esters tested) might be an artifact of oil droplets flocculating and/or coalescing. However, the former is unlikely due to the nature of the

light scattering equipment used, the droplet size analyzer (LS 320), makes use of agitation to spread out individual oil droplets for analysis. Furthermore, as 20% O/W emulsions are continually being diluted, thus flocculated oil droplets would be dispersed, breaking the flocculate; single coalesced oil droplets would thus be the dominate nature of droplets at the time of analysis.

Not all lactose esters yielded consistent stability profiles, with greater differences in oil droplet sizes and relative stabilities changing greatly as we moved away from LML. LMO and LMM used at 0.5% generated emulsions with oil droplet dispersions that changed greatly over time. Emulsions made with 0.5% LMD initially produced a bimodal oil droplet distribution with a large narrow peak in a range similar to LML, Tween-20 and Ryoto L-1695. As time elapsed, however, the droplet size distributions became wider and even more bimodal due to increased coalescence of the smallest oil droplets. LMD stabilized emulsions followed a similar droplet size distribution as LML over 5 days albeit more exaggerated, resulting in stable emulsions, though not as stable as those made with Tween-20 and Ryoto L-1695. Although LMM at a 0.5% concentration initially showed a large portion of very small oil droplet diameters of $\leq$0.5 μm, a bimodal oil droplet distribution remained over time.

LMO also showed small oil droplets and bimodality. LMO, though, had a very large increase in the proportion of oil droplets between 0.5 and 10 μm at 0.5% concentration during the course of the test. There was also a clear bimodal or multi-modal oil droplet size distribution growing toward larger droplets suggesting coalescence, over time. This might be an indicator of the lower hydrophobic moiety of LMO compared to its hydrophilic moiety which may result in unfavorable absorption on

the O/W interface leading to a great loss of stability over time. These similarities suggest that while the HLB values of LMO and LMM both are similar to each other, and the more successful LML and LMD, they do not accurately indicate the ability of each lactose ester to interface at the oil and water surfaces of oil droplets effectively, consequently leading to larger oil droplet diameters over time and the loss of emulsion stability. It is true that LMM and LMO stabilized emulsions did contain small oil droplets initially, however, as previously reported in the destabilization rates study, LMM was not an effective emulsifier, being surpassed in all trials by LMD and LML.

Studies consistently showed that emulsions tend to develop large oil droplets and large oil droplet distributions when low concentrations of sugar esters are added to an emulsion (Neta and others 2012; Garg and others 2010). Not only concentration, this is not surprising, higher interfacial concentrations of lactose esters facilitated smaller droplet sizes and prevented coalescence of droplets, leading to emulsion stability during the storage times.

D (3,2) data results and variances also yielded some date of interest and some of an abstruse nature. Additional means were used to understand droplet size effects as the D (3,2) data were not forthcoming in assertation of concreate result. As one might expect, there was an increase in average droplet sizes observed in emulsions with decreased emulsifier usage concentrations (0.25% and 0.1%) for each of the lactose esters. For each decrease in concentration, emulsion oil droplet sizes increased along with the development of greater oil droplet distributions which additionally became predominantly bimodal.

As part of this research, we investigated the effectiveness of CMC values for estimating initial emulsifier potential for lactose esters. Of the esters tested, Ryoto L-1695 and LML which have different sugar headgroup for a fixed hydrophobic group, had the CMC values of 0.44 mM and 0.72 mM, respectively. This difference is expected, as typically the hydrophobicity of the sugar headgroup of lactose esters can be considered to explain the variations observed in the CMC and HLB values as well. Giulietti and Bernardo (2012) reported that the sugars in order of highest solubility in water were fructose, sucrose, glucose followed by lactose. In general, less water solubility implies more hydrophobicity of sugar headgroups (Garofalakis and others 2000). Emulsifiers having greater hydrophobicity are theoretically more surface-active and thus facilitate micelle formation, exhibiting low CMC values and greater emulsification properties (Rao and McClements 2011). Lactose is thus more hydrophobic than the other mentioned simple sugars and may form more stable emulsions via its better ability to influence surface-interface stability. Garofalakis and others (2000) reported that emulsifiers with more hydrophobic headgroups, such as lactose, exhibited better surface activity than those with greater hydrophilic headgroups, such as sucrose. However, in this study, the lactose monolaurate showed a higher CMC value than Ryoto L-1695, which has sucrose as its headgroup. Zhang and others (2014) reported CMC values of lactose esters containing decanoate and laurate as slightly above the CMC values of Ryoto L-1695 (Table 4.1). Thus, we need to look at the effectiveness of the fatty acid chain lengths with lactose and if the resulting emulsification properties are correlated to the CMC values.

This study clearly shows that the increasing chain lengths of the fatty acids in the respective esters significantly influences the HLB values with a decrease in HLB value

due to increasing hydrophobicity. With HLB decreasing, the CMC also decreases with the increasing hydrophobicity resulting from the fatty acid chain lengths for a fixed hydrophilic group (Fig 4). As expected, LMM showed lower HLB and CMC values than LMO, LMD and LML since it has a longer fatty acid chain length. Yanke and others (2004) demonstrated that as fatty acid chain lengths were increased, sucrose monopalmitate was more hydrophobic than sucrose monolaurate, exhibiting lower CMC values of >28 μM. It stands to reason that in a like manner the hydrophobicity of LMM should make it a better 20% O/W emulsifier than the other lactose esters, as it should be more likely to be adsorbed at the O/W interface, resulting in lower CMC values as compared with LMO, LMD and LML. However, LMD and LML formed more stabilized emulsions, even with HLB values higher than those of LMM which would normally suggest poorer performance. The implication from these findings is that while HLB and CMC values may be an important characteristic for evaluating the activity of a non-ionic emulsifier; HLB and CMC values do not assure suitability for a specific application as an emulsifier. It may be that HLB and CMC values do not adequately describe the careful balance of hydrophobicity to hydrophily that truly dictate emulsifiers effectiveness at emulsion stability.

Conclusions

Results suggest that the chain length of the fatty acid ester significantly influences the emulsification properties of lactose esters in 20% soybean O/W emulsions. Lactose esters made with decanoic and lauric acid chains have a tendency toward higher effectiveness and stability in forming emulsions, whereas esters containing octanoate and

myristate are not able to maintain the stability of their emulsions. There is a linear decrease in both HLB and CMC values with an increase in fatty acid chain lengths. Both HLB and CMC parameters may be important characteristics for evaluating the activity of a non-ionic emulsifier; however, just because an emulsifier has an appropriate HLB and CMC value does not guarantee its appropriateness for a specific application as an emulsifier. It will be interesting to see how LML and LMD behave as emulsifiers in a salad dressing, or other food system, at the concentrations tested in this research (specifically 0.5%). Future research can also compare these two lactose esters to other commonly used food grade non-ionic emulsifiers to determine actually viability of use.

References

1. **Zhang X, Song F, Taxipalati M, Wei W, Feng F** (2014) Comparative study of surface-active properties and antimicrobial activities of disaccharide monoesters. PLoS ONE 9(12): e114845.
2. **Becerra N, Toro C, Zanocco AL, Lemp E, Günther G** (2008) Characterization of micelles formed by sucrose 6-O-monoesters. Colloids and Surfaces A: Physicochemical and Engineering Aspects 327(1–3): 134–139.
3. **Yanke L, Shufen Z, Qinghui W, Jinzong Y** (2004) Relationship of solubility parameters to interfacial properties of sucrose esters. Colloids and Surfaces A: Physicochemical and Engineering Aspects 248(1–3): 127–133.
4. **Piao J, Adachi S** (2006) Stability of O/W emulsions prepared using various monoacyl sugar alcohols as an emulsifier. Innovative Food Science & Emerging Technologies 7(3): 211–216.

5. Soultani S, Ognier S, Engasser J-M, Ghoul M (2003) Comparative study of some surface active properties of fructose esters and commercial sucrose esters. Colloids and Surfaces A: Physicochemical and Engineering Aspects 227(1–3): 35–44.

6. Ferrer M, Comelles F, Plou FJ, Cruces MA, Fuentes G, et al. (2002) Comparative surface activities of di- and trisaccharide fatty acid esters. Langmuir 18(3): 667–673.

7. Garofalakis G, Murray BS, Sarney DB (2000) Surface activity and critical aggregation concentration of pure sugar esters with different sugar headgroups. Journal of Colloid Interface Science 229(2): 391–398.

8. Walsh MK, Bombyk RA, Wagh A, Bingham A, Berreau LM (2009) Synthesis of lactose monolaurate as influenced by various lipases and solvents. Journal of Molecular Catalysis B: Enzymatic 60(3–4): 171–177.

9. Neta N do AS, Santos JCS, Sancho S de O, Rodrigues S, Gonçalves LRB, et al. (2012) Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions. Food Hydrocolloids 27(2): 324–331.

10. Chiralt A (2005) Food emulsions. Food Engineering–Vol. II. Oxford.

11. McClements DJ (2005) Food emulsions: Principles, practices, and techniques. CRC Press.

12. Formulaction (2009) Stability of various beverage emulsions. Food Application Paper 1-7. http://www.titanex.com.tw/doc/tecsupport/ANF-Turbiscan-application%20paper%20on%20%20beverage%20emulsions.pdf

13. Bai L, McClements D (2016) Extending emulsion functionality: Post-homogenization modification of droplet properties. Processes. Multidisciplinary Digital Publishing Institute 4: 17.

14. **Trujillo-Cayado LA, Natera A, García MC, Muñoz J, Alfaro MC, et al.** (2015) Rheological properties and physical stability of ecological emulsions stabilized by a surfactant derived from cocoa oil and high pressure homogenization. Grasas y Aceites 66(3): e087.

15. **Kaombe DD, Lenes M, Toven K, Glomm WR** (2013) Turbiscan as a tool for studying the phase separation tendency of pyrolysis oil. Energy Fuels 27(3): 1446–1452.

16. **Huck-Iriart C, Candal RJ, Herrera ML** (2011) Effect of processing conditions and composition on sodium caseinate emulsions stability. Procedia Food Science. 1: 116–122.

17. **Garg N, Martini S, Britt DW, Walsh MK** (2010) Emulsifying properties of lactose-amines in oil-in-water emulsions. Food Research International 43(4): 1111–1115.

18. **BeckmanCoulter** (2011) Instruction for use LS 13 320 laser diffraction particle size analyzer. Beckman Coulter Inc. https://www.beckmancoulter.com/wsrportal/techdocs?docname=B05577AB.pdf

19. **Szűts A, Szabó-Révész P** (2012) Sucrose esters as natural surfactants in drug delivery systems—A mini-review. International Journal of Pharmaceutics 433: 1–9.

20. **Ferrer M, Comelles F, Plou FJ, Cruces MA, Fuentes G, et al.** (2002) Comparative surface activities of di- and trisaccharide fatty acid esters. Langmuir 18(3): 667–673.

21. **Patist A, Bhagwat SS, Penfield KW, Aikens P, Shah DO** (2000) On the measurement of critical micelle concentrations of pure and technical-grade nonionic

surfactants. Journal of Surfactants and Detergents 3(1): 53–58.

22. **Suradkar YR, Bhagwat SS** (2006) CMC determination of an odd carbon chain surfactant (C_{13} E_{20}) mixed with other surfactants using a spectrophotometric technique. Journal of Chemical & Engineering Data 51(6): 2026–2031.
23. **Hait SK, Moulik SP** (2001) Determination of critical micelle concentration (CMC) of nonionic surfactants by donor-acceptor interaction with iodine and correlation of CMC with hydrophile-lipophile balance and other parameters of the surfactants. Journal of Surfactants and Detergents 4(3): 303–309.
24. **Walsh MK, Bombyk RA, Wagh A, Bingham A, Berreau** LM (2009) Synthesis of lactose monolaurate as influenced by various lipases and solvents. Journal of Molecular Catalysis B: Enzymatic 60(3–4): 171–177.
25. **El Rassi Z** (1995) Carbohydrate analysis : high performance liquid chromatography and capillary electrophoresis. Elsevier.
26. **Ritthitham S** (2009) Synthesis of sucrose fatty acid esters as catalyzed by alkaline protease AL 89 and *Candida antarctica* lipase B in hydrophilic solvents. Aalborg University.
27. **Tadros TF** (2013) Emulsion formation and stability. Wiley-VCH Verlag GmbH & Co. KGaA.
28. **Whitehurst RJ** (2004) Emulsifiers in food technology. Blackwell Publishing Ltd.
29. **Smith J** (1991) Food additive user's handbook. Interprint Ltd.
30. **Pawlik A, Kurukji D, Norton I, Spyropoulos F** (2016) Food-grade pickering emulsions stabilised with solid lipid particles. Food & Function 7(6): 2712–21.
31. **Giulietti M, Bernardo A** (2012) Crystallization by antisolvent addition and

cooling, Crystallization - Science and Technology, Dr. Andreeta M (Ed.), InTech

32. **Rao J, McClements DJ** (2011) Food-grade microemulsions, nanoemulsions and emulsions: Fabrication from sucrose monopalmitate & lemon oil. Food Hydrocolloids 25(6): 1413–1423.

Table 4.1. Properties of emulsifiers including HLB and CMC

Emulsifier	Composition	MW	HLB[a]	HLB[b]	CMC (mM)	Reported CMC (mM)
Tween-20	Sorbitan laurate ester ($C_{26}H_{50}O_{10}$)	1227.5	16.7	15.6	0.07	0.042 (Patist and others 2000)
Ryoto L-1695	Sucrose monolaurate ester ($C_{24}H_{44}O_{12}$)	524	16	15	0.44	0.42 (Zhang and others 2014)
LMO	Lactose monooctanoate ester ($C_{20}H_{36}O_{12}$)	468.54	16.9	15.8	0.96	0.76 (Zhang and others 2014)
LMD	Lactose monodecanoate ester ($C_{22}H_{40}O_{12}$)	496.55	16	14.8	0.89	0.56 (Zhang and others 2014)
LML	Lactose monolaurate ester ($C_{24}H_{44}O_{12}$)	524.28	15.7	14.1	0.72	0.31 (Zhang and others 2014)
LMM	Lactose monomyristate ester ($C_{26}H_{48}O_{12}$)	552.66	14.1	13.4	0.56	NR

MW, molecular weight; HLB, hydrophile-lipophile balance; CMC, critical micelle concentration; NR, not reported

[a] The formula HLB= 7 + ∑ (hydrophilic values) - ∑ (lipophilic values) was used (McClements 2005)

[b] The formula HLB= [(L/T) * 20] was used (Ritthitham 2009)

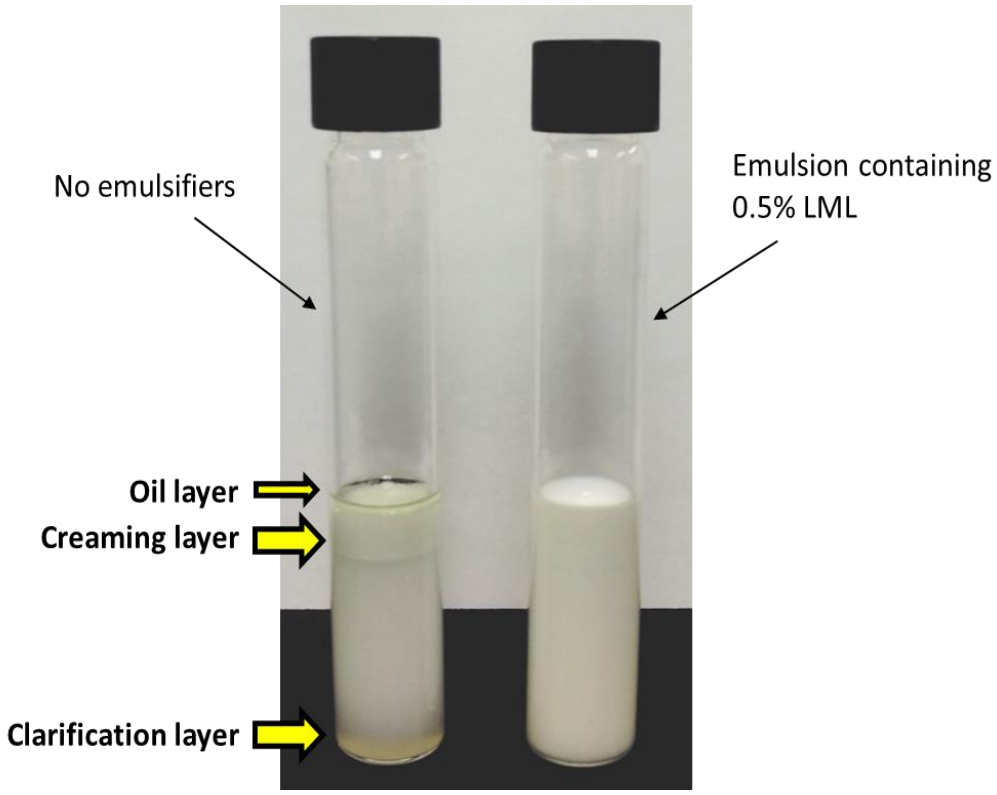

Figure 4.1. Clarification layer (0.5-10 mm) from the bottom of each tube tested for 20% O/W emulsion (A) with no emulsifiers and (B) the emulsion prepared with LML after 5 days

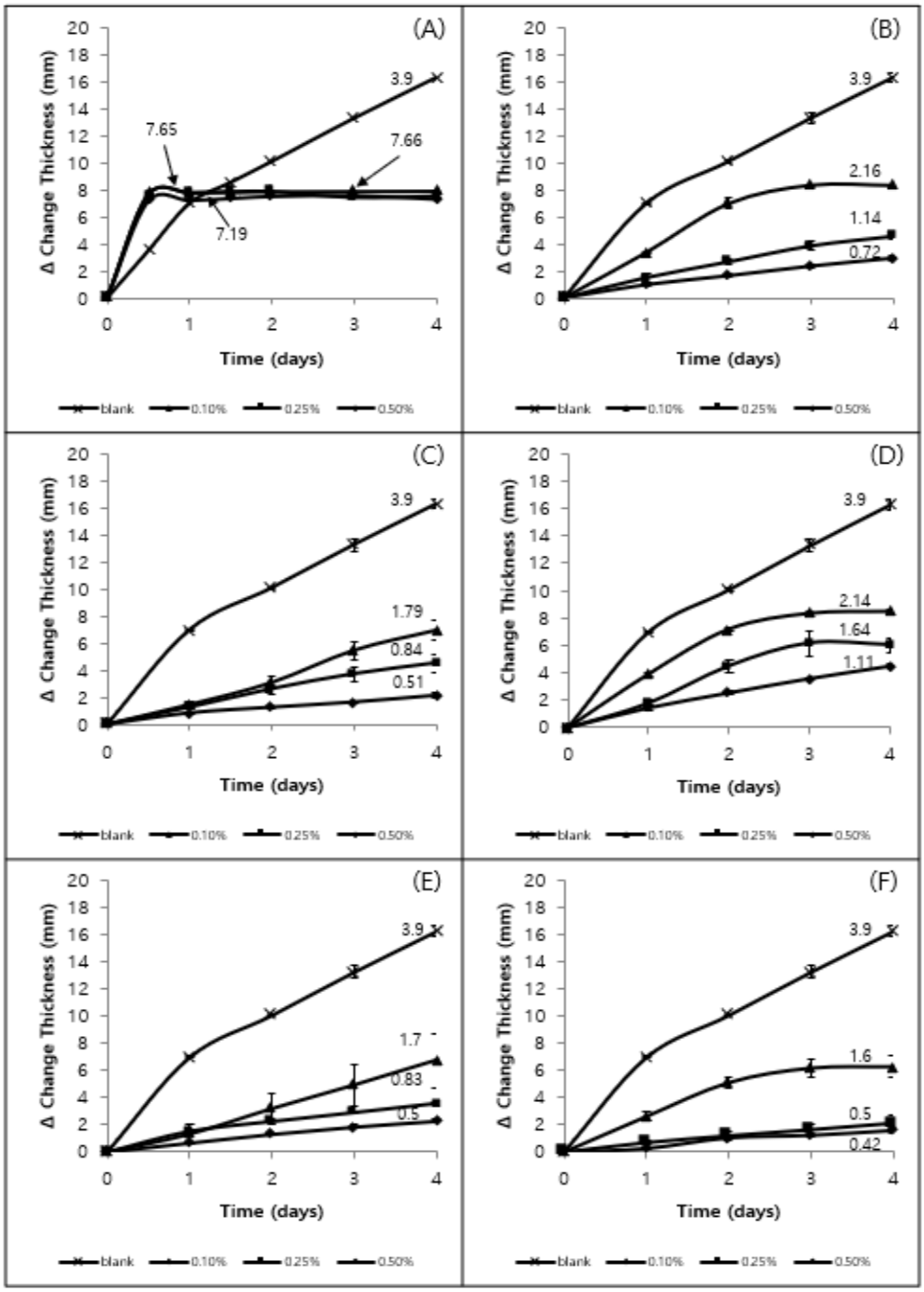

Figure 4.2. Change in thickness (mm) of clarification layer of the emulsions at 25 °C: The rate of destabilization (at the bottom of the tube from 0.5-10 mm) of the clarification layer of the emulsions formulated with (A) LMO; (B) LMD; (C) LML; (D) LMM; (E) Tween-20; (F) Ryoto L-1695 at concentrations of (▲) 0.1%, (■) 0.25% and (◆) 0.5% was compared to negative control (x) over 5 days period. The error bars indicate the standard errors.

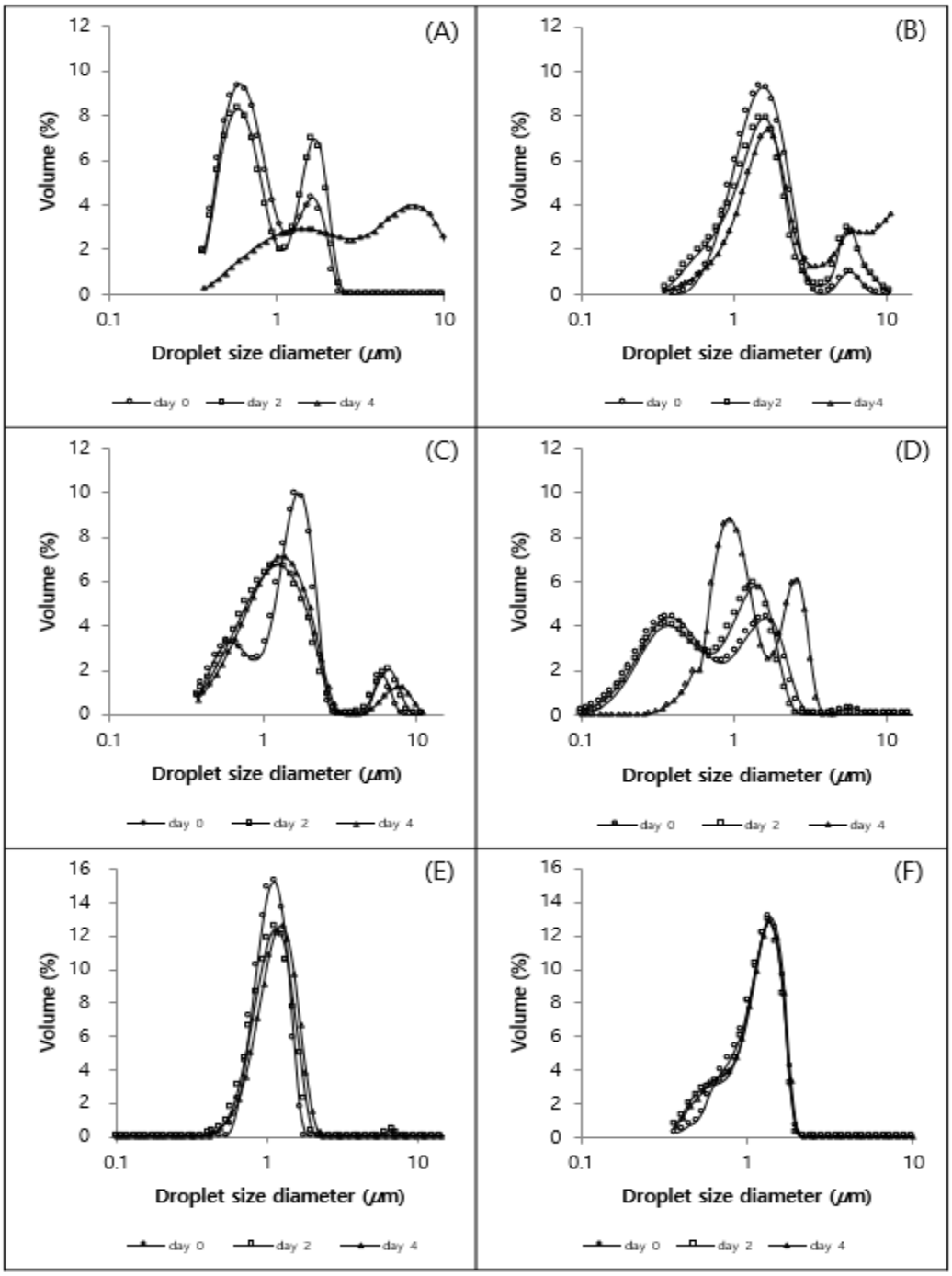

Figure 4.3. Oil droplet size diameter (μm) distribution with volume (%) of the emulsions at 25 ℃: Droplet size diameter (μm) distribution with respect to percentage of the volume (%) at (A) 0.5% LMO; (B) 0.5% LMD; (C) 0.5% LML; (D) 0.5% LMM; (E) 0.5% Tween-20; (F) 0.5% Ryoto L-1695 on (○) day 0, (□) day 2 and (△) day 4 were investigated.

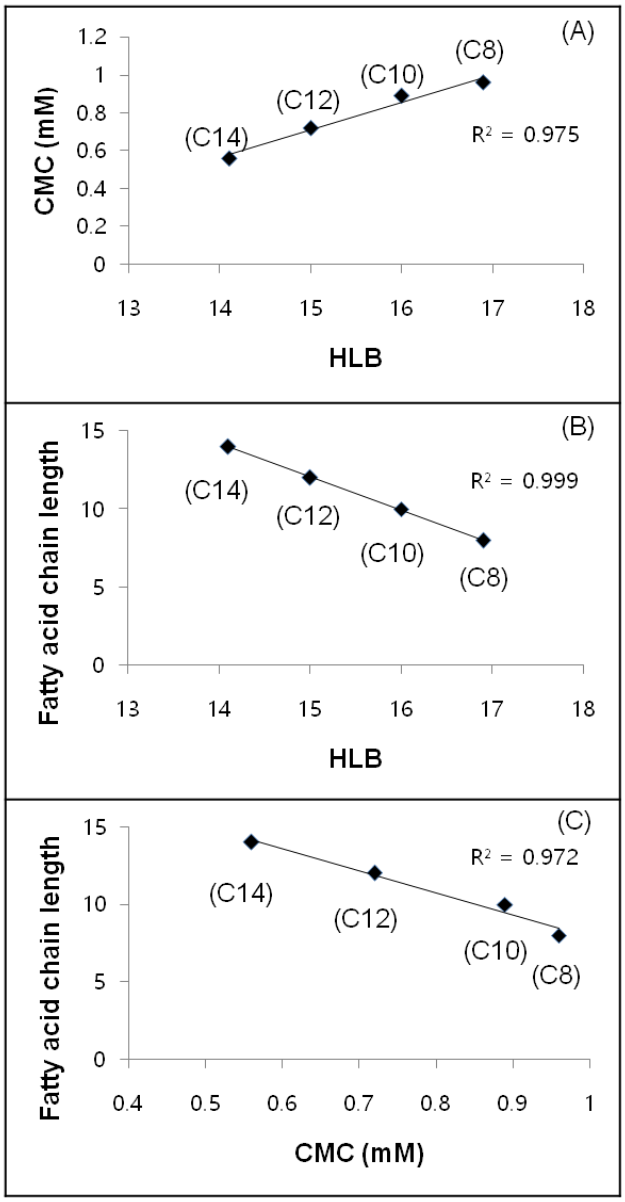

Figure 4.4. Comprehensive comparison of HLB, CMC values and fatty acid esters of lactose: Influence of the chain length of the fatty acid ester on the HLB and CMC value of water at 25 °C, for lactose esters containing octanoate (C8), decanoate (C10), laurate (C12) and myristate (C14).

CHAPTER 5

ANTI-LISTERIAL ACTIVITY OF LACTOSE FATTY ACID ESTERS IN MILK

Abstract

Sugar esters have been shown to inhibit pathogens in foods. The anti-listerial activities of the novel esters, lactose monodecanoate (LMD) and lactose monolaurate (LML) were tested in different milk samples with variable fat and temperature treatments. Both LMD and LML in these tests demonstrated anti-listerial properties against a 5-strain cocktail of *listeria* in milk, though the observed log reductions in the LMD milk samples were higher than those in the LML at all tested temperatures (5, 24 ℃ and 37 ℃). LMD in 1% and 2% fat milk was found to be the most effective microbial inhibitory ester with absence of viable bacterial growth on days 3 and 4, respectively.

Introduction

Food-borne infection, caused by *Listeria monocytogenes,* is a major public health problem worldwide, resulting in millions of severe food-poisoning cases each year. *L. monocytogenes* has been isolated from food sources, such as high moisture dairy products, raw meat, ready-to-eat food (RTE) and food processing environments (Coroneo and others 2016; Pesavento 2010). The anti-listerial effect of sugar esters has traditionally be measured and reported as minimum inhibitory concentrations (MIC) (Lee and others 2017; Chen and others 2014; Wagh and others 2012; Nobmann and others 2009). Sugar

esters are non-ionic emulsifiers used in a variety of applications in the food, pharmaceutical, and personal care industries (Kralova and Sjöblom 2009). A limited number of studies on the microbial inhibitory activity of these esters in food systems have been conducted (Chen and others 2014; Xiao and others 2011; Yang and others 2003).

Lactose based esters, a class of sugar esters, are important as they are environmentally friendly and can be synthesized using renewable resources (Walsh and others 2009). One recent study by Chen and others (2014) showed that the anti-listerial activity of LML in dairy products against *L. monocytogenes* was affected by the fat content (0.5%, 1% and 3.25%) at 37 ℃. Milk products, though, can come in even higher fat quantities than these (such as half-and-half, 12% milk fat and whip cream, 36% milk fat) so this study investigated the microbial inhibitory properties of lactose esters at higher fat values. Also, there was not any data in the literature on the log reductions of *L. monocytogenes* resulting from sugar esters use below 37 ℃. In this study, the anti-listerial effect of LMD and LML by determining the log reductions in milk at various fat content and temperatures was investigated in order to evaluate the use of lactose esters in food systems.

Materials and Methods

Bacterial Strains

The bacterial strains used are displayed in Table 5.1. Different clinical isolates of *Listeria* (C1-056, J1-177, N1-277, N3-013 and R2-499) were obtained from Dr. Martin Wiedmann, director of the International Life Sciences Institute North American Database at Cornell University.

Materials and Equipments

A high-performance liquid chromatography (HPLC) (Beckman System Gold 125 Solvent Module, Ontario, Canada) equipped with Luna 5 lm C18 100 A ° (250 mm X 4.6 mm, Phenomenex, Torrance, CA, USA), an evaporative light scattering detector (Agilent Technologies, Santa Clara, CA, USA), incubator shaker (Beckman, USA), spectrophotometer (Beckman, Portland, OR, USA) and Ultra-turrax T25 (Janke and Kunkel, Staufen, Germany) were provided by Utah State University. Lactose (Proliant, IA, USA) and acetonitrile (HPLC grade, Thermo Fisher, PA, USA) were also prepared. Brain heart infusion (BHI) media, lipase TM3 (immobilized from *Thermomyces lanuginosus*), Whatman glass microfiber filters, molecular sieves (3A), 2-methyl-2-butanol (2M2B) (dried using 10% 3A molecular sieves), dimethyl sulfoxide (DMSO) and 1, 5 ml macro cuvettes were supplied by Sigma (Aldrich, MO, USA). Vinyl decanoate and vinyl laurate were from TCI (Portland, OR, USA). Fat free milk (1% fat), low fat milk (2% fat), whole milk (3.5% fat) and whip cream (36% fat) were obtained from Gossner Foods Inc (Logan, UT, USA).

Synthesis of Lactose Esters

Enzymatic synthesis of LML was performed according to Walsh and others (2009). Synthesis of LMD was conducted using lactose, vinyl decanoate, molecular sieves and immobilized lipase enzyme TM3. For a 60 ml reaction in 2M2B, 3 g of lactose, 6 g of dried molecular sieves, 870 µl of vinyl decanoate (lactose to fatty acid ratio of 1:1) and 1.8 g TM3 were combined. The reactions were assembled in a 100 ml glass bottle and incubated at 55 ℃ and 90 rpm for 2 days. The amount of LMD

synthesized was determined using HPLC with the evaporative light scattering detector set at 60 ℃ with a nitrogen gas pressure of 3.55 bar. There was a gradient from 10% acetonitrile–water (40:60, v/v) to 95% acetonitrile–water (95:5, v/v) as the mobile phase.

Purification of Lactose Esters

For ester purification, the 2M2B reaction was filtered through a Whatman glass microfiber filter then dried in a hood for 48 hrs. The dry solid of LML was suspended in a 50% hexane, 50% water solution while the dry solid of LMD was suspended in a 50% ethanol, 50% water solution. These were then placed in a separatory funnel. The lower aqueous layer was drained into a beaker and dried in a hood for 48 hrs. After completely drying, the product powder was suspended in hexane, and then centrifuged for 15 min at room temperature at 2000 x g and the supernatant analyzed via HPLC for the presence of di- tri- or higher saccharides. The hexane extraction was repeated until only the monoester was present in the pellet. The purities of the lactose esters were confirmed to be greater than 85% by HPLC analysis.

Microbial Inhibitory Studies in Milk

Making a 5-strain cocktail of *Listeria* including C1-056, J1-177, N1-277, N3-013, and R2-499 was prepared. The 5 stocks were stored individually at -80 °C, and each individual freezer stock (20 µl) was added to 15 ml of BHI media. The *Listeria* strains were grown at 37 °C and 200 rpm for 24 hrs. Aliquots (2 ml) from each strain were combined in a test tube to develop the 5-strain stock cocktail. Aliquots (315 µl) of the stock cocktail were grown in BHI media (12 ml), and incubated with shaking at 37 °C for 4 hrs. Aliquots of the 5-strain stock cocktail were kept at -80 °C. The growing cultures

were monitored by optical density measurements at 600 nm (OD600) and diluted with fresh media to reach an OD600 of 0.2, which was determined by plating on BHI agar to be equivalent to between 10^5 and 10^6 cfu/ml. After the optical density was standardized at 0.2, an aliquot of the culture, 100 µl was mixed with 10 ml of fresh media, and then centrifuged (4000 rpm, 15 min, 4 ℃). The resulting pellets from the centrifugation were then resuspended in 10 ml of fresh 0.1% phosphate-buffered saline (PBS). This allowed the treatment cultures to be standardized for each test.

Identical stock solution concentrations (93.75 mg/ml) of each of LMD and LML were prepared in 100% DMSO. Ester stock solutions were diluted into sterilized milk to give a final DMSO concentration 2.5% as described by Lee and others (2017). Sterile milk samples with various levels of fat were also prepared by mixing fat free milk (1% fat) with heavy whipping cream (36% fat). Each of the various fat levels was achieved by calculating how much total fat was needed in each for a given volume of 100 ml. Once each samples' fat content was calculated, the required amount of cream to achieve that level was added with the remaining volume being filled by 1% fat milk. To these were added either LML or LMD, which was then blended at 18,000 rpm (Ultra-turrax T25) for 1 min to obtain a homogeneous solution. The different fat levels used for each lactose ester are detailed below, along with the temperature at which these solutions were later to be incubated (Table 5.2). The lactose esters in a stock solution were then added to each well for a final concentration 5 mg/ml and each well contained a total of 10 ml, each containing 10^5 to 10^6 cfu/ml of the listeria cocktail prepared as described above. Controls contained the same concentration of DMSO as the treatments. These were then incubated at 5, 24 ℃ and 37 ℃, respectively. Each combination of milk fat level, lactose ester

treatment, and microbial inoculation were tested a total of eight times, two tests of four samples, then they were compared to control to confirm results. Survival and growth of *L. monocytogenes* was monitored daily for 6 days to determine the log reduction by plate counts. A paired T-test was used to compare the treatments with the controls at each concentration to determine if the treatments were significantly different from the controls.

Results

LMD was evaluated for anti-listerial effect at different milk fat contents (1%, 2%, 3.5%, 7%, 8%, 9%, 10%, 11%, 14% and 36%) at 37 ℃ with a final concentration of 5 mg/ml ester in each sample (Fig 5.1 and Fig 5.2). BHI growth media was also tested along with the milk samples for comparison. When testing the antimicrobial activity of LMD in growth media at 37 ℃, 3 and 8 log reductions in cells were observed after 1 and 2 days respectively with no viable cells on day 3. With 1% fat milk, 4.5 and 8.2 log reductions were observed on days 1 and 3. With 2% fat milk, 2 and 6.5 log reductions were observed on days 1 and 3. With 3.5% fat milk, 2 and 6 log reductions were observed at days 1 and 3. With 7% milk fat, 1.8 and 4.8 log reductions were observed at days 1 and 3. With 8% milk fat, 1.6 and 2.3 log reductions were observed at days 1 and 3. LMD in 1% and 2% fat milk was found to be the

most effective microbial inhibitory ester since it showed absence of viable bacterial growth on day 3 and 4, respectively. Additionally, milk samples with fat concentrations up to 9% showed measurable and significant log reductions of *L. monocytogenes* over 6 days. There was no observed inhibition of the *L. monocytogenes* in 10%, 11%, 14% and 36% milk fat with LMD at 37 °C.

The anti-listerial effectiveness of LMD was also tested at 5 ℃ at different fat contents (1%, 2% and 7%) (Fig 5.3). Results of LMD at this temperature were as follows: in 1% fat milk, 2.6 and 2.4 log reductions in cells were observed after 5 and 6 days respectively; in 2% fat milk fat, 2.2 and 3 log reductions were observed on days 5 and 6; and finally, at 7% milk fat no significant log reductions were observed.

The anti-listerial effectiveness of LMD was also tested at 24 ℃ at different fat contents (1%, 2% and 10%) (Fig 5.4). Results of LMD at this temperature were as follows: in 1% fat milk, 6.7 and 7.5 log reductions in cells were observed after 5 and 6 days respectively; in 2% fat milk, 4.8 and 5.6 log reductions were observed on days 5 and 6; and finally, at 10% milk fat no significant log reductions were observed.

LML also was evaluated for anti-listerial effect at different milk media fat contents (1%, 2%, 3.5%, 7%, 8% and 36%) at 37 ℃ with a final concentration of 5 mg/ml ester in each sample (Fig 5.5 and Fig 5.6). BHI growth media was also tested along with the milk samples for comparison. When testing the antimicrobial activity of LML in growth media at 37 ℃, 3 and 7.2 log reductions in cells were observed after 1 and 2 days respectively with no viable cells on day 3. With 1% fat milk, 2.1, 3.7, and 7.4 log reductions were observed on days 1, 3 and 6. With 2% fat milk 5 log reductions were observed on day 6. With 3.5% milk fat, 3 log reductions were observed at day 6. Additionally, milk samples with fat concentrations up to 3.5% showed measurable and significant log reductions of *L. monocytogenes* over 6 days, however LML was not shown to be bactericidal in the same samples. There was no observed inhibition of the *L. monocytogenes* in 7%, 8% and 36% milk fat.

The anti-listerial effectiveness of LML was also tested at 5 ℃ and 24 ℃, respectively, at different fat contents (1% and 2%) (Fig 5.7 and Fig 5.8). Results of LML at 5 °C and 24 °C showed that there was no observed inhibition of the *L. monocytogenes* in 1% and 2% milk fat.

Discussion

Previous data (Chapter 3) showed that the fatty acid chain length of lactose esters significantly influenced the microbial inhibitory activity of these esters towards Gram-positive bacteria in growth media. The tests showed minimum inhibitory concentrations (MIC) for LMD and LML for *L. monocytogenes* above 3 mg/ml (≤5.7 mM). In a similar study, Nobmann and others (2009) reported that the lauric ether of methyl α-D-glucopyranoside and the lauric ester of methyl α-D-mannopyranoside showed the greatest activity against *L. monocytogenes* at concentrations of 0.04 mM.

This study was mainly focused on determining the anti-listerial activities of both LMD and LML, and how fat content of milk and temperature influence these activities. One recent study (Chen and others 2014) showed that *L. monocytogenes* is affected by a concentration of ≤5 mg/ml LML at different fat contents (0.5%, 1% and 3.25%) in milk at 37 ℃. Milk products, though, can come in even higher fat quantities than these (such as half-and-half and cream) so additional testing was performed at these higher levels. Also, there was not any data in the literature on the log reductions of *L. monocytogenes* resulting from sugar esters use below 37 ℃, so the tests were performed on LMD and LML at 5 and 24 ℃ as well as at 37 ℃.

An addition of either LMD or LML for each milk sample was first dissolved in 2.5% DMSO. Previous research has noted that the solvent (DMSO) used to dissolve these esters was not inhibitory to *L. monocytogenes* (Lee and others 2017) whereas Chen and others (2014) showed that 50% ethanol itself was inhibitory. Additional research on the microbial inhibitory activity of these esters in food systems without needing to first dissolve in DMSO is needed. In the current absence of such a method (using the lactose esters without a solvent), DMSO was added.

Both LMD and LML demonstrated anti-listerial properties in tested milk samples, though the observed log reductions in the LMD milk samples were greater than those with LML at all tested temperatures (5, 24 ℃ and 37 ℃). This suggests that LMD may not partition into the fat phase as readily as LML and thus remains more available in the liquid phase for microbial inhibitory activity. Additionally, LMD (but not LML) showed listerial inhibitory action at all temperatures including 5 ℃, a standard milk refrigerated storage temperature. LMD, as a milk additive, may help further combat contamination of *L. monocytogenes*, a pathogen which has long proven a bother for food processors due to its ability to grow even at refrigerated temperatures. No longer content to just slow listerial growth, listerial load would be reduced or eliminated in food systems with less than 9% fat at 37 °C and at 5 °C with less than 2% fat.

It is also interesting to note the cut off in effectiveness on anti-listerial activity for LMD seems so sudden, with 9% showing effect and 10% not. While these fat levels are close together, these results may suggest that the effective cut off for listerial inhibitory action is indeed in this range and is rather sudden. Additional research into the mechanics of action and specific fat levels may be warranted to better understand this observation.

Future testing could investigate this potential action as part of understanding the mechanisms for anti-listerial activity. Loss of antimicrobial activity in higher fat milk samples may be related to multiple interactions within the milk. To begin with, having more milk fat may cause more ester to be bound at the fat water interaction phases leading to less ester available for anti-listerial activity in the water phase of the milk. It has been also suggested that fats in solutions contribute to biofilm formation, thus higher fat in milk may cause more biofilms to develop faster, protecting bacterial cells for antimicrobial agents (Brebbia and Echarri 2017). Either or both activities could account for the loss of anti-listerial activity seen the tested samples. The mechanism of action of these esters in food will be interesting to study since it has not been previously reported.

There are also several explanations for decreasing temperatures reducing anti-listerial activity of lactose esters as seen in this research. First, hydrophobic interactions are directly dependent on temperature with lower temperature decreasing or eliminating hydrophobic interactions. If the lactose esters' anti-listerial activity is dependent upon hydrophobic interactions, it is easy to understand that the reduction or loss of these reactions would necessarily decrease or eliminate anti-listerial properties as well. Moreover, physical changes occur at reduced temperatures, it is possible that listerial cell walls and membranes become less malleable or interactive at lower temperatures interfering with interactions with the outer environment including those that might be of an anti-listerial sort. Again, either or both would explain the observed results from this research.

Conclusions

Results suggest that lactose-based esters, a class of sugar esters, are important, as they are environmentally friendly and can be synthesized using renewable resources. Although both LMD and LML in these tests showed anti-listerial properties at different fat contents in milk medium at 37 ℃, specifically, LMD in 1% and 2% milk fat only showed listerial inhibitory action at other temperatures including 5 ℃ and 24 ℃ as well. LMD, as a food additive, may play a role in increasing the safety of foods as it has shown a significant ability to reduce listerial in milk samples at multiple fat and temperature levels. Additional research on the anti-listerial activity of these esters in determination other effects such pH is needed.

References

1. **Brebbia CA, Echarri V.** 2017. Water and society–WIT Transactions on ecology and the environment. WIT Press.
2. **Chen Y, Nummer B, Walsh MK.** 2014. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Lett Appl Microbiol. 58:156–162.
3. **Coroneo V, et al.** 2016. Detection of Virulence Genes and Growth Potential in *Listeria monocytogenes* Strains Isolated from Ricotta Salata Cheese. J Food Sci. 81:M114–M120.
4. **Kralova I, Sjöblom J.** 2009. Surfactants used in food industry: A Review. J Dispers Sci Technol. 30(9): 1363–1383.
5. **Lee S-M, Sandhu G, Walsh MK**. 2017. Growth inhibitory properties of lactose fatty acid esters. Saudi J Biol Sci. 24:1483–1488.

6. **Nobmann P, Smith A, Dunne J, Henehan G, Bourke P.** 2009. The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. Int J Food Microbiol. 128:440–445.
7. **Pesavento G, Ducci B, Nieri D, Comodo N, Lo Nostro A.** 2010. Prevalence and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. Food Control. 21:708–713.
8. **Wagh A, Shen S, Shen FA, Miller CD, Walsh MK.** 2012. Effect of lactose monolaurate on pathogenic and nonpathogenic bacteria. Appl Environ Microbiol. 78:3465–8.
9. **Walsh MK, Bombyk RA, Wagh A, Bingham A, Berreau LM.** 2009. Synthesis of lactose monolaurate as influenced by various lipases and solvents. J Mol Catal B Enzym. 60:171–177.
10. **Xiao D, et al.** 2011. Sucrose monolaurate improves the efficacy of sodium hypochlorite against *Escherichia coli* O157:H7 on spinach. Int J Food Microbiol 145:64–68. Int J Food Microbiol. 145:64–8.
11. **Yang C-M, Luedecke LO, Swanson BG, Davidson PM.** 2003. Inhibition of microorganisms in salad dressing by sucrose and methylglucose fatty acid monoesters. J Food Process Preserv. 27:285–298.

Table 5.1. Series of microorganisms involved in the study

No.	Microorganisms	ATCC no./serovar	Gram reaction[a]	Growth medium
1	*Listeria monocytogenes*	FSL C1-056	+	BHI
2	*Listeria monocytogenes*	FSL J1-177	+	BHI
3	*Listeria monocytogenes*	FSL N3-013	+	BHI
4	*Listeria monocytogenes*	FSL R2-499	+	BHI
5	*Listeria monocytogenes*	FSL N1-227	+	BHI

[a] +, positive

Table 5.2. An outline of the different milk fat levels at 5, 24 °C and 37 °C used for each lactose ester

37 °C		
Lactose ester	Concentration	Fat content in milk (%)
LMD	5 mg/ml	1, 2, 3.5, 7, 8, 9, 10, 11, 14, 36
LML	5 mg/ml	1, 2, 3.5, 7, 8, 36
5 °C		
Lactose ester	Concentration	Fat content in milk (%)
LMD	5 mg/ml	1, 2, 3.5, 7
LML	5 mg/ml	1, 2
24 °C		
Lactose ester	Concentration	Fat content in milk (%)
LMD	5 mg/ml	1, 2, 3.5, 7, 10
LML	5 mg/ml	1, 2

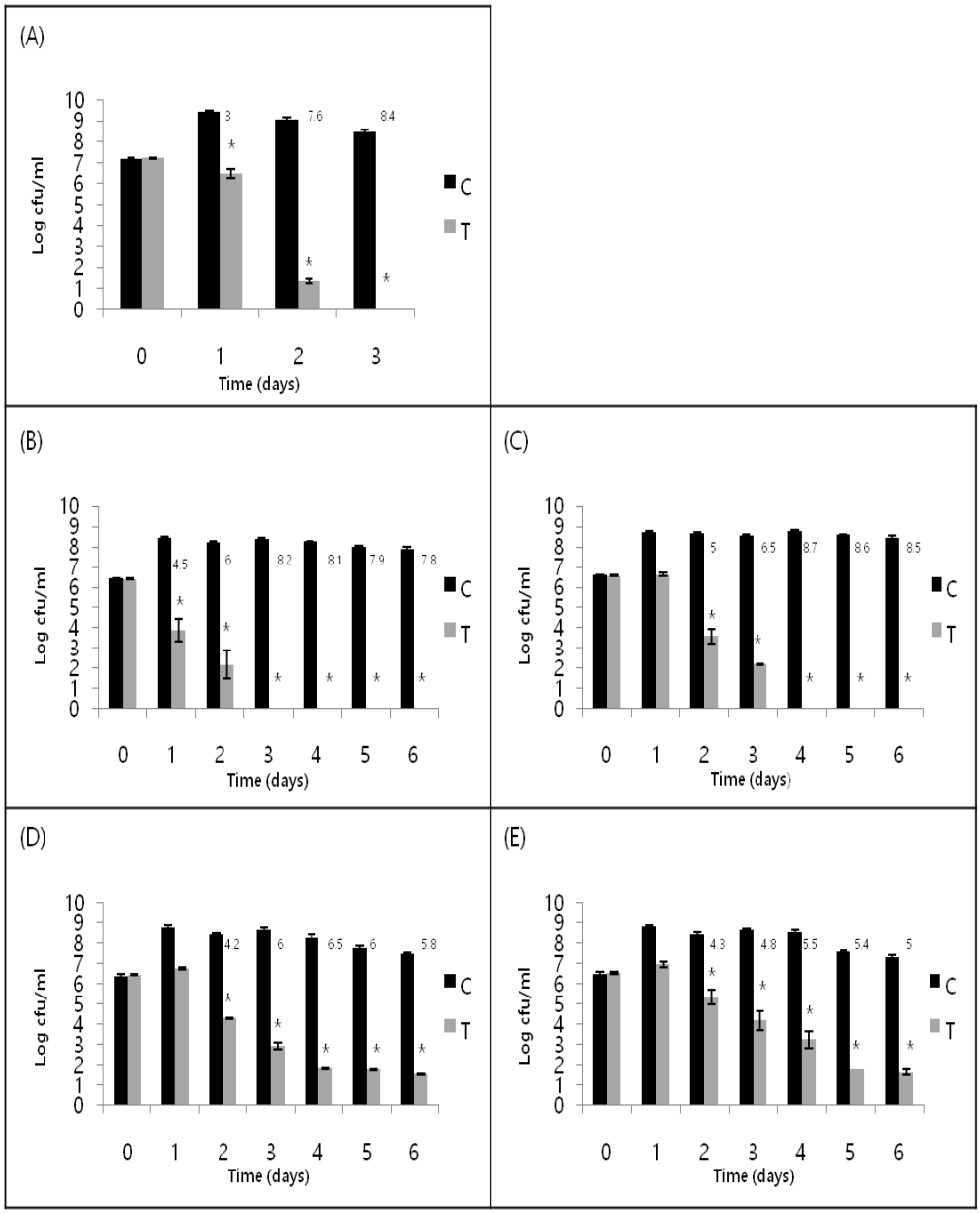

Figure 5.1. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LMD over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) BHI media (B) 1% fat milk (C) 2% fat milk (D) 3.5% fat milk and (E) 7% fat milk at 37 ℃. The numbers above the treatment bars represent the log reductions.

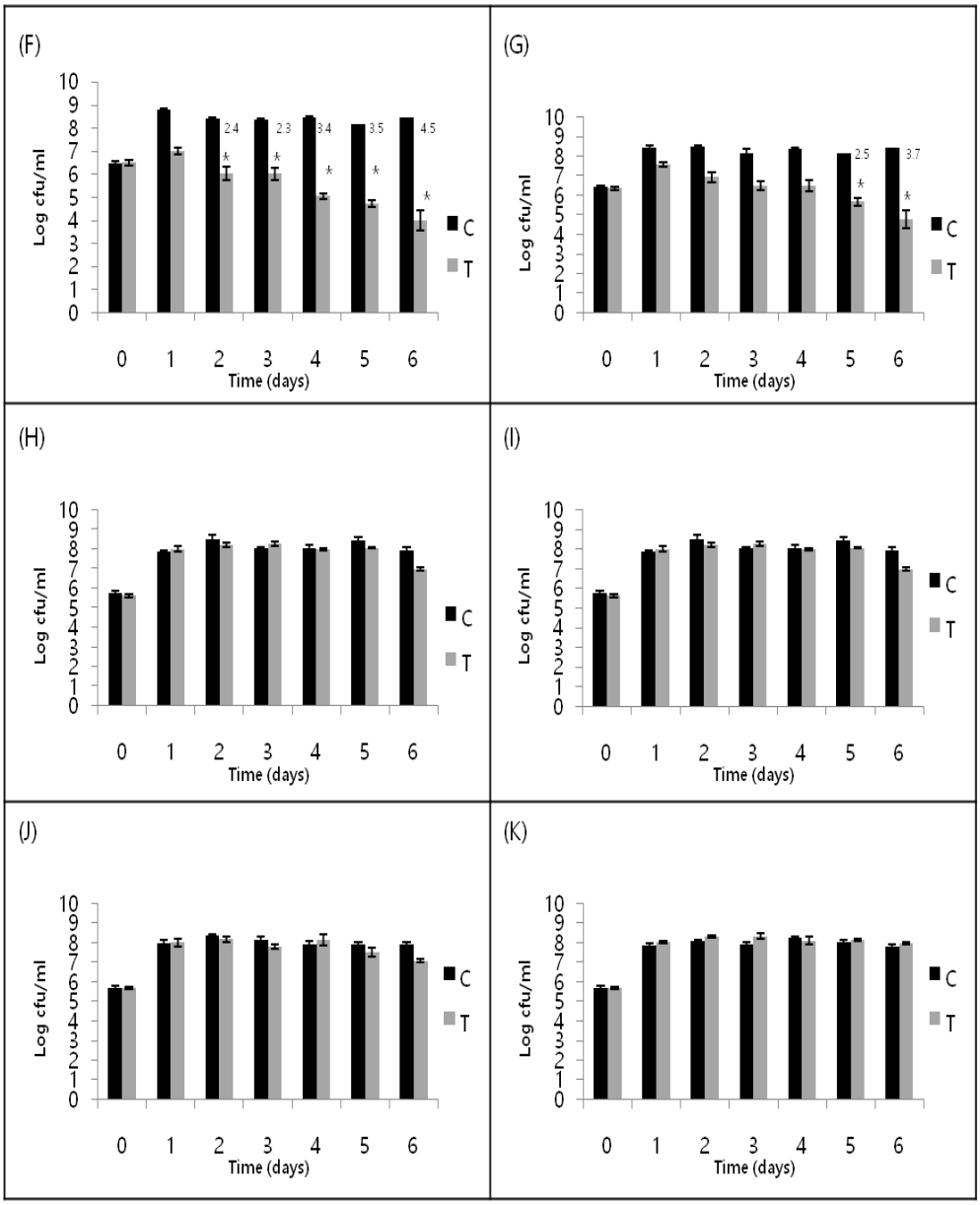

Figure 5.2. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LMD over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (F) 8% fat milk (G) 9% fat milk (H) 10% fat milk (I) 11% fat milk (J) 14% fat milk and (K) 36% fat milk at 37 ℃. The numbers above the treatment bars represent the log reductions.

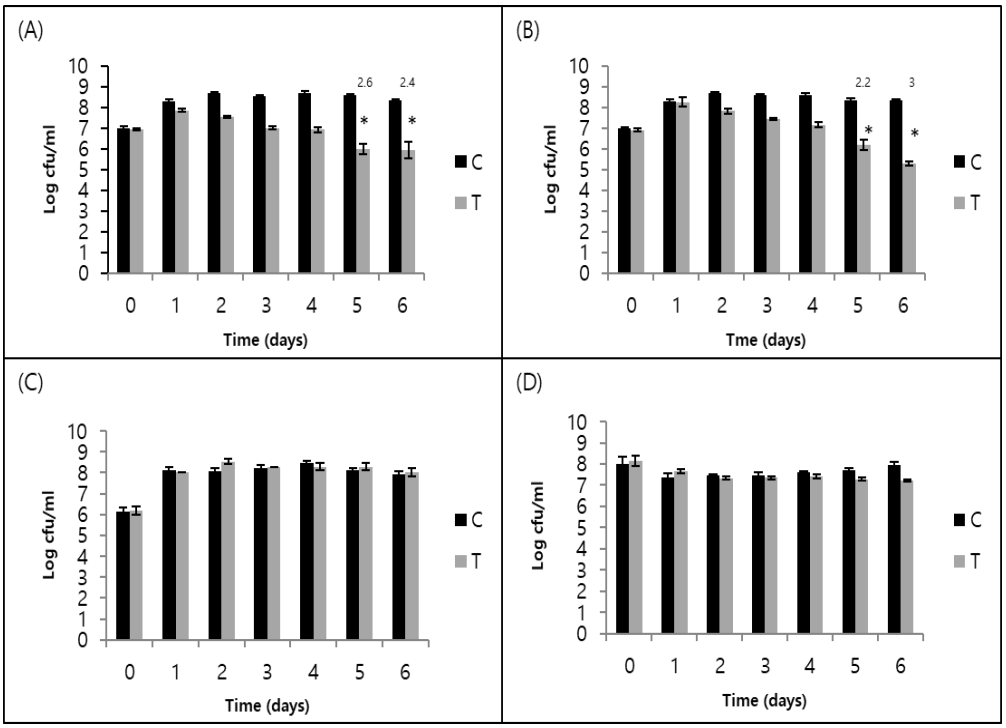

Figure 5.3. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LMD over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) 1% fat milk (B) 2% fat milk and (C) 3.5% fat milk and (D) 7% fat milk at 5 ℃. The numbers above the treatment bars represent the log reductions.

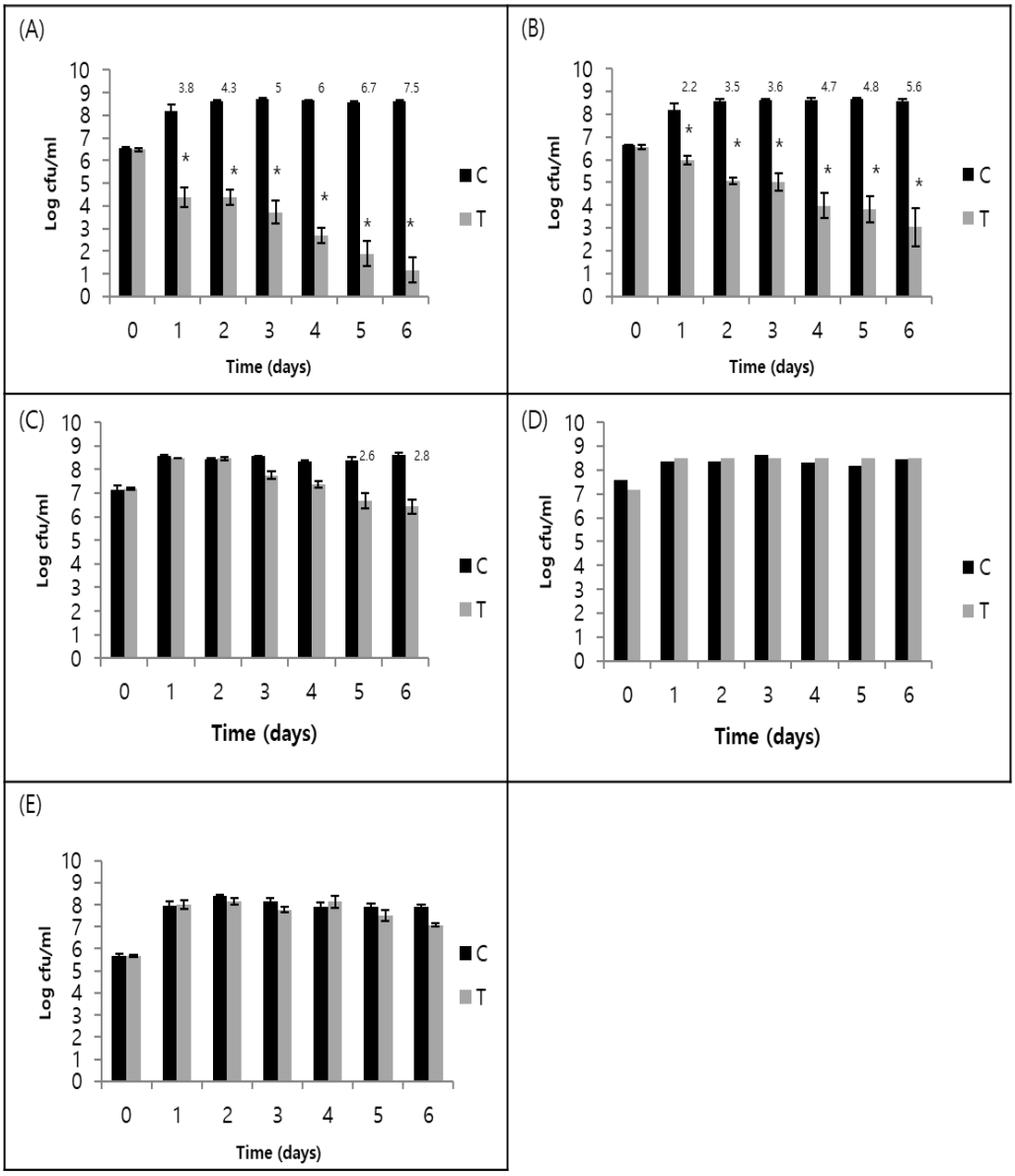

Figure 5.4. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LMD over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) 1% fat milk (B) 2% fat milk, (C) 3.5% fat milk, (D) 7% fat milk and (E) 10% fat milk at 24 ℃. The numbers above the treatment bars represent the log reductions.

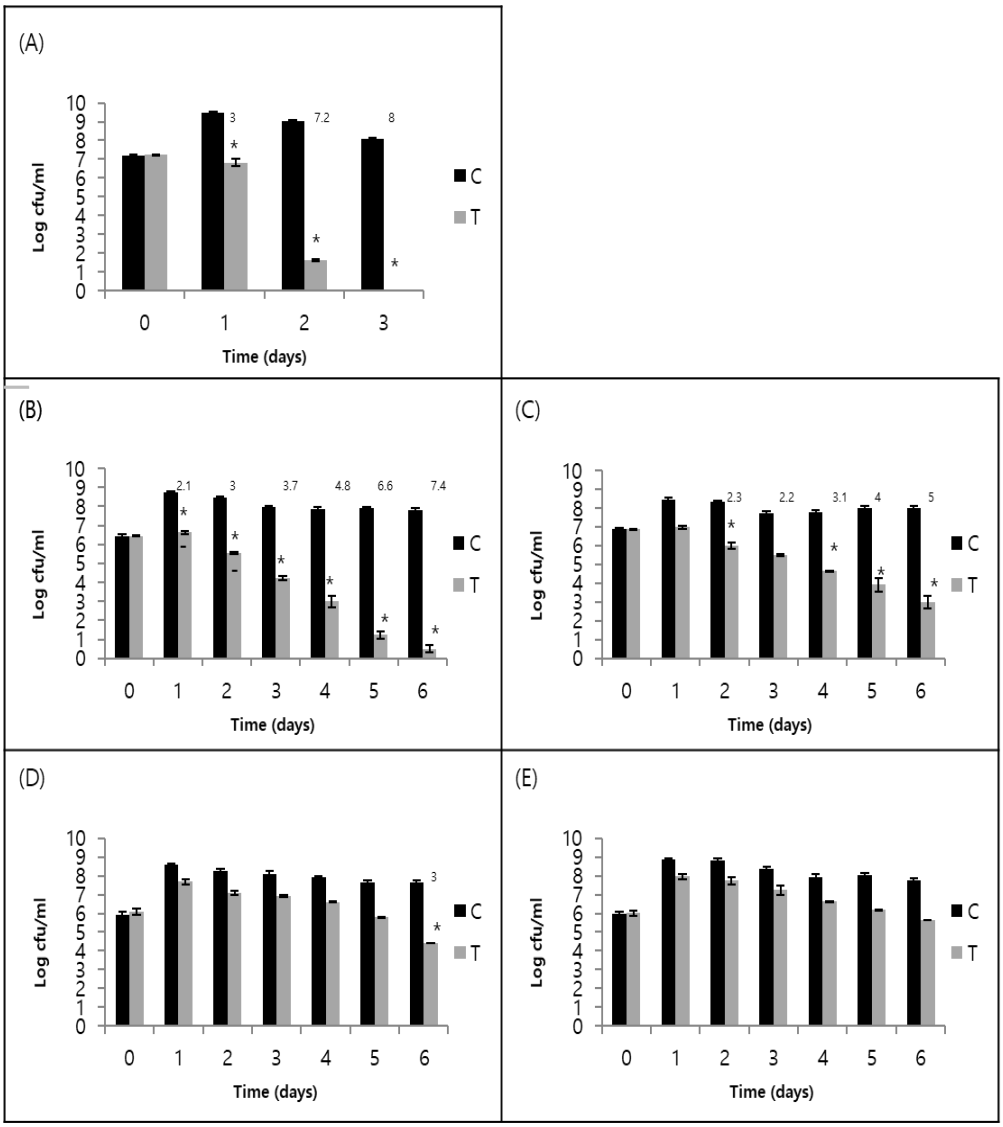

Figure 5.5. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LML over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) BHI media (B) 1% fat milk (C) 2% fat milk (D) 3.5% fat milk and (E) 7% fat milk at 37 ℃. The numbers above the treatment bars represent the log reductions.

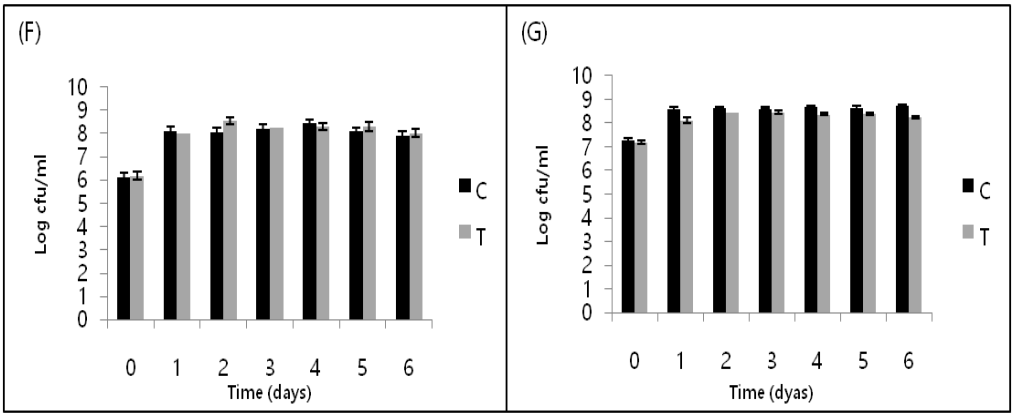

Figure 5.6. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LML over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (F) 8% fat milk and (G) 36% fat milk at 37 ℃.

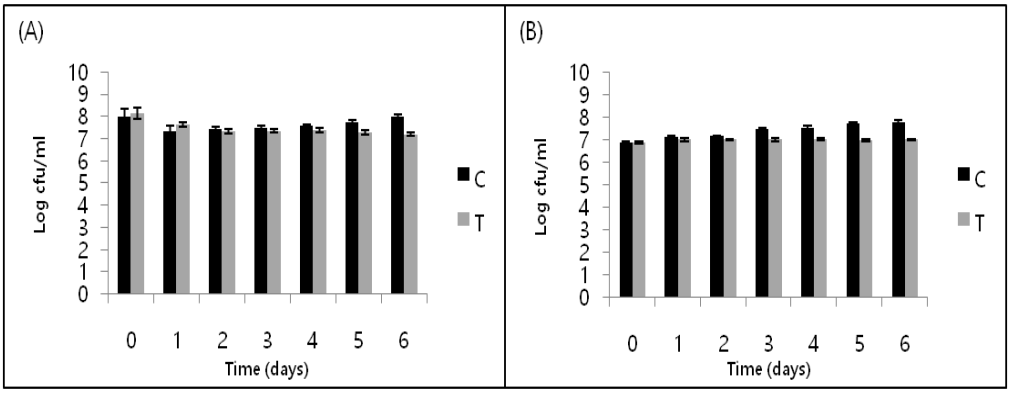

Figure 5.7. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LML over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) 1% fat milk and (B) 2% fat milk at 5 ℃.

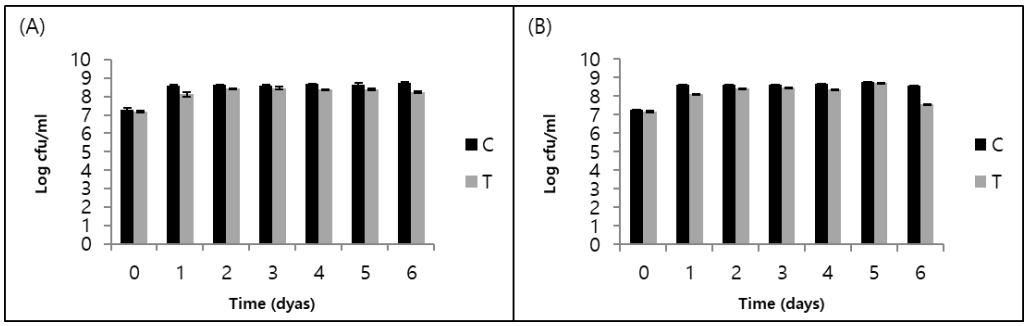

Figure 5.8. Average log cfu/ml results of a 5-strain cocktail of *L. monocytogenes* at 5 mg/ml LML over time. The black bars are the controls and the light grey bars are the treatments. The error bars represent the standard deviations and the asterisks indicate a significant difference from the control. (A) 1% fat milk and (B) 2% fat milk at 24 ℃.

CHAPTER 6

CONCLUSIONS

Fatty acid chain lengths used in enzymatically synthesizing lactose esters have significant influences on microbial inhibitory and bactericidal activity of the lactose esters towards Gram-positive bacteria but not Gram-negative bacteria such as *E. coli* O157:H7. LML and LMD were the most effective esters, dissolved in both DMSO and ethanol, exhibiting MIC values of ≤1 to ≤5 mg/ml for each Gram-positive bacteria tested (*Bacillus cereus* and *Streptococcus suis*) and MBC values of ≤3 to ≤5 mg/ml for the same bacteria. My data were in agreement with studies carried out by Nobmann and others (2009) which showed MIC values in the range of 0.04 mM to 0.31 mM for glucose fatty acid ether (containing lauric acid and decanoic acid) against *Streptococcus aureus* and *Listeria* strains. Zhang and others (2014) were even more comparable to my studies in showing how sugar monoesters containing octanoate to laurate exhibit a broad spectrum of antimicrobial activities. This evidence supported my study into LML and LMD as food additives which may play a role in increasing the safety of foods through their higher antimicrobial ability. Limitations to this work, and areas for future research, involve the solvents used to dissolve these esters and the possible influences these solvents have on the microbial inhibitory activity of these lactose esters. Future studies need to separate these factors by studying the inhibitory activity of these lactose esters in food systems without the need to first dissolve them in either DMSO or ethanol.

In this research, HLB values (14.1 to 16.9) related to CMC values (0.56 mM to 0.96 mM) and formed linear relationships with each other for each chain length of fatty

acid in the lactose esters tested. These results are comparable to the HLB values (13.1 to 14.5) and CMC values (0.56 mM to 0.96 mM) of lactose esters reported by Zhang and other (2014). Specifically, LML and LMD have a CMC of 0.72 mM (HLB; 15.7) and 0.89 mM (HLB; 16), respectively. LML and LMD individually acted as emulsifiers when used at a concentration of 0.5% in 20% O/W emulsions, and are comparable to Tween-20 and Ryoto L-1695, a commercially available food grade emulsifier commonly in use in foods. Emulsion destabilization rates, as measured by the increasing size of the clarification layers which form from the process of coalescence and emulsion component separation, were assessed for my emulsions, prepared using 0.5% lactose ester. The best result was LML (0.5 mm/d) which had the most stable emulsion as shown by the lowest destabilization rate. LMD (0.72 mm/d) showed the second lowest emulsion destabilization rates followed by LMM (1.1 mm/d) and LMO (7.19 mm/d) respectively. In addition, in my study the 0.5% LMD and LML stabilized emulsions had significant populations of droplets in the 0.5-5 μm size range remaining after an elapsed time. This suggests a strong stabilizing influence by these lactose esters on the emulsions by preventing composite oil droplets from coalescence into larger droplets which would lead to emulsion destabilization. Similar results are reported by Neta and others (2012). Lactose based esters are important as they are environmentally friendly, can be synthesized using renewable resources and demonstrate viable emulsifier potential. This research is limited in emulsifier ability as the samples were only analogs for food systems. Future research should look at LML or LMD as emulsifiers in other food systems at the suggested usage rate 0.5% to test actual food emulsifier viability.

LMD showed listerial inhibitory action at all temperatures including 5 ℃, a standard milk refrigerated storage temperature, and 37 ℃, an optimal incubation temperature for *Listeria* strains. This result is in oppositional contrast to the results reported by Chen and other (2014), who showed that lactose esters, including lauric acid, demonstrates microbial inhibitory effects at 37 ℃, but not 5 ℃. Additionally, LMD in 1% and 2% fat milk was found to be the most effective microbial inhibitory ester tested in this research, as it caused an absence of viable bacterial growth on day 3 and 4, respectively when grown at 37 ℃. Milk fat content and temperature are not the only influential parameters that can influence anti-listerial activity, other side effects like pH may play significant roles as well. Further research should look into these other parameters and determine consequential effects they may cause when used in conjunction with my lactose esters. Additionally, the influences of lactose esters in dairy products taste, acceptance and overall functionality is unknown. Sensory investigations and dairy product performance evaluations will also need to be performed.

References

Chen Y, Nummer B, Walsh MK. 2014. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Lett Appl Microbiol 58:156–162.

Neta NDAS, Santos JCS, Sancho SDO, Rodrigues S, Gonçalves LRB, Rodrigues LR, Teixeira JA. 2012. Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions. Food Hydrocoll 27:324–331.

Nobmann P, Smith A, Dunne J, Henehan G, Bourke P. 2009. The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives

against *Listeria* spp. and food spoilage microorganisms. Int J Food Microbiol 128:440–445.

Zhang X, Song F, Taxipalati M, Wei W, Feng F. 2014. Comparative study of surface-active properties and antimicrobial activities of disaccharide monoesters. PLoS One 9:e114845.

APPENDICES

APPENDIX A

FOR CHAPTER 3

Table A.1. Literature review: Antimicrobial effects of various sugar esters

Ref	Esters	Organism	Effect	Medium/test/ any solvent	Etc.
Zhang 2014	synthesized different 12 sugar monoesters	*S. aureus, E. coli 0157:H7, Candida albicans*	sugar fatty acid monoesters containing C_8, C_{10} and C_{12} alkyl chain showed antimicrobial activity.	growth media/ MIC/media	methyl α-D-glycoside monoesters were the most effective. sugar monoesters were inhibitory against *S. aureus* than *E. coli*/media
Chen 2014	synthesized lactose monolaurate	*L. monocytogenes*	bactericidal against *L. monocytogenes*	milk, yogurt and cheese/MIC, MBC/ ethanol 50%	
Wagh 2012	synthesized lactose monolaurate	various Gram positive and Gram negative	bactericidal against *L. monocytogenes* and *Mycobacteria*	growth media/MIC, MBC/ethanol 50% (mM)	
Nobmann 2009	synthesized sugar esters and ethers (11 types), commercial monolau	*Listeria species, E.coli Salmonella EnterobacterPseudomonas*	Synthesized lauric ether of methyl α-D-glucopyranoside and lauric ester of methyl α-D-mannopyra	growth media/MIC (mM)	increase in lag time between concentrations of a compound was observed markedly for compound

	rin, monocaprylin, lauric acid, capric acid		noside showed strong inhibitory effects (0.04 mM) against Gram positive bacteria.		effficacies.(decrease in growth rate was more gradual)
Habulin 2008	commercial and synthesized sucrose and fructose palmitate and laurate	*Bacillus cereus* *E. coli* K12	strong inhibition (75-96%) against *B. cereus* with sucrose laurate at 1% concentration at 3 days limited (10%) inhibitation against *E. coli* with all esters	growth media/Inhibition %/different liquid media containing meat peptones	activity of the lipase increases with the chain length of the fatty acid for synthesis of sugar esters.
Piao 2006	various synthesized erythritol and xylitol esters	various Gram positive and negative, yeast	strong inhibitory effect with monomyristoyl xylitol (most effective) monolaurate against *B. cereus*. All esters were ineffective against *E.*	growth media and plates/MIC(mg/L)/dissolved in hot water, added to media	Both the number and orientation of the hydroxyl group of the monoacyl sugar alcohols are inhibitory as well as the acyl chain length

			coli, S. cerevisiae		
Ferrer 2005	various synthesized sugar esters	various Gram positive and negative	sucrose and maltose laurate inhibited *Bacillus* at 0.5%. Limited inhibitation (26%) against *E. coli* at 0.4%.	growth media/screening of antimicrobial properties, MIC/different nutritive broth	effect of sugar head group, length of the fatty acid and degree of substitution, but sucrose dilaurates and 6-O-lauroylglucose were not inhibitory effects
Devulapalle 2004	maltose laurate, maltotriose laurate, sucrose laurate	*Streptococcus sobrinus*	All esters suppressed the growth at 0.05-2% concentration of esters	growth media (BHI/ liquid BHI broth solid) and plates/concentration (mM) vs glucosyltransferase activity/media, liquid media	
Yang 2003	sucrose and glucose esters	spoilage organisms *Z. bailii* and *L fructivorans*	1% sucrose esters of laurate (C_{12}), myristate (C_{14}) or palmitate (C_{16}) inhibited the growth	salad dressing/log reduction/growth media	sucrose monoesters were usually more inhibitory than methylglucose monoesters of the same fatty acid

			of the organisms and were more effective than 0.1% sodium benzoate		
Watanabe 2000	23 different synthesized sugar esters	*Streptococcus mutans*	Galactose and fructose laurates inhibited growth at ≤0.05% (even in the presence of 0.2%, 2% sucrose from S. mutans)	Microbial media/OD 620nm (fructose laurate: no cell growth, sucrose monolaurate: no grown inhibitory)/MIC (μg/mg) (galactose laurate: 50, fructose laurate: 100)	The configuration of the hydroxyl group is an essential for antibacterial activity. They determined the effect of chain length of the aliphatic side group.

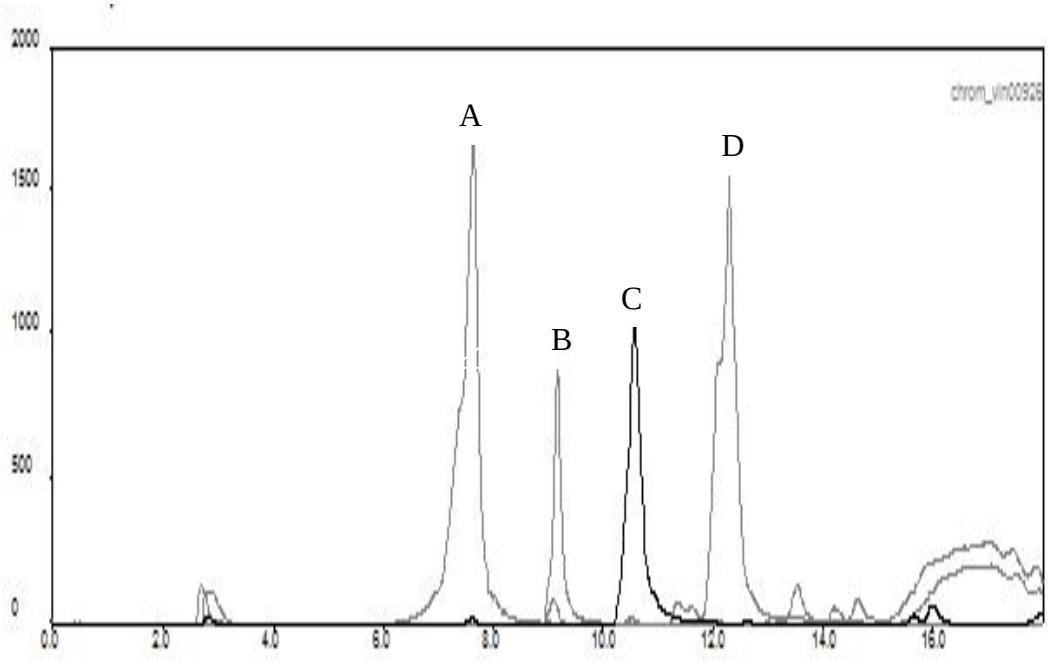

Figure A.1. Overlay of HPLC chromatograms of lactose esters synthesized in 2M2B with lipase from *Thermomyces lanuginosus* (TM3). Peaks are: A) LMO; B) LMD; C) LML; D) LMM.

Figure A.2. Pictures for purified lactose monoesters. Pictures are: A) LMO; B) LMD; C) LML; D) LMM.

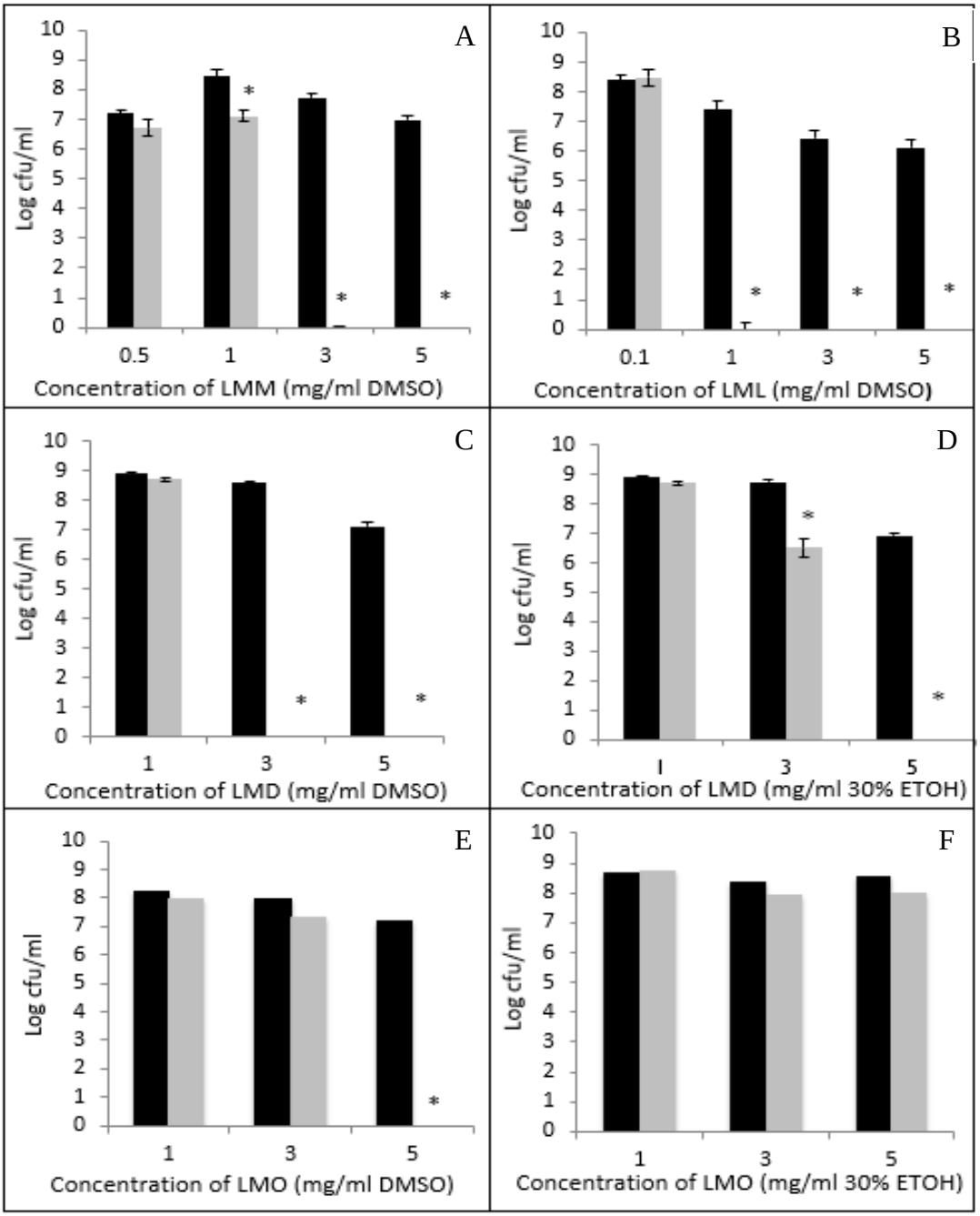

Figure A.3. Average log cfu/ml results of *B. cereus* after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: *B. cereus* treated with LMM/DMSO; B: *B. cereus* treated with LML/DMSO; C: *B. cereus* treated with LMD/DMSO; D: *B. cereus* treated with LMD/30% ETOH; E: *B. cereus* treated with LMO/DMSO; F: *B. cereus* treated with LMO/30% ETOH.

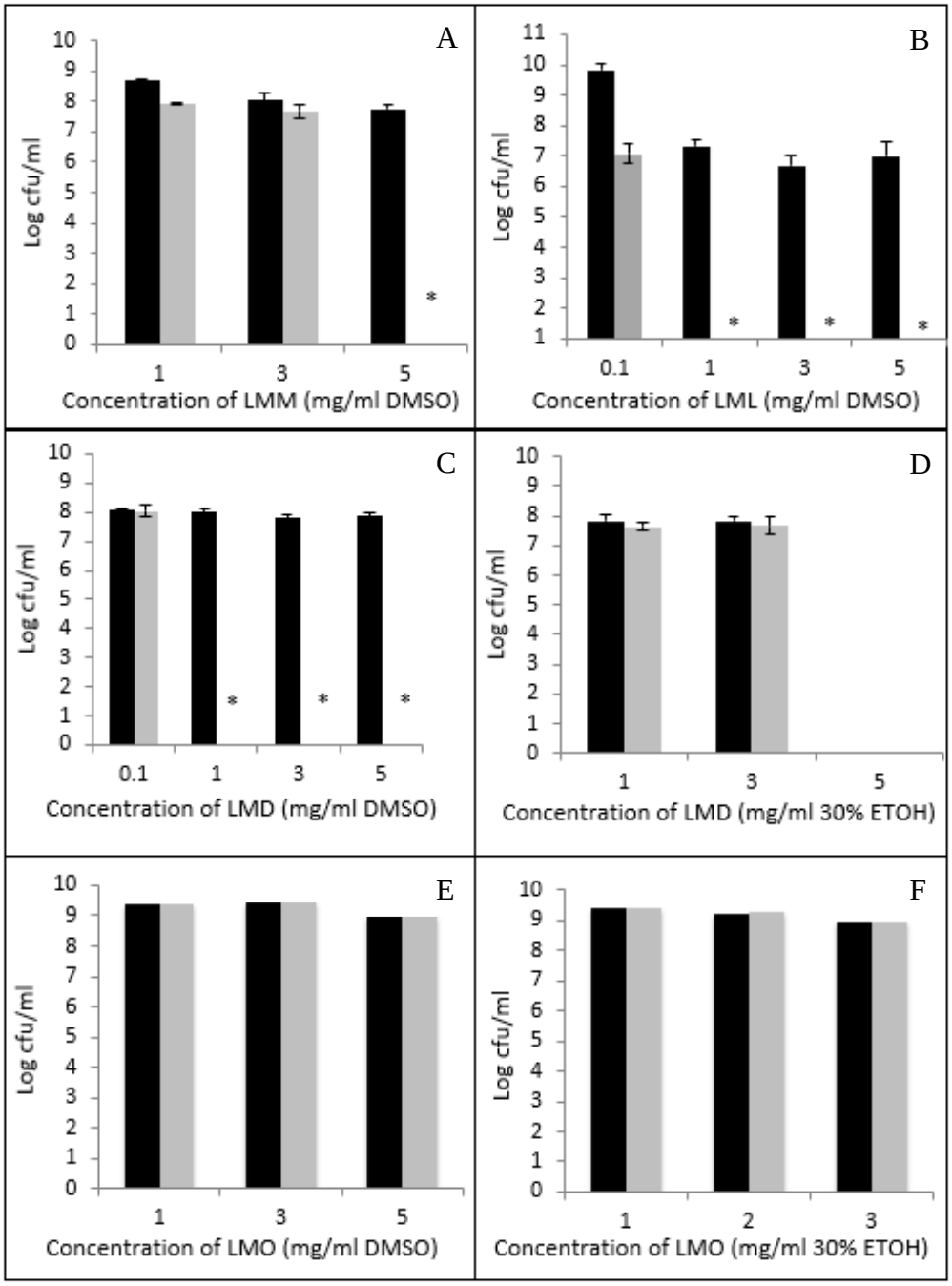

Figure A.4. Average log cfu/ml results of M. KMS after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: M. KMS treated with LMM/DMSO; B: M. KMS treated with LML/DMSO; C: M. KMS treated with LMD/DMSO; D: M. KMS treated with LMD/30% ETOH; E: M. KMS treated with LMO/DMSO; F: M. KMS treated with LMO/30% ETOH.

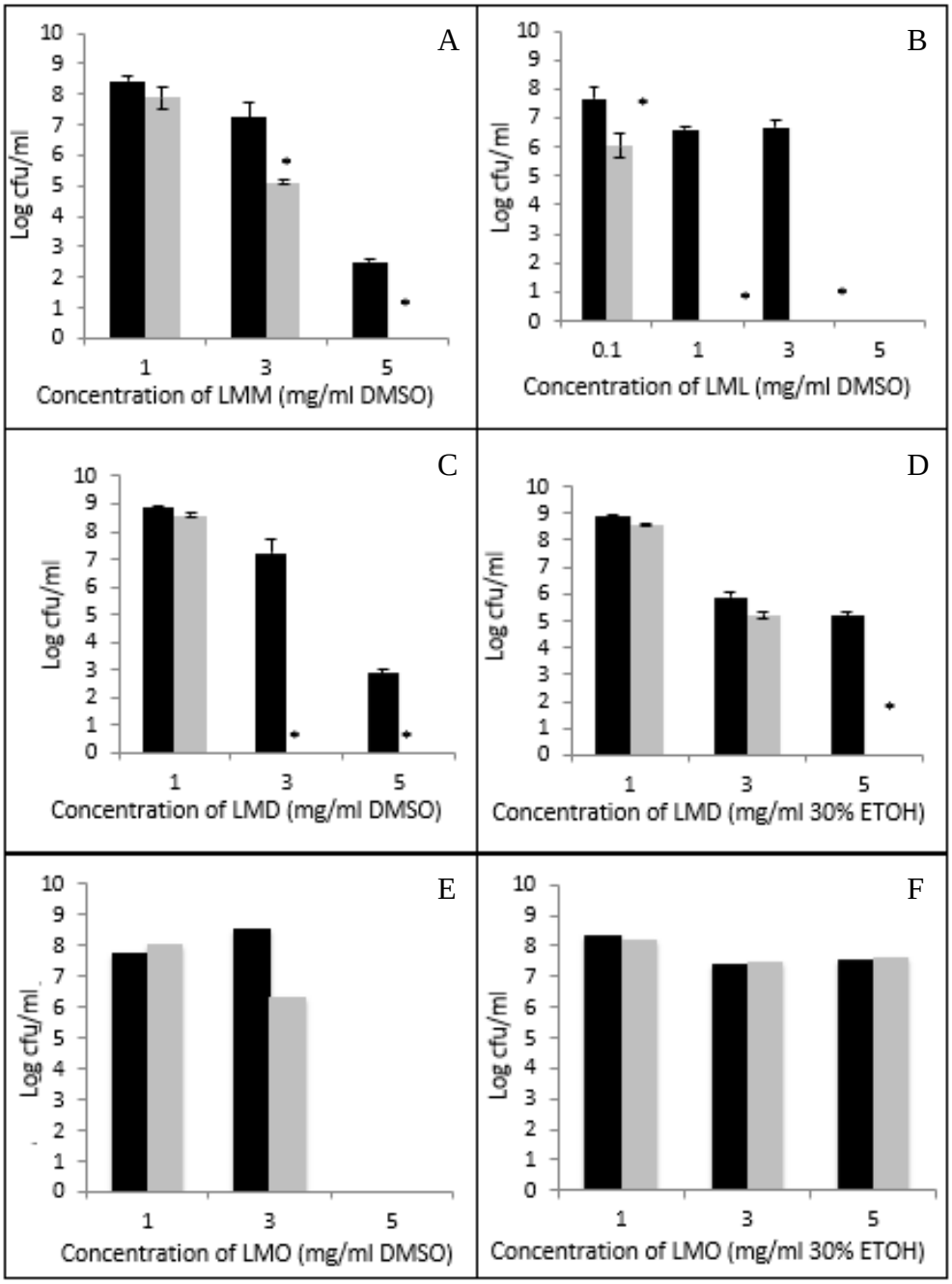

Figure A.5. Average log cfu/ml results of *S. suis* after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: *S. suis* treated with LMM/DMSO; B: *S. suis* treated with LML/DMSO; C: *S. suis* treated with LMD/DMSO; D: *S. suis* treated with LMD/30% ETOH; E: *S. suis* treated with LMO/DMSO; F: *S. suis* treated with LMO/30% ETOH.

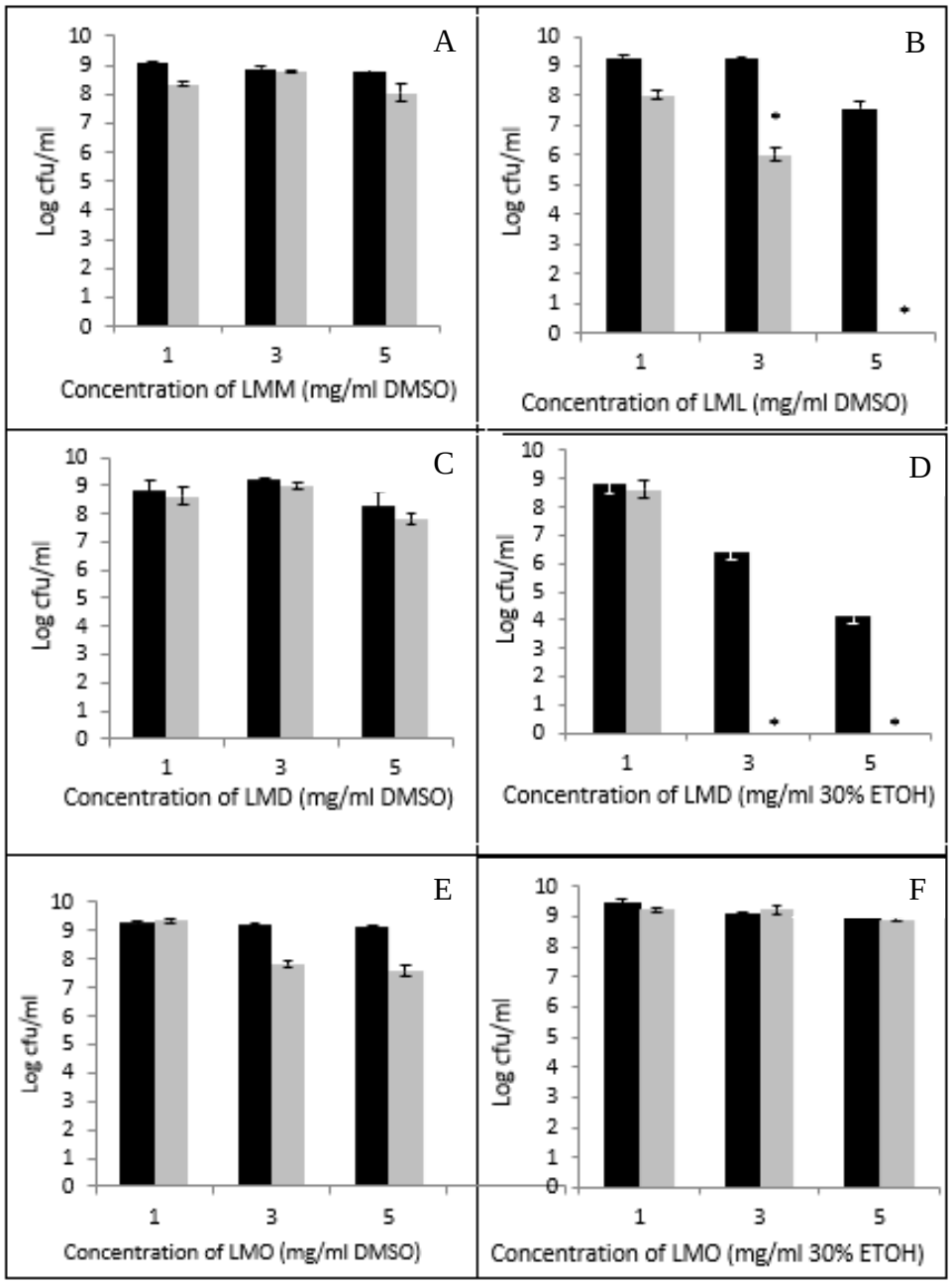

Figure A.6. Average log cfu/ml results of *L. monocytogenes* after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: *L. monocytogenes* treated with LMM/DMSO; B: *L. monocytogenes* treated with LML/DMSO; C: *L. monocytogenes* treated with LMD/DMSO; D: *L. monocytogenes* treated with LMD/30% ETOH; E: *L. monocytogenes* treated with LMO/DMSO; F: *L. monocytogenes* treated with LMO/30% ETOH.

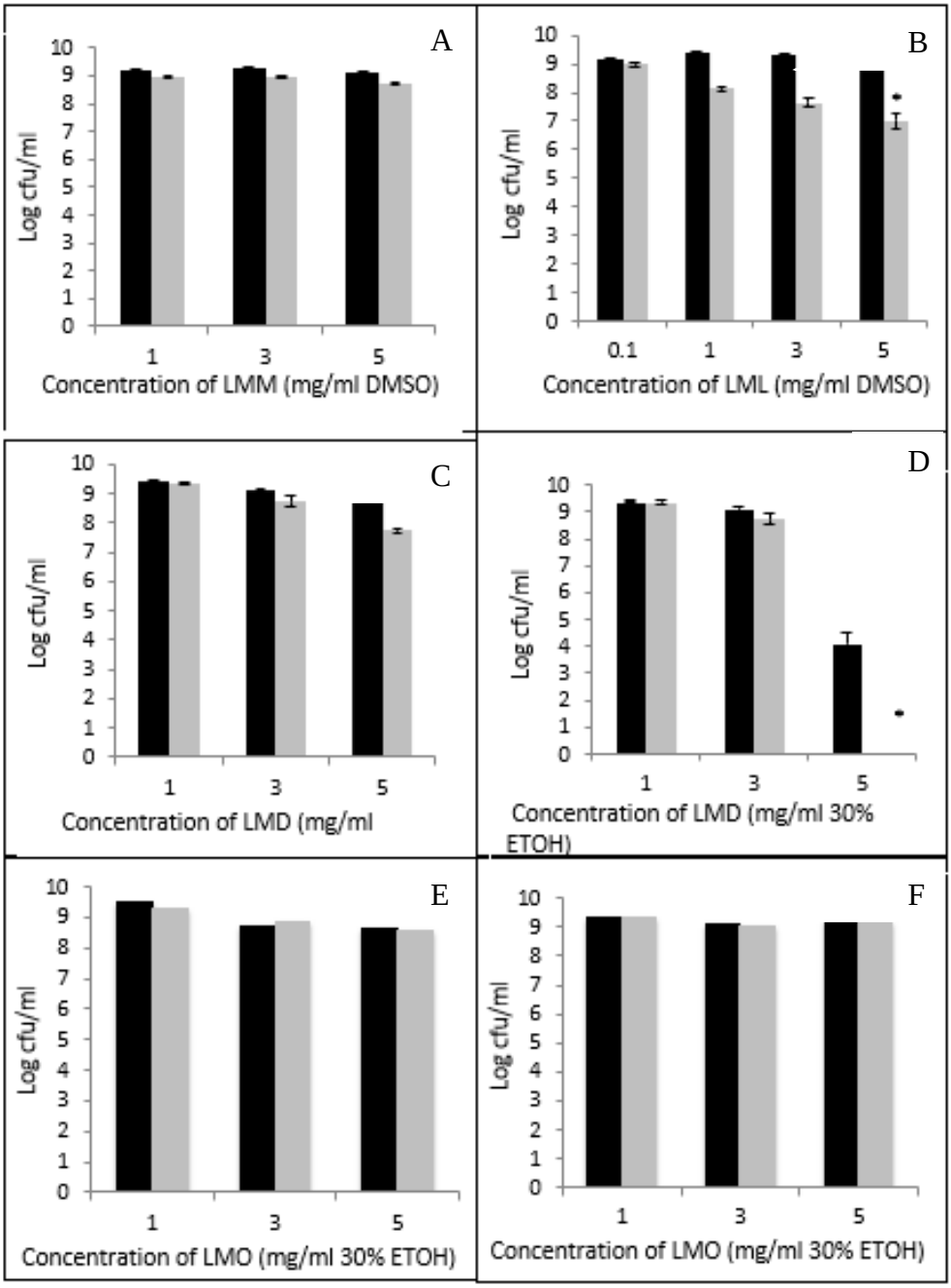

Figure A.7. Average log cfu/ml results of *E. faecalis* after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: *E. faecalis* treated with LMM/DMSO; B: *E. faecalis* treated with LML/DMSO; C: *E. faecalis* treated with LMD/DMSO; D: *E. faecalis* treated with LMD/30% ETOH; E: *E. faecalis* treated with LMO/DMSO; F: *E. faecalis* treated with LMO/30% ETOH.

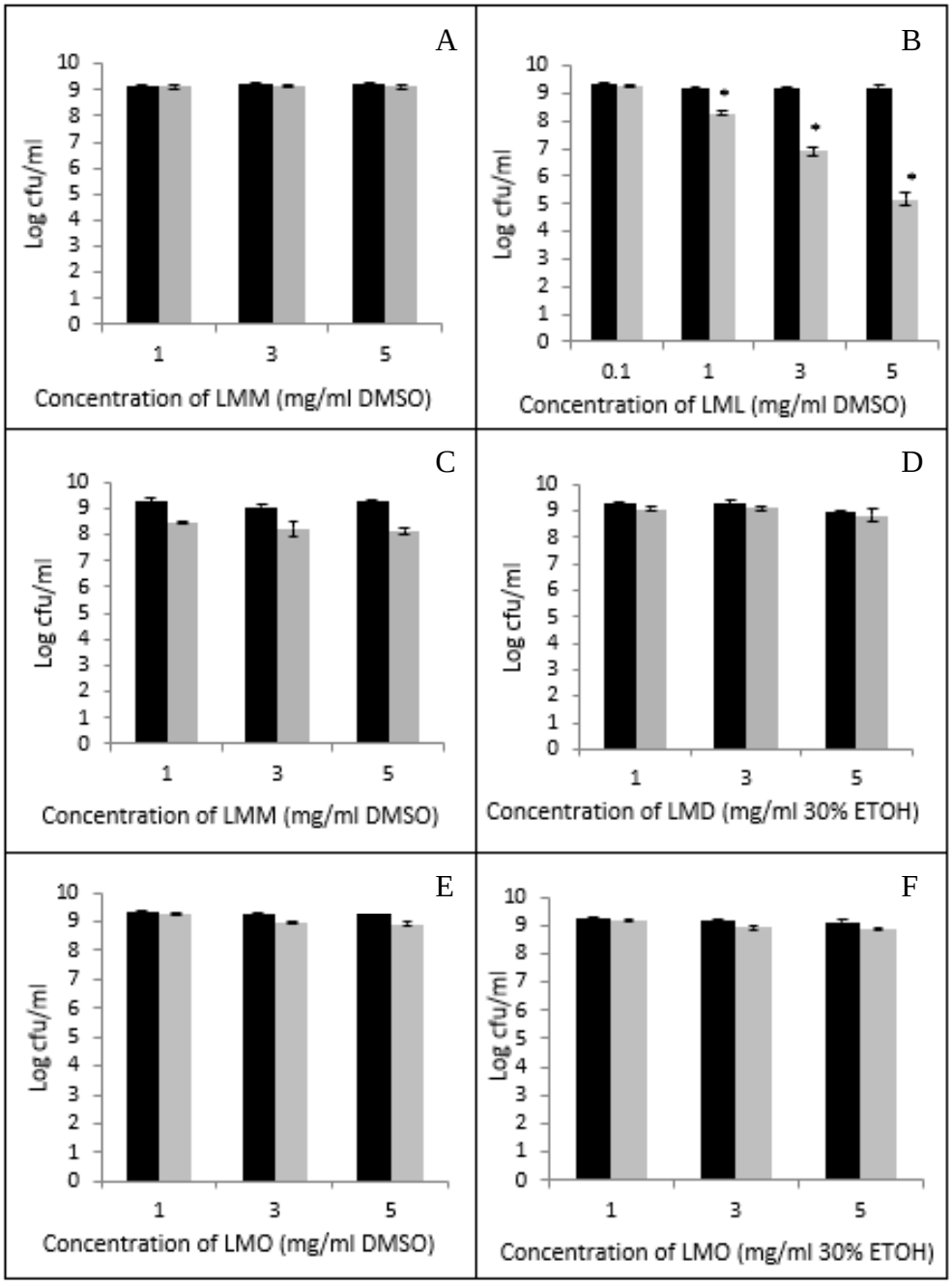

Figure A.8. Average log cfu/ml results of *S. mutans* after 24 hrs of incubation at 37 ºC. The black bars indicate the controls and light bars are treatments. Error bars represent the standard errors and asterisks indicate significant difference from the control. A: *S. mutans* treated with LMM/DMSO; B: *S. mutans* treated with LML/DMSO; C: *S. mutans* treated with LMD/DMSO; D: *S. mutans* treated with LMD/30% ETOH; E: *S. mutans* treated with LMO/DMSO; F: *S. mutans* treated with LMO/30% ETOH.

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ORIGINAL ARTICLE

Growth inhibitory properties of lactose fatty acid esters

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KEYWORDS

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Abstract Sugar esters are biodegradable, nonionic surfactants which have microbial inhibitory properties. The influence of the fatty acid chain length on the microbial inhibitory properties of lactose esters was investigated in this study. Specifically, lactose monooctanoate (LMO), lactose monodecanoate (LMD), lactose monolaurate (LML) and lactose monomyristate (LMM) were synthesized and dissolved in both dimethyl sulfoxide (DMSO) and ethanol. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined in growth media. LML was the most effective ester, exhibiting MIC values of < 0.05 to < 5 mg/ml for each Gram-positive bacteria tested (*Bacillus cereus*, *Mycobacterium KMS*, *Streptococcus suis*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Streptococcus mutans*) and MBC values of < 3 to < 5 mg/ml for *B. cereus*, *M. KMS*, *S. suis*, and *L. monocytogenes*. LMD showed MIC and MBC values of < 1 to < 5 mg/ml for *B. cereus*, *M. KMS*, *S. suis*, *L. monocytogenes*, and *E. faecalis*, with greater inhibition when dissolved in ethanol. LMM showed MIC and MBC values of < 1 to < 5 mg/ml for *B. cereus*, *M. KMS*, and *S. suis*. LMO was the least effective showing a MBC value of < 5 mg/ml for only *B. cereus*, though MIC values for *S. suis* and *L. monocytogenes* were observed when dissolved in DMSO. *B. cereus* and *S. suis* were the most susceptible to the lactose esters tested, while *S. mutans* and *E. faecalis* were the most resilient and no esters were effective on *Escherichia coli O157:H7*. This research showed that lactose esters esterified with decanoic and lauric acids exhibited greater microbial inhibitory properties than lactose esters of octanoate and myristate against Gram-positive bacteria.

Abbreviations: DMSO, dimethyl sulfoxide; ETOH, ethanol; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; LMO, lactose monooctanoate; LMD, lactose monodecanoate; LML, lactose monolaurate; LMM, lactose monomyristate

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1. Introduction

Sugar esters are nonionic surfactants used in a variety of applications in the food, pharmaceutical, and personal care industries. The microbial inhibitory activity of sugar esters has been studied. Although it has been shown that sugar esters inhibit bacterial growth, there is a lack of consensus as to which bacteria are most susceptible. While some studies have shown inhibitory effects of Gram-negative bacteria (Ferrer et al., 2005; Habulin et al., 2008; Zhang et al., 2014; Smith et al., 2008), others have shown inhibition of only Gram-positive bacteria (Wagh et al., 2012; Piao et al., 2006). Studies have shown that esters containing laurate were inhibitory against both Gram-positive and Gram-negative bacteria (Smith et al., 2008; Nobmann et al., 2009; Zhang et al., 2014). A study on the microbial inhibitory activity of lactose monolaurate showed low minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for *Listeria monocytogenes* and *Mycobacterium* sp. strain KMS, and no inhibitory activity against Gram-negative bacteria (Wagh et al., 2012).

The nature and number of fatty acid chains esterified to sugars can be variable, yielding a broad range of hydrophilic-lipophilic balances and microbial inhibitory activities (Szuts and Szabó-Révész, 2012). Previous research showed that fatty acid derivatives such as monolaurin are highly inhibitory and more inhibitory than lauric acid (Smith et al., 2008; Nobmann et al., 2009). Others have reported that sugar monoesters of decanoic, myristic and palmitic acids were microbial inhibitory (Piao et al., 2006; Habulin et al., 2008; Zhang et al., 2014). There was one study investigating the microbial inhibition of sugar octanoate esters which showed no inhibitory effects (Zhang et al., 2014).

Of the carbohydrate fatty acid esters previously investigated, sucrose esters have been the most thoroughly studied (Nobmann et al., 2009). Other oligosaccharide esters of laurate, including maltose, fructose and galactose have been synthesized and have generally been shown to be very effective microbial inhibitory agents (Nobmann et al., 2009; Watanabe et al., 2000; Devulapalle et al., 2004; Habulin et al., 2008), whereas hexose laurate did not suppress microbial growth significantly (Watanabe et al., 2000).

While many studies examine the microbial inhibition of sugar esters in terms of MIC values, few studies have determined the MBC values of sugar esters. In this study we synthesized novel lactose esters including lactose monooctanoate (LMO), lactose monodecanoate (LMD) and lactose monomyristate (LMM). The microbial inhibitory properties of these esters (MIC and MBC) in microbial growth media, and the previously synthesized ester, lactose monolaurate (LML) (Wagh et al., 2012) were determined against Gram-positive (*Bacillus cereus*, *Mycobacterium KMS*, *Streptococcus suis*, *L. monocytogenes*, *Enterococcus faecalis* and *Streptococcus mutans*) bacteria and the Gram-negative bacteria, *Escherichia coli* O157:H7. Furthermore, we also determined MIC and MBC values of the esters dissolved in two solvents, DMSO and ethanol. This allowed us to ascertain the role of the solvents in the microbial inhibitory activity.

Table 1 Microorganisms and growth media used in this study.

Microorganism	ATCC no./serovar	Gram reaction[a]	Growth medium
Bacillus cereus	13061	+	BHI
Mycobacterium sp. strain KMS	NA	+	LB
Streptococcus suis	89/1591	+	BHI
Listeria monocytogenes	EGDe	+	BHI
Listeria monocytogenes	FSL J1-177	+	BHI
Listeria monocytogenes	FSL N3-013	+	BHI
Listeria monocytogenes	FSL R2-499	+	BHI
Listeria monocytogenes	FSL N1-227	+	BHI
Enterococcus faecalis	V538	+	BHI
Streptococcus mutans	FSL R2-499	+	BHI
Escherichia coli O157:H7	EDL 931	−	LB

NA = not available.
[a] +, positive; −, negative.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used are listed in Table 1. *E. faecalis* V538 and *L. monocytogenes* EGDe were received from Dr. Andy Benson of the University of Nebraska, Lincoln. Different clinical isolates of *Listeria* (FSL J1-177, FSL N3-013, FSL R2-499 and FSL N1-227) were obtained from Dr. Martin Wiedmann, director of the international Life Sciences Institute North American Database at Cornell University. *S. suis* 89/1591 was received from Dr. Richard Higgins of University of Montreal, Qubec, Canada. *M. KMS* was isolated by Utah State University from treatment soils in Champion International Superfund Site, Libby, Montana. *B. cereus* ATCC 13061, *S. mutans* ATCC 25175 and *E. coli* O157:H7 EDL 931 stains were obtained from ATCC (Manassas, VA).

2.2. Materials and equipment

Materials and equipment included a high-performance liquid chromatography (HPLC) (Beckman System Gold 125 Solvent Module, Ontario, Canada) equipped with Luna 5 μm C18 100 Å (250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) and an evaporative light scattering detector (Agilent Technologies, Santa Clara, CA, USA), incubator shaker, spectrophotometer (Beckman, USA), 48 microtitre well plates (Becton Dickinson, NJ, USA), brain–heart infusion (BHI) media, Lauria–Bertani (LB) media, granulated agar (BD, New Jersey, USA), lactose (Proliant, Iowa, USA), vinyl laurate, vinyl myristate, vinyl decanoate, vinyl octanoate (TCI, Portland OR, USA), lipase TM2 (immobilized from *Thermomyces lanuginose*), Tween 80, Whatman glass microfiber filters, molecular sieves (3A), 2-methyl-2-butanol (2M2B) (dried using 10% 3A molecular sieves), dimethyl sulfoxide (DMSO) (Sigma Aldrich, MO, USA), ethanol, and acetonitrile (HPLC grade, Thermo Fisher, PA, USA).

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2.3. Lactose ester synthesis and purification

Enzymatic synthesis of LML was performed according to Walsh et al. (2009). Synthesis of LMO was conducted using lactose, vinyl octanoate, molecular sieves and immobilized lipase enzyme TM2. For a 60 mL reaction in 2M2B, 3 g of lactose, 6 g of dried molecular sieves, 1.7 mL of vinyl octanoate (lactose to fatty acid ratio of 1:2.1) and 1.8 g TM2 were combined. The reactions were assembled in a 100 mL glass bottle and incubated at 60 °C and 90 rpm for 2 days. The amount of LMO synthesized was determined using HPLC with the evaporative light scattering detector set at 60 °C with a nitrogen gas pressure of 3.55 bar. There was a gradient from 10% acetonitrile–water (40:60, v/v) to 100% acetonitrile–water (95:5, v/v) as the mobile phase. Synthesis of LMM and LMD was done as described above for LMO using the same molar ratios of lactose to fatty acid (vinyl myristate or vinyl decanoate).

For ester purification, the 2M2B reaction was filtered through a Whatman glass microfiber filter then dried in a hood for 24 h. The dry solids were suspended in 60% ethanol, 40% water (60 °C) and placed in a separatory funnel. The lower aqueous layer was drained into a beaker and dried in a hood for 24 h. After completely drying, the product powder was suspended in acetone, and then centrifuged for 15 min at room temperature at 2000g and the supernatant analyzed via HPLC for the presence of di- tri- or higher saccharides. The acetone extraction was repeated until only the monoester was present in the pellet.

2.4. Microbial inhibitory studies

Stock solutions of LMO (60 mg/ml) and LMD (25 mg/ml) were prepared in 30% ethanol:water. Stock solutions of LML (60 mg/ml) were prepared in 50% ethanol:water and 100% DMSO. Stock solutions of LMO and LMD (60 mg/ml) were prepared in 100% DMSO. LMM was not soluble in 60% ethanol:water hence a stock (60 mg/ml) was prepared in 100% DMSO. Controls were 30% ethanol:water, 50% ethanol:water and 100% DMSO. Ester stock solutions were diluted into growth media to give final ethanol concentrations ranging from 0.5% to 10% and final DMSO concentrations ranging from 2% to 8%. All seven stocks of esters and controls were tested on the bacteria listed in Table 1.

Analysis of microbial inhibitory activities of LMO was performed by making a 5 strain cocktail of *L. monocytogenes* including C1-056, J1-177, N1-277, N3-013, and R2-499. The individual 5 stocks were stored at −80 °C, and each individual freezer stock (20 µl) was added to 15 ml of BHI media. The *Listeria* strains were grown at 37 °C and 200 rpm for 24 h. Aliquots (2 ml) from each strain were combined in a test tube to develop the 5-strain stock cocktail. Aliquots, 315 µl, of the stock cocktail were grown in BHI media (12 ml), and incubated with shaking at 37 °C for 4 h. Aliquots of the 5-strain stock cocktail were kept at −80 °C.

Stock solutions of the other bacteria were maintained at −80 °C before use. Aliquots of bacterial stock solutions (300 µl) were grown in 15 ml media at 37 °C, 200 rpm for 24 h. Aliquots of the overnight growths (300 µl) were added to 12 ml media and grown again at 37 °C, 200 rpm for 4 h before use. The growing cultures were monitored by optical density measurements at 660 nm (OD_{600}) and diluted with fresh media to reach an OD_{600} of 0.2 which was approximately 1×10^8 cfu/ml. An aliquot of the culture, 100 µl, was mixed with 10 ml fresh media containing 0.1% Tween 80. The ester stock ester solutions were added to each well for final concentrations of 0.05, 0.1, 0.5, 1, 3, and/or 5 mg/ml and each well contained a total of 0.5 ml. Controls contained the same concentration of ethanol or DMSO as the treatments. Each treatment and control was performed in triplicate and replicated three times. A paired *T*-test was used to compare the treatments with the controls at each concentration to determine if the treatments were significantly different from the controls. All controls and treatments were plated on appropriate agar and incubated at 37 °C for 24 h to obtain plate counts. The MIC of each compound was determined as the lowest concentration which showed a significant difference in the number of cells in treatments as compared to those in controls as determined by plate counts. Similarly, the MBC of each compound for each organism was reported as the minimum concentration of ester at which there was no cell growth as determined by plate counts.

3. Results

3.1. Minimum inhibitory concentrations (MIC) of lactose esters

In our earlier work, we showed that the novel lactose ester, LML (in 50% ethanol:water) was antimicrobial towards *L. monocytogenes* and *M. KMS*, but had no activity against Gram-negative bacteria (Wagh et al., 2012). In this study, additional lactose esters, LMO, LMD, and LMM were synthesized, and along with LML, were dissolved in both ethanol and DMSO, and tested for microbial inhibitory activity against Gram-positive bacteria and *E. coli O157:H7*. The control samples contained the same concentration of solvent as the treatments.

MIC values of the lactose esters against various Gram-positive bacteria are listed in Table 2. LML was found to be the most effective microbial inhibitory ester since it showed MIC values (< 0.05 to < 5 mg/ml) for each Gram-positive bacteria tested in each solvent. On average, there were lower MIC values with LML/ETOH for *M. KMS*, *L. monocytogenes* and *E. faecalis*. The MIC for LML/DMSO with *E. faecalis* was 5 mg/ml, which was the highest MIC value for LML among the bacteria tested.

MIC values of LMD/DMSO ranged from < 1 to < 3 mg/ml for *B. cereus*, *M. KMS* and *S. suis*. The MIC for LMD/DMSO for *L. monocytogenes*, *E. faecalis* and *S. mutans* was above 5 mg/ml. MIC values for LMD/ETOH ranged from < 3 to < 5 mg/ml with no MIC values for *S. mutans*. Ethanol itself was inhibitory, specifically with *M. KMS* which showed no cells in the control or treatment with 5 mg/ml LMD/ETOH (corresponding to 10% ethanol), therefore, no MIC could be determined. LMD/ETOH inhibited the growth of *L. monocytogenes* and *E. faecalis* while LMD/DMSO showed no inhibitory effects on these bacteria.

LMM in DMSO showed inhibitory activity against *B. cereus*, *M. KMS* and *S. suis* with MIC values between < 1 mg/ml and < 5 mg/ml. However, MIC values for LMM with *L. monocytogenes*, *E. faecalis* and *S. mutans* were > 5 mg/ml.

LMO/ETOH showed no inhibitory effect at concentrations up to 5 mg/ml but LMO/DMSO was inhibitory to *B. cereus*, *S. suis* and *L. monocytogenes*. *S. suis* and *L. monocytogenes*

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Table 2 Minimum inhibitory concentrations of lactose esters as both mg/ml and mM concentrations. Esters were tested at concentrations up to 5 mg/ml.

	LMO DMSO	LMD DMSO	LMD ETOH	LML DMSO	LML ETOH	LMM DMSO
B. cereus	< 5 mg/ml < 10.7 mM	< 3 mg/ml < 6.0 mM	< 3 mg/ml < 6.0 mM	< 1 mg/ml < 1.9 mM	< 1 mg/ml < 1.9 mM	< 1 mg/ml < 1.8 mM
M. KMS	No	< 1 mg/m < 2.0 mM	X[1]	< 1 mg/ml < 1.9 mM	< 0.05 mg/ml[2] < 0.095 mM	< 5 mg/ml < 9.0 mM
S. suis	< 3 mg/ml < 6.4 mM	< 3 mg/ml < 6.0 mM	< 5 mg/ml < 10.1 mM	< 1 mg/ml < 1.9 mM	< 1 mg/ml < 1.9 mM	< 3 mg/ml < 5.4 mM
L. monocytogenes	< 3 mg/ml < 6.4 mM	No	< 3 mg/ml < 6.0 mM	< 3 mg/ml < 5.7 mM	< 0.1 mg/ml[2] < 0.19 mM	No
E. faecalis	No	No	< 5 mg/ml < 10.1 mM	< 5 mg/ml < 9.5 mM	< 1 mg/ml < 1.9 mM	No
S. mutans	No	No	No	< 1 mg/ml < 1.9 mM	< 3 mg/ml < 5.7 mM	No

No = No growth inhibition.
[1] X = No growth in treatment or control at 5 mg/ml.
[2] Data obtained from Wagh et al. (2012).

Table 3 Minimum bactericidal concentration of lactose esters as both mg/ml and mM concentrations. Esters were tested at concentrations up to 5 mg/ml. The log reductions of the treatment samples compared to the controls are given as log values.

	LMO DMSO	LMD DMSO	LMD ETOH	LML DMSO	LML ETOH	LMM DMSO
B. cereus	< 5 mg/ml < 10.7 mM 7 log	< 3 mg/ml < 6.0 mM 9 log	< 5 mg/ml < 10.1 mM 7 log	< 1 mg/ml < 1.9 mM 7 log	< 5 mg/ml > 9.5 mM 8 log	< 3 mg/ml < 5.4 mM 8 log
M. KMS	No	< 1 mg/ml < 2.0 mM 8 log	X[1]	< 1 mg/ml < 1.9 mM 7 log	< 1 mg/ml[2] < 1.9 mM 4 log	< 5 mg/ml < 9.0 mM 8 log
S. suis	X[1]	< 3 mg/ml < 6.0 mM 7 log	< 5 mg/ml < 10.1 5 log	< 1 mg/ml < 1.9 mM 7 log	< 5 mg/ml < 9.5 mM 8 log	< 5 mg/ml < 9.0 mM 2 log
L. monocytogenes	No	No	< 3 mg/ml < 6.0 mM 6 log	< 5 mg/ml < 9.5 mM 8 log	< 5 mg/ml[2] < 1.9 mM 5 log	No
E. faecalis	No	No	< 5 mg/ml < 10.1 mM 4 log	No	No	No
S. mutans	No	No	No	No	No	No

No = No MBC value.
[1] X = No growth in treatment or control at 5 mg/ml.
[2] Data obtained from Wagh et al. (2012).

were more sensitive with MIC values < 3 mg/ml than *B. cereus* with an MIC value < 5 mg/ml. No ester dissolved in either DMSO or ethanol showed microbial inhibitory activity against the Gram-negative bacteria tested (*E. coli* O157:H7).

3.2. Minimum bactericidal concentrations (MBC) of lactose esters

MBC of the lactose esters are reported in Table 3 as well as the log reductions in the treatments as compared to the controls. No esters showed bactericidal activity against *S. mutans*. Out of the 4 compounds tested, LML was the only lactose ester to exert a bactericidal effect against *B. cereus*, *M. KMS*, *S. suis* and *L. monocytogenes* in both solvents used. MBC values of LML/DMSO were < 1 mg/ml for *B. cereus*, *M. KMS*, and *S. suis*. MBC concentrations of LML were lower in DMSO compared to ethanol for *B. cereus* and *S. suis*.

In tests against the Gram-positive bacteria, LMD/ETOH showed broad antimicrobial activity against *B. cereus*, *S. suis*, *L. monocytogenens* and *E. faecalis* with MBC values between < 3 mg/ml and < 5 mg/ml. However, LMD/DMSO was not shown to be bactericidal to *L. monocytogenes* or *E. faecalis* at concentrations up to 5 mg/ml. Furthermore, bactericidal activity of ethanol was shown against *M. KMS*, with no cells growing in the control or treatment at 10% ethanol as stated earlier for the MIC values. LMM/DMSO was effective against *B. cereus*, *M. KMS* and *S. suis* with MBC values between < 3 and < 5 mg/ml.

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LMO/ETOH showed no bactericidal effects up to concentrations of 5 mg/ml whereas LMO/DMSO was only shown to have bactericidal activity against *B. cereus* at < 5 mg/ml. DMSO was itself inhibitory towards *S. suis* with no growth in the treatment of controls with LMO/DMSO containing 8% DMSO, therefore no MBC could be determined. *S. mutans* and *E. faecalis* were observed to be the most resilient among the bacteria tested and *B. cereus* was the most susceptible. Only LMD/ETOH was observed to be bactericidal against *E. faecalis*.

4. Discussion

Carbohydrate fatty acid derivatives are biodegradable, non-toxic and non-skin irritant surfactants with microbial inhibitory activity (Szuts and Szabó-Révész, 2012). The microbial inhibitory properties of these derivatives are increasingly of interest and many of these compounds have been shown to inhibit Gram-positive rather than Gram-negative bacteria (Piao et al., 2006; Wagh et al., 2012). This study evaluated both microbial inhibitory and bactericidal properties of lactose esters. LML was shown to be the most effective lactose ester in preventing microbial growth, yielding the lowest MIC values in the range of < 0.05 mg/ml to < 5 mg/ml (0.095 mM to < 9.53 mM) against each Gram-positive bacteria tested. Moreover *B. cereus* and *S. suis* appeared to be the most susceptible with MIC values obtained for each ester tested, and the lowest MIC value was obtained with LML/ETOH and *M. KMS* (< 0.05 mg/ml or < 0.095 mM). With regard to previous studies of bacterial inhibition with lactose esters, LML/ETOH showed inhibitory activity against *L. monocytogenes* at concentrations of 0.1 mg/ml (0.19 mM) (Wagh et al., 2012). Similar microbial inhibitory effects of LML were observed in another study in which LML/ETOH inhibited the growth of *L. monocytogenes* in milk, low fat yogurt and cheese at < 5 mg/ml (Chen et al., 2013).

It is known that the identity of the sugar group attached to the ester plays a role in modulating the antimicrobial activity (Smith et al., 2008; Nobmann et al., 2009). The antimicrobial effect of sugar esters has traditionally be measured and reported as MIC values, with no MBC values given. Smith et al. (2008) and Nobmann et al. (2009) reported MIC values in the range of 0.04 mM to 0.31 mM for lauric methyl D-glucopyranoside and lauric ester of methyl α-D-mannopyranoside with *S. aureus* and *Listeria* strains. Watanabe et al. (2000) also showed inactivation of *S. mutans* by both galactose laurate and fructose laurate, with MIC values of 0.05 mg/ml and 0.2 mg/ml respectively, whereas hexose laurate did not suppress microbial growth. In a similar study, inhibitory effects of the sugar esters 6′-O-lauroylmaltose, 6′-O-lauroylsucrose, and 6″-O-lauroylmaltotriose were observed against *Streptococcus sobrinus*, with MIC values of 0.1 mg/ml (Devulapalle et al., 2004). Therefore, laurate sugar esters have previously been shown to be microbial inhibitory against Gram-positive bacteria.

The importance of the fatty acid was investigated in this study using octanoatic, decanoic, lauric, and myristic acids esterified to lactose. LMM and LMD were effective in controlling the growth of *B. cereus*, *M. KMS* and *S. suis*. Previous research showed that erythritol and xylitol monomyristoyl suppressed *Bacillus* growth with MIC values between 6.3 μg/ml and 12.5 μg/ml (Piao et al., 2006), which are lower than reported here. As for short chain esters, Zhang et al. (2014) reported that sucrose and glucose octanoate had no inhibitory effect against *S. aureus* and *E.coli* H7:O157. In contrast, we showed LMO/DMSO to have microbial inhibitory activity against *B. cereus*, *S. suis* and *L. monocytogenes* with MIC values ranging from 3 mg/ml to 5 mg/ml respectively.

Zhang et al. (2014) reported that sucrose and glucose monodecanoate showed inhibitory effects against *S. aureus* at 4 mg/ml and 3 mg/ml, respectively. In a similar study, Smith et al. (2008) and Nobmann et al. (2009)reported that a glucose fatty acid ether containing decanoic acid showed the greatest activity against *S. aureus* and *Listeria* at concentrations of 0.04 mM but was effective against *E. coli* at 20 mM. In this study, we showed that LMD had MIC values for all bacteria tested except *S. mutans*, although MIC values were solvent dependent for *M. KMS*, *L. monocytogenes* and *E. faecalis*.

Our previous research (Wagh et al., 2012) showed that LML was not inhibitory to Gram-negative bacteria, *E. coli O157:H7*, *Salmonella enterica* or *Klebsiella pneumonia* and this study showed that the other esters (LMO, LMD and LMM) were not inhibitory to *E. coli* O157:H7 (data not shown). On the other hand, there are a limited number of studies showing microbial inhibitory properties of sugar esters against Gram-negative bacteria. Ferrer et al. (2005) and Habulin et al. (2008) both reported limited inhibition of *E. coli* by sucrose monolaurate with MIC values of 4 mg/ml and 6.25 mg/ml respectively. Zhang et al. (2014) showed that methyl α-D-glucopyranoside monolaurate was effective in inhibiting the growth of both *S. aureus* and *E. coli* O157:H7 at a concentration of 0.188 mg/ml.

Compared to the amount of literature on the microbial inhibitory properties of sugar esters, there is very little information about the effects of the solvent used. Previous studies on microbial inhibitory activities of sugar esters involved dissolving sugar esters into an ethanol solution (Smith et al., 2008; Nobmann et al., 2009; Wagh et al., 2012; Chen et al., 2013) or DMSO (Ferrer et al., 2005) before diluting into growth media. Others have added esters directly into growth media (Devulapalle et al., 2004; Piao et al., 2006). All the esters used in the current study were soluble in a 50% ethanol solution except LMM; therefore, we only tested LMM in DMSO. Previous studies with LML showed that final ethanol concentrations greater then 7.5% were microbial inhibitory towards *L. monocytogenes* (Chen et al., 2013). In this study we found that 10% ethanol was antimicrobial to *M. KMS* and 8% DMSO was antimicrobial/inhibitory to *S. suis*. The effect of the solvent on the cell growth can be observed by the log reductions in Table 3, specifically for *S. suis* with LMM/DMSO and LMO/DMSO.

In general, MIC values of the LML/ETOH treatments were lower than the LML/DMSO treatments suggesting compounding stress of both LML and ethanol leads to growth inhibition as suggested by Chen et al. (2013). Similar results are seen with LMD/ETOH, where MIC values were obtained for *L. monocytogenes* and *E. faecalis*, but not with LMD/DMSO. Conversely, the MBC values of LML/DMSO were lower or equal to the LML/ETOH values. Therefore, the effect of ethanol on the MBC values is not understood.

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5. Conclusions

Results suggest that the chain length of the fatty acid ester significantly influences the microbial inhibitory and bactericidal activity of lactose esters towards Gram-positive bacteria. Lactose esters containing decanoate and laurate were more microbial inhibitory than esters containing octanoate and myristate. No esters inhibited the growth of the Gram-negative bacteria *E. coli O157:H7*. The solvent used to dissolve the esters influenced the microbial inhibitory activity of some bacteria. Ethanol (>7.5%) and DMSO (<8%) inhibited the growth of *L. monocytogenes* and *S. suis* respectively. Additional research on the microbial inhibitory activity of these esters in food systems without the need to prior dissolve in either ethanol or DMSO is needed.

Acknowledgement

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References

Chen, Y., Nummer, B.A., Walsh, M.K., 2013. Antilisterial activity of lactose monolaurate in milk, drinkable yogurt and cottage cheese. Lett. Appl. Microbiol. 58, 156–162. http://dx.doi.org/10.1111/lam.12169.

Devulapalle, K.S., de Segura, A.G., Ferrer, A., Alcalde, M.G., Mooser, G., Plou, F.J., 2004. Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyltransferase activity. Carbohydrate Res. 339, 1029–1034.

Ferrer, M., Soliveri, J., Plou, F.J., Lopez-Cortez, N.L., Reyes-Duarte, D.R., Christensen, M., Dopa-Patino, J.L., Ballestros, A., 2005. Synthesis of sugar esters in solvent mixtures by lipases from thermomyces lanuginosus and candida antarctica B, and their antimicrobial properties. Enzyme Microb. Technol. 36, 391–398. http://dx.doi.org/10.1016/j.enzmictec.2004.02.009.

Habulin, M., Sabeder, S., Knez, Z., 2008. Enzymatic synthesis of sugar fatty acid esters in organic solvent and in supercritical carbon dioxide and their antimicrobial activity. J. Supercrit. Fluids 45, 338–345. http://dx.doi.org/10.1016/j.supflu.2008.01.002.

Nobmann, P., Smith, A., Dunne, J., Henehan, G., Bourke, P., 2009. The anitmicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. Int. J. Food Microbiol. 128 (3), 440–445. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.10.008.

Piao, J., Kawahara-Aoyama, Y., Inoue, T., Adachi, S., 2006. Bacteriostatic activities of monoacyl sugar alcohols against thermophilic sporeformers. Biosci. Biotechnol. Biochem. 70, 263–265.

Smith, A., Nobmann, P., Henehan, G., Bourke, P., Dunne, J., 2008. Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives. Carbohydrate Res. 343, 2557–2566. http://dx.doi.org/10.1016/j.carres.2008.07.012.

Szűts, A., Szabó-Révész, P., 2012. Sucrose esters as natural surfactants in drug delivery systems. Int. J. Pharm. 433, 1–9. http://dx.doi.org/10.1016/j.ijpharm.2012.04.076.

Wagh, A., Shen, S., Shen, F.A., Miller, C.D., Walsh, M.K., 2012. Effect of lactose monolaurate on pathogenic and nonpathogenic bacteria. Appl. Environ. Microbiol. 78 (9), 3465–3668. http://dx.doi.org/10.1128/AEM.07701-11.

Walsh, M.K., Bombyk, R.A., Wagh, A., Bingham, A., Berreau, L., 2009. Synthesis of lactose monolaurate as influenced by various lipases and solvents. J. Mol. Catal. B Enzym. 60 (3–4), 171–177. http://dx.doi.org/10.1016/j.molcatb.2009.05.003.

Watanabe, T., Katayama, S., Matsubara, M., Honda, Y., Kuwahara, M., 2000. Antibacterial carbohydrate monoesters suppressing cell growth of *Streptococcus mutans* in the presence of sucrose. Curr. Microb. 41, 210–213.

Zhang, X., Wei, W., Cao, X., Feng, F., 2014. Characterization of enzymatically prepared sugar medium-chain fatty acid monoesters. J. Sci. Food Agric. 95 (8), 1631–1637. http://dx.doi.org/10.1002/jsfa.6863.

APPENDIX B

FOR CHAPTER 4

Table B.1. Standard deviations and standard errors of LMO with respect to each concentration of fat over 5 days

Times (days)	LMO		
	0.1%	0.25%	0.5%
0	0	0	0
1	7.76	7.23	6.05
2	7.84	7.53	7.29
3	7.86	7.6	7.43
4	7.88	7.73	7.52
	STDEV		
0	0	0	0
1	0.77	0.47	0.76
2	1.71	0.79	0.31
3	0.14	0.38	0.06
4	0.1	1.67	0.73
	STERR		
0	0	0	0
1	0.26	0.16	0.25
2	0.57	0.26	0.1
3	0.05	0.13	0.02
4	0.03	0.56	0.24

Table B.2. Standard deviations and standard errors of LMD with respect to each concentration over 5 days

Times (days)	LMD		
	0.1%	0.25%	0.5%
0	0	0	0
1	3.35	1.5	0.97
2	6.96	2.67	1.63
3	8.32	3.85	2.33
4	8.31	4.56	2.92
	STDEV		
0	0	0	0
1	0.2	0.52	0.22
2	0.7	0.42	0.22
3	0.11	0.57	0.32
4	0.08	0.41	0.36
	STERR		
0	0	0	0
1	0.12	0.31	0.13
2	0.41	0.24	0.13
3	0.06	0.33	0.19
4	0.05	0.24	0.21

Table B.3. Standard deviations and standard errors of LML with respect to each concentration over 5 days

Times (days)	LML		
	0.1%	0.25%	0.5%
0	0	0	0
1	1.44	1.25	0.84
2	3.07	2.61	1.26
3	5.45	3.72	1.65
4	6.93	4.52	2.15
	STDEV		
0	0	0	0
1	0.76	0.31	0.12
2	1.09	0.8	0.24
3	1.55	1.18	0.24
4	1.85	1.52	0.43
	STERR		
0	0	0	0
1	0.45	0.18	0.07
2	0.64	0.47	0.14
3	0.91	0.69	0.14
4	1.09	0.89	0.26

Table B.4. Standard deviations and standard errors of LMM with respect to each concentration over 5 days

Times (days)	LMM		
	0.1%	0.25%	0.5%
0	0	0	0
1	1.44	1.25	0.84
2	3.07	2.61	1.26
3	5.45	3.72	1.65
4	6.93	4.52	2.15
	STDEV		
0	0	0	0
1	0.76	0.31	0.12
2	1.09	0.8	0.24
3	1.55	1.18	0.24
4	1.85	1.52	0.43
	STERR		
0	0	0	0
1	0.45	0.18	0.07
2	0.64	0.47	0.14
3	0.91	0.69	0.14
4	1.09	0.89	0.26

Table B.5. Standard deviations and standard errors of Tween-20 with respect to each concentration over 5 days

Times (days)	Tween-20		
	0.1%	0.25%	0.5%
0	0	0	0
1	1.31	1.49	0.65
2	3.16	2.2	0.29
3	4.88	2.84	1.83
4	6.69	3.5	2.25
	STDEV		
0	0	0	0
1	1.13	0.34	0.1
2	1.9	0.42	0.16
3	2.67	0.09	0.22
4	3.53	0.14	0.24
	STERR		
0	0	0	0
1	0.65	0.14	0.06
2	1.1	0.17	0.09
3	1.54	0.04	0.13
4	2.04	0.06	0.14

Table B.6. Standard deviations and standard errors of Ryoto L-1695 with respect to each concentration over 5 days

Times (days)	Ryoto L-1695		
	0.1%	0.25%	0.5%
0	0.00	0.00	0.00
1	2.60	0.67	0.27
2	5.08	1.15	1.05
3	6.15	1.57	1.24
4	6.25	2.03	1.60
	STDEV		
0	0.00	0.00	0.00
1	1.73	0.14	0.36
2	3.36	0.03	0.12
3	3.94	0.00	0.08
4	3.72	0.08	0.14
	STERR		
0	0.00	0.00	0.00
1	1.02	0.10	0.21
2	1.98	0.02	0.07
3	2.32	0.00	0.05
4	2.19	0.06	0.08

Table B.7. Average D (3,2) of emulsions (at 25 ºC) prepared using LMO (0.1%, 0.25%, 0.5%), LMD (0.1%, 0.25%, 0.5%), LML (0.1%, 0.25%, 0.5%), LMM (0.1%, 0.25%, 0.5%), Tween-20 (0.1%, 0.25%, 0.5%) and Ryoto L-1695 (0.1%, 0.25%, 0.5%) from day 0 to day 4 with standard deviations

Treatments	Time (days)				
	Day 0	Day 1	Day 2	Day 3	Day 4
LMO 0.1%	1.94±0.05	1.69±0.19	1.26±0.36	0.8±0.20	1.23±0.04
LMO 0.25%	1.69±0.54	2.08±0.21	1.55±0.19	1.6±0.07	1.26±0.02
LMO 0.5%	1.86±0.24	0.89±0.03	0.81±0.03	0.7±0.11	0.71±0.07
LMD 0.1%	1.46±0.08	1.44±0.18	1.42±0.04	1.4±0.04	1.37±0.03
LMD 0.25%	0.96±0.03	1.07±0.16	0.81±0.08	0.93±0.03	1.24±0.27
LMD 0.5%	0.9±0.01	0.97±0.13	0.89±0.02	0.94±0.08	0.93±0.07
LML 0.1%	1.24±0.13	1.30±0.06	1.10±0.07	0.97±0.09	1.10±0.16
LML 0.25%	1.85±0.14	1.76±0.2	1.52±0.20	1.53±0.09	1.26±0.33
LML 0.5%	0.98±0.10	1.07±0.14	1.04±0.15	0.94±0.11	0.97±0.17
LMM 0.1%	0.95±0.24	1.00±0.24	1.09±0.22	0.81±0.16	1.12±0.12
LMM 0.25%	0.98±0.15	0.89±0.17	1.00±0.10	0.75±0.03	0.81±0.05
LMM 0.5%	0.95±0.06	0.83±0.03	0.94±0.24	0.76±0.02	0.87±0.15
Tween-20 0.1%	1.57±0.24	1.49±0.27	1.43±0.22	1.39±0.17	1.37±0.26
Tween-20 0.25%	1.29±0.13	1.52±0.22	1.34±0.09	1.08±0.39	0.96±0.20
Tween-20 0.5%	1.09±0.07	1.13±0.03	1.15±0.04	1.09±0.03	1.04±0.02
Ryoto L-1695 0.1%	1.79±0.03	1.73±0.09	1.76±0.01	1.69±0.05	1.65±0.01
Ryoto L-1695 0.25%	1.08±0.11	1.05±0.14	1.07±0.11	1.06±0.11	1.14±0.08
Ryoto L-1695 0.5%	1.09±0.06	1.13±0.07	1.05±0.09	0.92±0.20	1.11±0.08

Table B.8. Literature reviews: HLB and CMC values depend on the type of sugar used for the head group and the chain length of the fatty acid

Ref.	Sugar esters	HLB	CMC	
			surface tension	dye micellization
Zhang 2014	sugar laurate ester sugar decanoate ester sugar octanoate ester L-1695(sucrose laurate)	13.0 13.8 14.5 12.4	0.31-0.45 0.56-0.60 0.66-0.78 0.42	X
Suradkar 2006	$C_{13}E_{20}$ Tween-20 Tween-80	X	0.024 0.029 0.021	0.052 0.096 0.093
Piao 2006	monoacyl sugar alcohols (C_{10}, C_{12}, C_{14}, C_{16}) Tween-20 (C_{12})	7.2-16.7 16.7	X	X
Yanke 2004	sucrose stearate sucrose palmitate sucrose laurate	X	0.3097-0.7964 0.0742-0.1996 0.4342-0.5479	X
Soultani 2003	pure monoester blends of mono- and difructose esters sucrose esters (P1670, S1670, SP30, SP70)	8.1-10.8 6.3-8.4 6-16	0.04-0.22 0.06-0.1 0.01-0.06	X
Ferrer 2002	6-O-acyl-carbohydrate lactose esters (C_{14} and C_{16})	X	0.002-0.25 0.043-0.011	X
Hait 2001	Tweens (20, 30, 60, 80)	X		1.15-5.57
Garofalakis 2000	sugar monoesters of xylose, galactose, sucrose, lactose (C_{12}-C_{16}) + NaCI, KCI, CaC_{l2} commercial monoesters	X	0.0041-0.21 0.004-6.5	X
Patist 2000	Tween (20, 22, 40, 60, 80), triton X-100, Brij (35, 58, 78), $C_{12}(EO)_5$, $C_{12}(EO)_8$	X	0.0018-0.080	0.0071-0.2

HLB, hydrophile-lipophile balance; CMC, critical micelle concentration

Table B.9. The SAS analysis of clarification layer thickness yielded to see the effect of lactose esters

Lineplot of Results 10:15 Thursday, January 18, 2018 1

The Mixed Procedure

Model Information	
Data Set	WORK.LACTOSE2
Dependent Variable	valueSQRT
Covariance Structure	Autoregressive Moving Average
Subject Effect	CONC*variable
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Satterthwaite

Class Level Information		
Class	Levels	Values
CONC	4	0 0.001 0.005 0.0025
variable	6	LMD LML LMM LMO Ryo Twe
Day	4	1 2 3 4

Dimensions	
Covariance Parameters	3
Columns in X	55
Columns in Z	0
Subjects	24
Max Obs Per Subject	4

Number of Observations	
Number of Observations Read	96
Number of Observations Used	96
Number of Observations Not Used	0

Iteration History			
Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-36.17257575	
1	2	-82.85831388	52.99177357
2	1	-83.75643807	10.08264988
3	2	-97.86280924	17.66111681
4	2	-113.78100150	0.18010650
5	2	-120.55998728	0.01005964
6	1	-120.77038697	0.00395333

Lineplot of Results 10:15 Thursday, January 18, 2018 2

The Mixed Procedure

Iteration History			
Iteration	Evaluations	-2 Res Log Like	Criterion
7	1	-121.32130858	0.00070557
8	1	-121.41305211	0.00003312
9	1	-121.41701401	0.00000009
10	1	-121.41702407	0.00000000

Convergence criteria met.

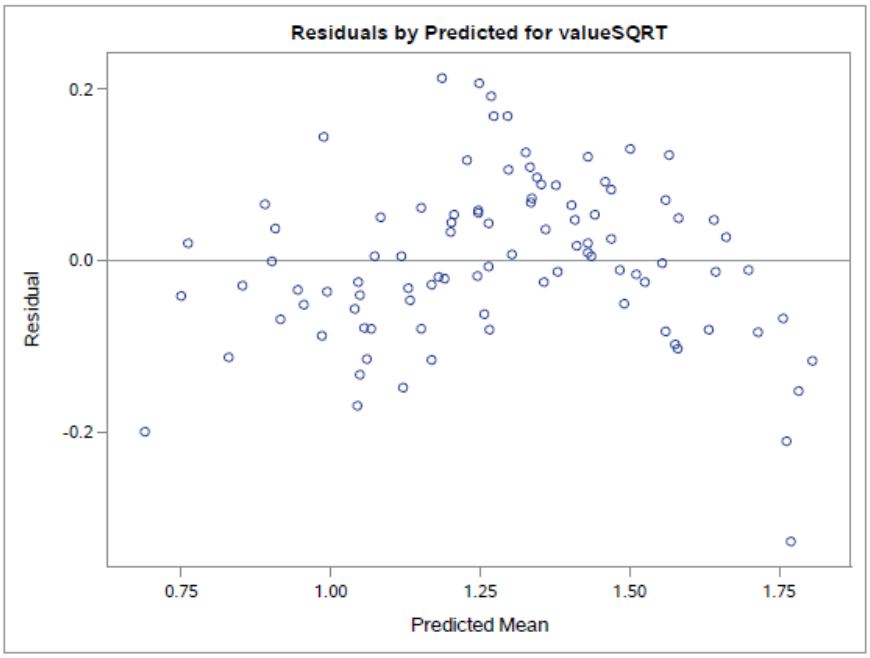
Covariance Parameter Estimates		
Cov Parm	Subject	Estimate
Rho	CONC*variable	0.8743
Gamma	CONC*variable	0.9271
Residual		0.01594

Fit Statistics	
-2 Res Log Likelihood	-121.4
AIC (smaller is better)	-115.4
AICC (smaller is better)	-115.0
BIC (smaller is better)	-111.9

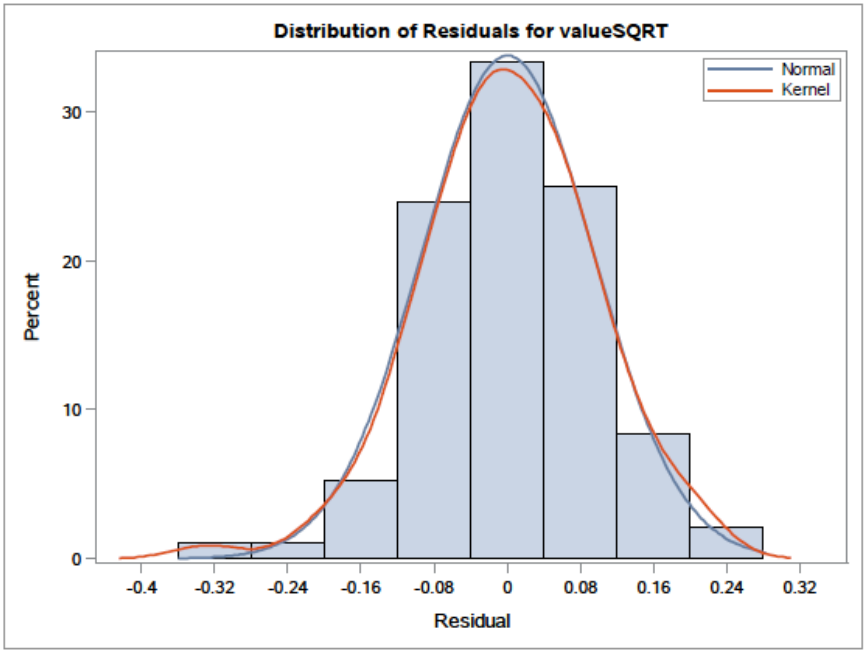
Null Model Likelihood Ratio Test		
DF	Chi-Square	Pr > ChiSq
2	85.24	<.0001

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
CONC	3	15.5	22.27	<.0001
variable	5	15.5	4.22	0.0129
Day	3	22.7	105.49	<.0001
CONC*Day	9	22.7	1.43	0.2327
variable*Day	15	22.7	2.63	0.0185

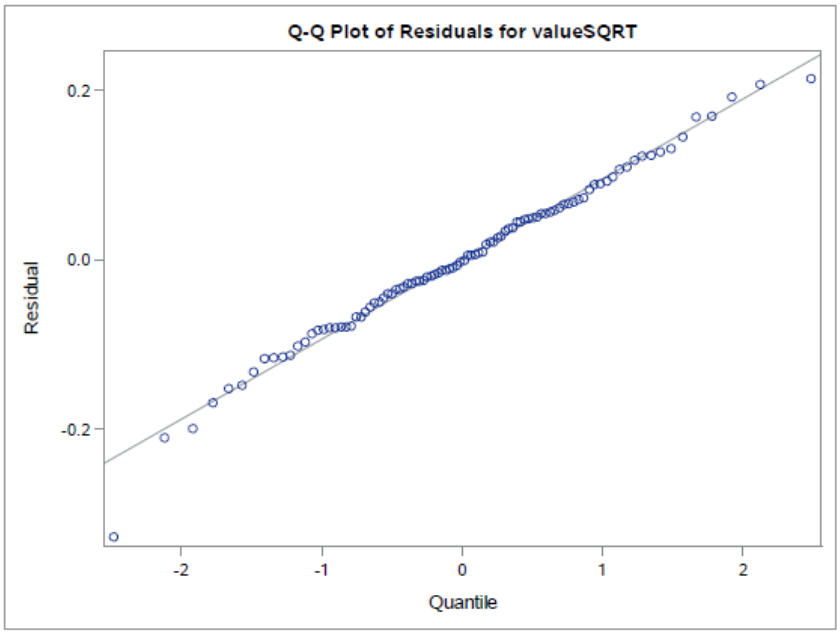
The Mixed Procedure



The Mixed Procedure



The Mixed Procedure



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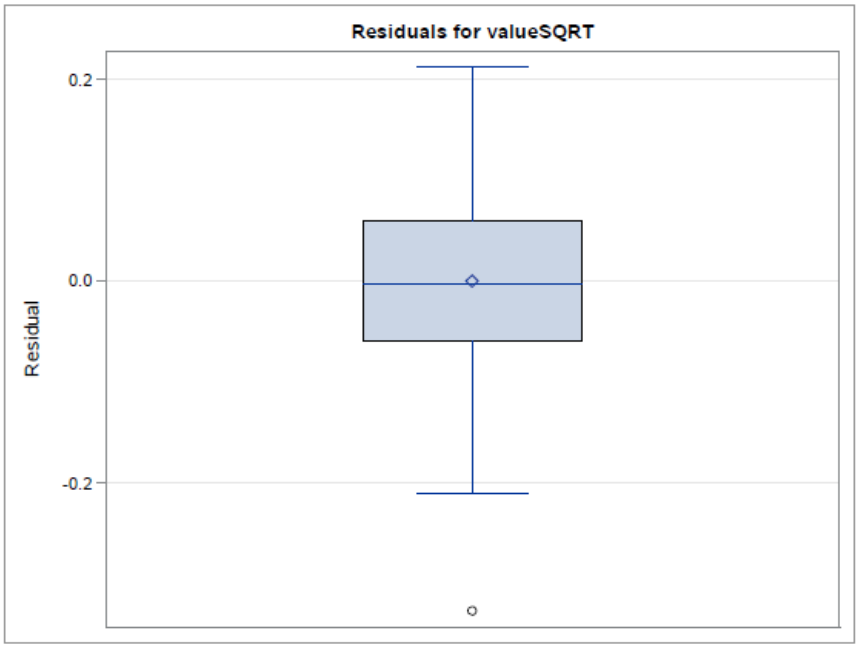


The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments			
N	96	Sum Weights	96
Mean	0	Sum Observations	0
Std Deviation	0.0943855	Variance	0.00890862
Skewness	-0.3105773	Kurtosis	0.83573634
Uncorrected SS	0.84631912	Corrected SS	0.84631912
Coeff Variation	.	Std Error Mean	0.00963318

Basic Statistical Measures			
Location		Variability	
Mean	0.00000	Std Deviation	0.09439
Median	-0.00228	Variance	0.00891
Mode	.	Range	0.54037
		Interquartile Range	0.11890

Tests for Location: Mu0=0				
Test	Statistic		p Value	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-1	Pr >= |M|	0.9188
Signed Rank	S	41	Pr >= |S|	0.8818

Tests for Normality				
Test	Statistic		p Value	
Shapiro-Wilk	W	0.987878	Pr < W	0.5293
Kolmogorov-Smirnov	D	0.041857	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.025799	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.192354	Pr > A-Sq	>0.2500

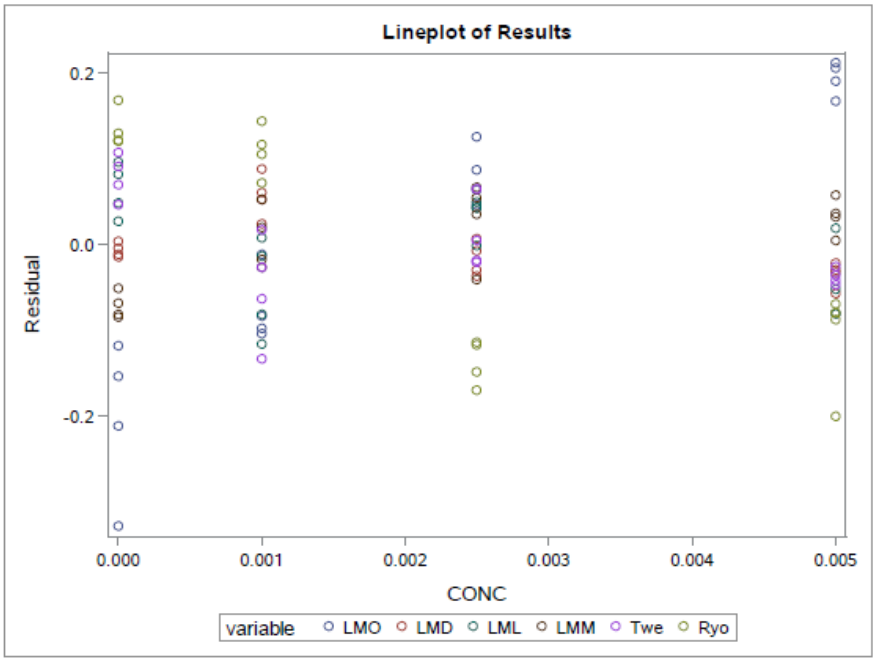
Quantiles (Definition 5)	
Quantile	Estimate
100% Max	0.21258233
99%	0.21258233
95%	0.16765938
90%	0.12117419
75% Q3	0.05938495
50% Median	-0.00228225
25% Q1	-0.05951997

The UNIVARIATE Procedure
Variable: Resid (Residual)

Quantiles (Definition 5)	
Quantile	**Estimate**
10%	-0.11529920
5%	-0.15262632
1%	-0.32778944
0% Min	-0.32778944

Extreme Observations			
Lowest		**Highest**	
Value	**Obs**	**Value**	**Obs**
-0.327789	4	0.167659	13
-0.211074	8	0.168634	84
-0.199608	81	0.191653	9
-0.169404	86	0.206376	5
-0.152626	12	0.212582	1

10:15 Thursday, January 18, 2018 9



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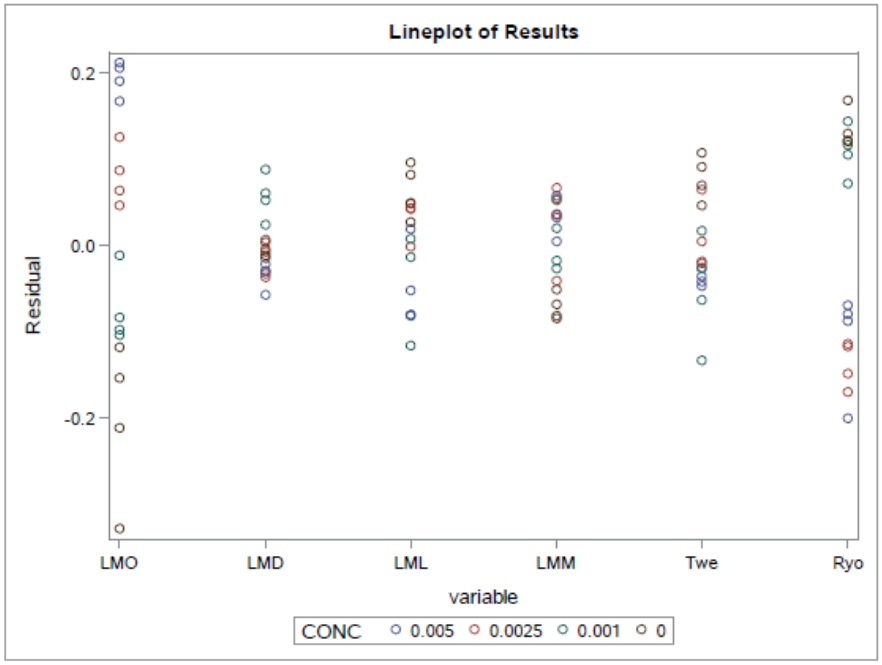

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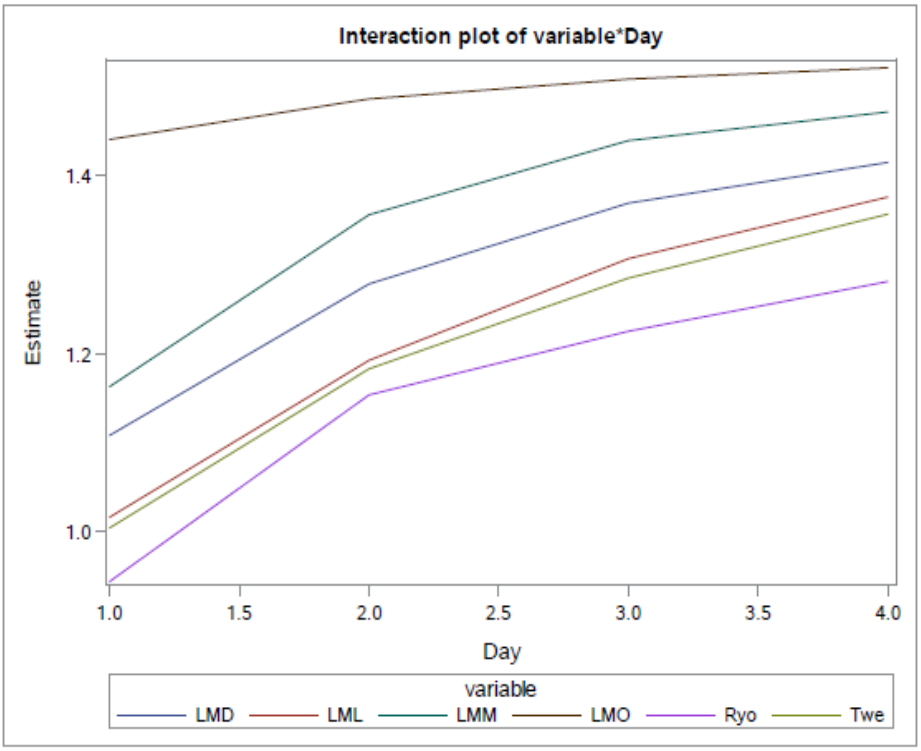


10:15 Thursday, January 18, 2018 12

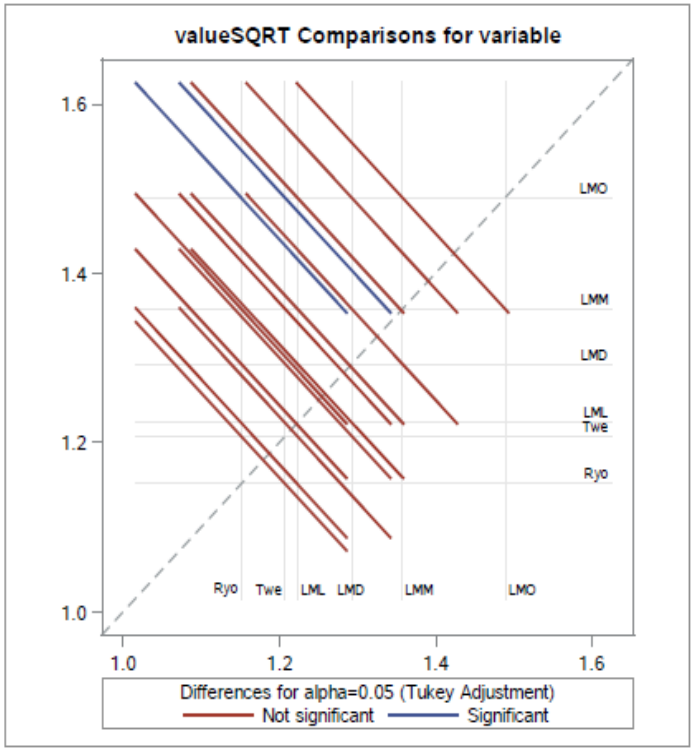
Average change in thickness (mm) for each Lactose Ester

Obs	Effect	CONC	variable	Day	Estimate	StdErr	DF	tValue	Probt
1	variable	_	Ryo	_	1.1514	0.05951	15.5	19.35	<.0001
2	variable	_	Twe	_	1.2076	0.05951	15.5	20.29	<.0001
3	variable	_	LML	_	1.2232	0.05951	15.5	20.56	<.0001
4	variable	_	LMD	_	1.2929	0.05951	15.5	21.73	<.0001
5	variable	_	LMM	_	1.3577	0.05951	15.5	22.81	<.0001
6	variable	_	LMO	_	1.4892	0.05951	15.5	25.03	<.0001

The PLM Procedure

Store Information	
Item Store	WORK.POLYFIT
Data Set Created From	WORK.LACTOSE2
Created By	PROC MIXED
Date Created	18JAN18:10:28:41
Response Variable	valueSQRT
Distribution	Normal
Class Variables	CONC variable Day
Model Effects	Intercept CONC variable Day CONC*Day variable*Day
Degrees of freedom Method	Satterthwaite

Class Level Information		
Class	Levels	Values
CONC	4	0 0.001 0.005 0.0025
variable	6	LMD LML LMM LMO Ryo Twe
Day	4	1 2 3 4



The PLM Procedure

Tukey Grouping for variable Least Squares Means (Alpha=0.05)			
LS-means with the same letter are not significantly different.			
variable	Estimate		
LMO	1.4892		A
			A
LMM	1.3577	B	A
		B	A
LMD	1.2929	B	A
		B	A
LML	1.2232	B	A
		B	
Twe	1.2076	B	
		B	
Ryo	1.1514	B	

Table B.10. Statistics for droplet size diameter measurements

"Analysis of raw droplet size D32 Data"
Determination of Covariance Structure

15:09 Thursday, January 18, 2018 1

The Mixed Procedure

Model Information	
Data Set	WORK.DROPLET
Dependent Variable	D32
Covariance Structure	Autoregressive Moving Average
Subject Effect	rep
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information		
Class	Levels	Values
time	5	0 1 2 3 4
type	6	1 2 3 4 5 6
concentration	3	0.1 0.25 0.5

Dimensions	
Covariance Parameters	3
Columns in X	168
Columns in Z	0
Subjects	270
Max Obs Per Subject	1

Number of Observations	
Number of Observations Read	270
Number of Observations Used	270
Number of Observations Not Used	0

Iteration History			
Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-56.49023767	
1	1	-56.49023767	0.00000000

Convergence criteria met but final hessian is not positive definite.

The Mixed Procedure

Covariance Parameter Estimates		
Cov Parm	Subject	Estimate
Rho	rep	0
Gamma	rep	0
Residual		0.02470

Fit Statistics	
-2 Res Log Likelihood	-56.5
AIC (smaller is better)	-50.5
AICC (smaller is better)	-50.4
BIC (smaller is better)	-39.7

Null Model Likelihood Ratio Test		
DF	Chi-Square	Pr > ChiSq
2	0.00	1.0000

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
time	4	180	18.83	<.0001
type	5	180	48.97	<.0001
time*type	20	180	7.23	<.0001
concentration	2	180	106.00	<.0001
time*concentration	8	180	4.94	<.0001
type*concentration	10	180	26.43	<.0001
time*type*concentrat	40	180	2.47	<.0001

"Analysis of raw droplet size D32 Data" 15:09 Thursday, January 18, 2018 3
Determination of Covariance Structure

The Mixed Procedure

Least Squares Means

Effect	"time"	"type"	"conc"	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0			1.2781	0.02139	180	59.76	<.0001
time	1			1.2795	0.02139	180	59.83	<.0001
time	2			1.1631	0.02139	180	54.39	<.0001
time	3			1.0756	0.02139	180	50.29	<.0001
time	4			1.1184	0.02139	180	52.30	<.0001
type		1		1.3383	0.02343	180	57.13	<.0001
type		2		1.1192	0.02343	180	47.77	<.0001
type		3		1.1604	0.02343	180	49.53	<.0001
type		4		0.8965	0.02343	180	38.27	<.0001
type		5		1.2950	0.02343	180	55.28	<.0001
type		6		1.2882	0.02343	180	54.99	<.0001
time*type	0	1		1.8307	0.05239	180	34.95	<.0001
time*type	0	2		1.1232	0.05239	180	21.44	<.0001
time*type	0	3		1.2083	0.05239	180	23.07	<.0001
time*type	0	4		0.9572	0.05239	180	18.27	<.0001
time*type	0	5		1.2250	0.05239	180	23.38	<.0001
time*type	0	6		1.3239	0.05239	180	25.27	<.0001
time*type	1	1		1.5534	0.05239	180	29.65	<.0001
time*type	1	2		1.1626	0.05239	180	22.19	<.0001
time*type	1	3		1.3169	0.05239	180	25.14	<.0001
time*type	1	4		0.9059	0.05239	180	17.29	<.0001
time*type	1	5		1.4376	0.05239	180	27.44	<.0001
time*type	1	6		1.3009	0.05239	180	24.83	<.0001
time*type	2	1		1.2060	0.05239	180	23.02	<.0001
time*type	2	2		1.0403	0.05239	180	19.86	<.0001
time*type	2	3		1.1962	0.05239	180	22.83	<.0001
time*type	2	4		0.9127	0.05239	180	17.42	<.0001
time*type	2	5		1.3300	0.05239	180	25.39	<.0001
time*type	2	6		1.2936	0.05239	180	24.69	<.0001
time*type	3	1		1.0348	0.05239	180	19.75	<.0001
time*type	3	2		1.0917	0.05239	180	20.84	<.0001
time*type	3	3		1.0463	0.05239	180	19.97	<.0001
time*type	3	4		0.7750	0.05239	180	14.79	<.0001
time*type	3	5		1.2847	0.05239	180	24.52	<.0001

The Mixed Procedure

Differences of Least Squares Means													
Effect	"time"	"type"	"conc"	"time"	"type"	"conc"	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
time	0			1			-0.00148	0.03024	180	-0.05	0.9610	Tukey	1.0000
time	0			2			0.1149	0.03024	180	3.80	0.0002	Tukey	0.0018
time	0			3			0.2024	0.03024	180	6.69	<.0001	Tukey	<.0001
time	0			4			0.1596	0.03024	180	5.28	<.0001	Tukey	<.0001
time	1			2			0.1164	0.03024	180	3.85	0.0002	Tukey	0.0015
time	1			3			0.2039	0.03024	180	6.74	<.0001	Tukey	<.0001
time	1			4			0.1611	0.03024	180	5.33	<.0001	Tukey	<.0001
time	2			3			0.08752	0.03024	180	2.89	0.0043	Tukey	0.0343
time	2			4			0.04472	0.03024	180	1.48	0.1410	Tukey	0.5777
time	3			4			-0.04280	0.03024	180	-1.41	0.1588	Tukey	0.6188
type		1			2		0.2191	0.03313	180	6.61	<.0001	Tukey	<.0001
type		1			3		0.1779	0.03313	180	5.37	<.0001	Tukey	<.0001
type		1			4		0.4418	0.03313	180	13.33	<.0001	Tukey	<.0001
type		1			5		0.04333	0.03313	180	1.31	0.1926	Tukey	0.7804
type		1			6		0.05011	0.03313	180	1.51	0.1322	Tukey	0.6567
type		2			3		-0.04120	0.03313	180	-1.24	0.2153	Tukey	0.8148
type		2			4		0.2227	0.03313	180	6.72	<.0001	Tukey	<.0001
type		2			5		-0.1758	0.03313	180	-5.31	<.0001	Tukey	<.0001
type		2			6		-0.1690	0.03313	180	-5.10	<.0001	Tukey	<.0001
type		3			4		0.2639	0.03313	180	7.96	<.0001	Tukey	<.0001
type		3			5		-0.1346	0.03313	180	-4.06	<.0001	Tukey	0.0010
type		3			6		-0.1278	0.03313	180	-3.86	0.0002	Tukey	0.0022
type		4			5		-0.3985	0.03313	180	-12.03	<.0001	Tukey	<.0001
type		4			6		-0.3917	0.03313	180	-11.82	<.0001	Tukey	<.0001
type		5			6		0.006778	0.03313	180	0.20	0.8381	Tukey	0.9999
time*type	0	1		0	2		0.7074	0.07408	180	9.55	<.0001	Tukey	<.0001
time*type	0	1		0	3		0.6223	0.07408	180	8.40	<.0001	Tukey	<.0001
time*type	0	1		0	4		0.8734	0.07408	180	11.79	<.0001	Tukey	<.0001
time*type	0	1		0	5		0.6057	0.07408	180	8.18	<.0001	Tukey	<.0001
time*type	0	1		0	6		0.5068	0.07408	180	6.84	<.0001	Tukey	<.0001
time*type	0	1		1	1		0.2772	0.07408	180	3.74	0.0002	Tukey	0.0620
time*type	0	1		1	2		0.6681	0.07408	180	9.02	<.0001	Tukey	<.0001
time*type	0	1		1	3		0.5138	0.07408	180	6.94	<.0001	Tukey	<.0001
time*type	0	1		1	4		0.9248	0.07408	180	12.48	<.0001	Tukey	<.0001

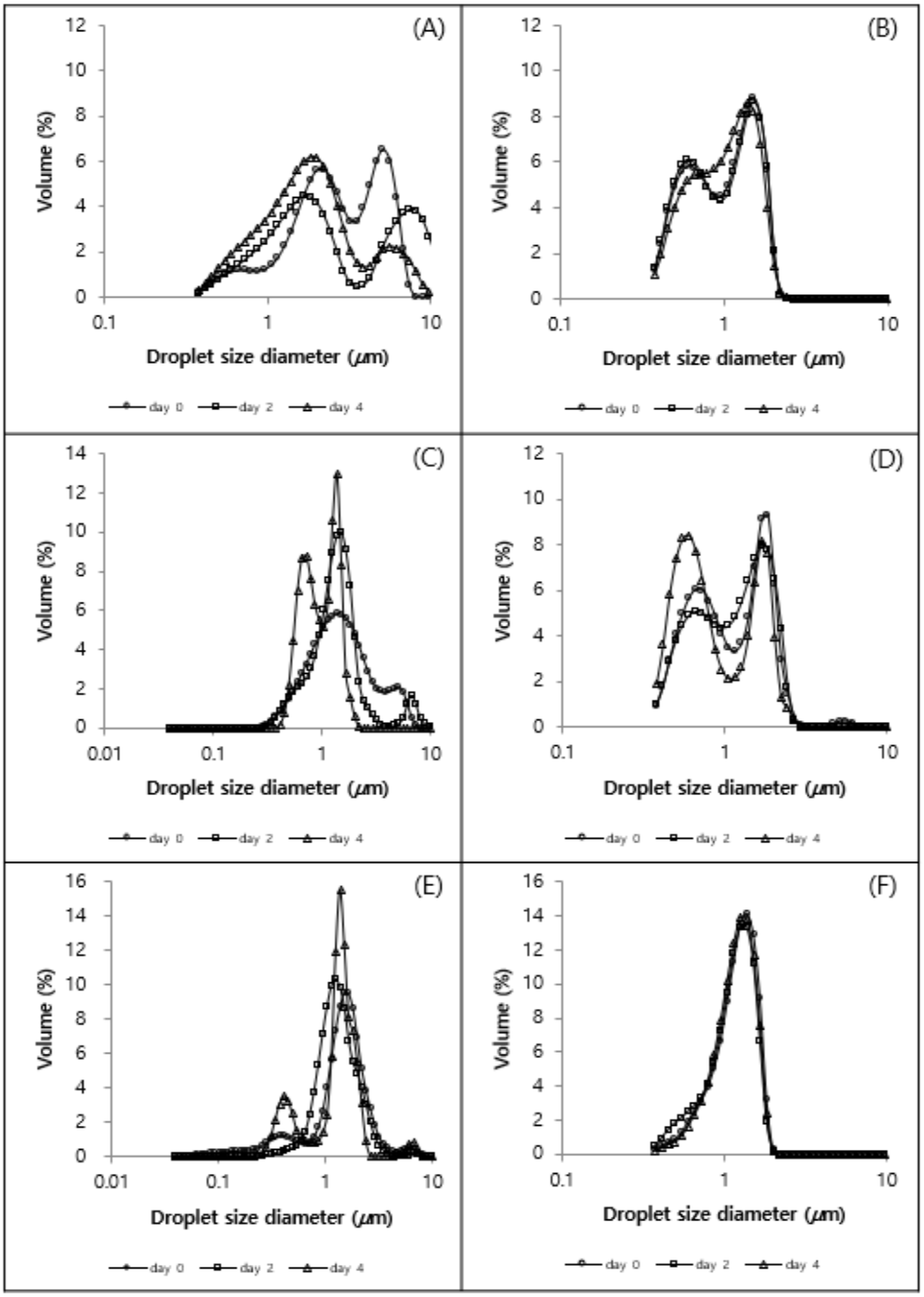


Figure B.1. Droplet size diameter (μm) distribution with respect to percentage of the volume (%) at (A) 0.25% LMO, (B) 0.25% LMD, (C) 0.25% LML, (D) 0.25% LMM, (E) 0.25% Tween-20 and (F) 0.25% Ryoto L-1695 emulsions formulated for (○) day 0, (□) day 2 and (△) day 4

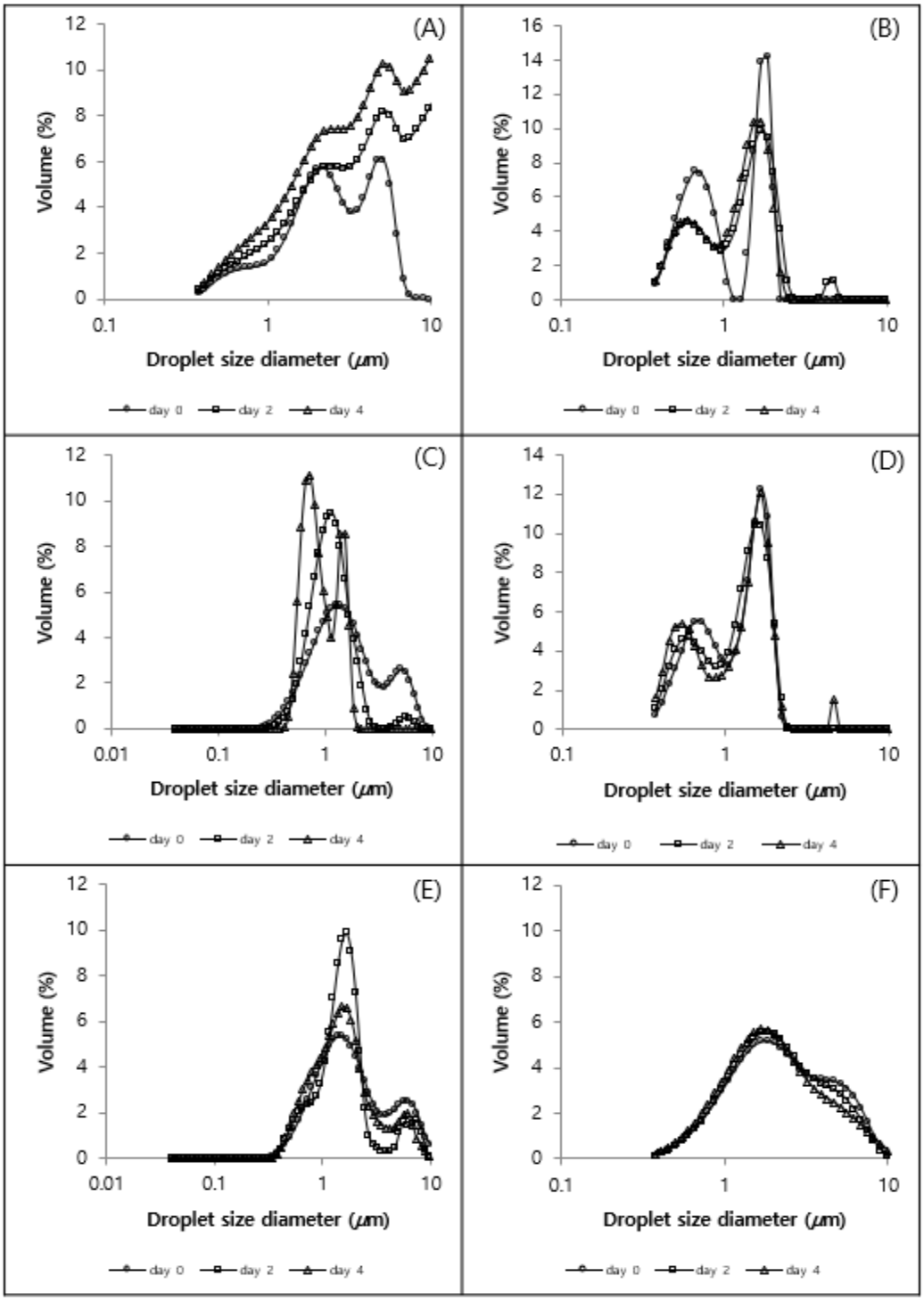


Figure B.2. Droplet size diameter (μm) distribution with respect to percentage of the volume (%) at (A) 0.1% LMO, (B) 0.1% LMD, (C) 0.1% LML, (D) 0.1% LMM, (E) 0.1% Tween-20 and (F) 0.1% Ryoto L-1695 emulsions formulated for (○) day 0, (□) day 2 and (△) day 4

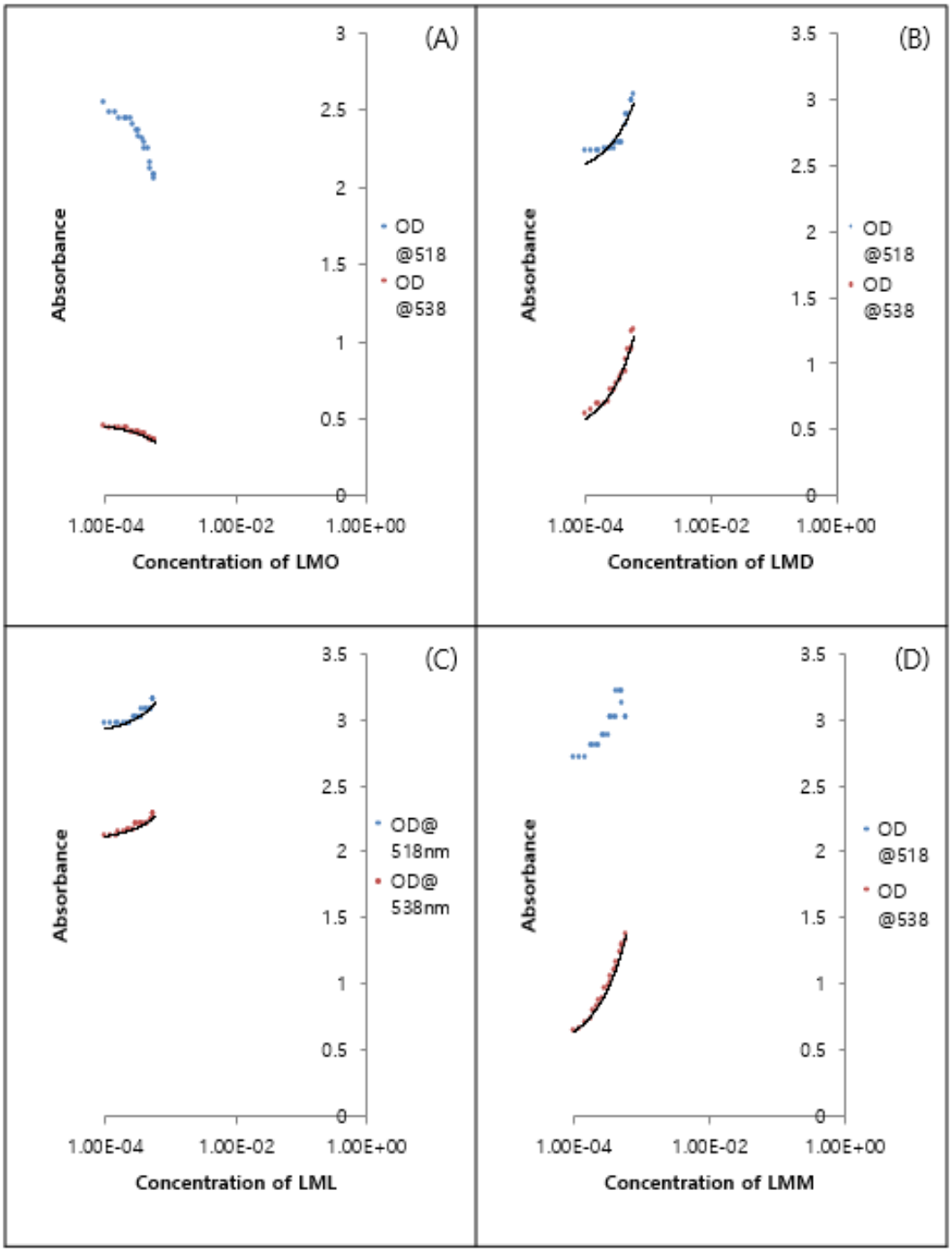


Figure B.3. Critical micelle concentration (CMC) determination of esters including LMO (CMC= 0.96 mM), LMD (CMC= 0.89 mM), LML (CMC= 0.72 mM) and LMM (CMC= 0.56mM) using the dye micellization method (absorbance at 518 nm and at 538 nm) Eosin Y concentration: 0.19 mM

CURRICULUM VITAE

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Personal Statement

I am a Ph.D. at Utah State University in the College of Agriculture and Applied Sciences program, a graduate research assistant in the department of Nutrition, Dietetics and Food Science. I have worked with Dr. Marie K. Walsh in development of lactose fatty acid esters containing octanoate, decanoate, laurate and myristate. My research showed that lactose monolaurate and lactose monodecanoate exhibited greater microbial inhibitory properties as well as emulsification activities. I research with an avid eagerness to know more about food science and coupled with a strong desire to carve out my own niche, in the areas of food additives and food safety. My background includes extensive laboratory skills, research and date collection on food science. A teaching certificate in Food Science includes a deeper understanding of the area.

Education

Aug. 2013. – May. 2018. Ph.D. Department of Nutrition, Dietetics and Food Science, College of Agriculture and Applied Sciences, Utah State University, logan, UT, USA

Aug. 2011. – Dec. 2011. Non-degree, Quincy College, MA, USA

Mar. 2005. – Feb. 2009. B.S. Department of Food Science and Technology, College of Fisheries Sciences, Pukyong National University, Busan, South Korea

Professional and Experiences

Utah State University (*Aug. 2013. – May. 2018.*)
Graduate Teaching Assistant (*Aug. 2015. – 2016.*)
• Food enzymes lab
• Food proteins lab

Graduate Research Assistant (*Aug. 2013. – May. 2018.*)
• Enzymatic synthesis of lactose esters
• Investigated the growth inhibitory properties of lactose esters
• Investigated the emulsification properties of lactose esters

• Investigated anti-listerial activity of lactose esters in milk
• Training in the use of lab equipment
• Completed HACCP program
• Completed Lab Safety Initial Training

Accomplishment

- Experience with microbial work (BSL 2+ lab); *Listeria* sp., *Bacillus cereus, Mycobacterium* sp., *Streptococcus* sp., *Enterococcus faecalis, E. coli* O157:H7, *Salmonella*
- Technical experience in high-performance liquid chromatography (HPLC), gas–chromatography (GC), thin layer chromatography (TLC), Turbiscan Classic-MA2000, Beckman Coulter droplet size analyzer, Ultra-turrax T25, microfluidizer, EpiShearProbe Sonicator, NMR, Enzyme exraction, PCR
- Experience with commercial enzyme work; *Rhizomucormehei, Thermomyces–lanuginosus*, *Pseudomonas cepacian*, *Candida antarctica*
- Statistical analysis experience with SAS program (taken the courses of linear regression and time series, design of experiments)
- Obtained teaching experience and BSL lab management abilities
- Collaboration research with the chemistry department at Utah State University

Ulsan Natural Science High School, Ulsan, South Korea
***Food Science Teacher* (*Feb. 2013. – Aug. 2013.*)**
• Planned and taught lessons in food science, food nutrition and food hygiene

Accomplishments

- Obtained valuable teaching experience for high school students
- Successfully supervised and assisted students

Pukyong National University
***Undergraduate Research Assistant, Food Microbiology lab* (*2007. – 2008.*)**
• Managed lab equipment and facilities
• Well trained in microbial detection in seawater

Honors and Awards
• ***June. 2017.* American Society for Microbiology (ASM) Outstanding Student Abstract Award of American Society for Microbiology "Anti-listerial Activity of Lactose Esters in Milk", New Orleans, LA, USA**
• *2017.* Travel Grant of Dep. of Nutrition, Dietetics, and Food Science, Utah State University, UT, USA
• *2016.* Team member for the Tangy Tomato product development competition, Food System Network Corporation, NY, USA
• *July. 2016.* Institute of Food Technologists (IFT) Poster presentation "Emulsification Properties of Lactose Fatty Acid Esters", Chicago, Il, USA
• *2016.* Travel Grant of Research Graduate Studies, Utah State University, UT, USA
• *2015. – 2016.* Invitation seminar "Synthesis of Lactose Esters and their Growth Inhibitory Properties", Department of Food Science, Pukyong National University, Busan, South Korea

- *Aug. 2015.* The 2015 Idaho Milk Processors' Association Annual Conference GRAND CHAMPION “EUREKA Marinade”, Sun Valley, ID, USA
- *2014. – 2015.* The Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Fellowship, the College of Agriculture and Applied Sciences, Utah State University, UT, USA
- *Aug. 2013. – 2018.* Graduate Research Assistantship, Department of Nutrition, Dietetics, and Food Science, Utah State University, UT, USA
- *2007. – 2008.* Award for Academic Achievement in Department of Food Science, Pukyong National University, Busan, South Korea

Certifications and Memberships

- *2016.* – Member, American Society of Microbiologists (ASM)
- *2015.* – Member, Institute of Food Technologists (IFT)
- *2014.* HACCP Certificate, Western Dairy Center, Utah State University, USA
- *2014.* Statistical Process Control Certificate, Western Dairy Center, Utah State University, USA
- *2014.* Safe Quality Foods Certificate, Western Dairy Center, Utah State University, USA
- *2014.* Blood-borne Pathogens Certificate, Bio Safety Level 2+, Environmental Health and Safety office, Utah State University, USA
- *2013.* – Member, Food Science Club, Utah State University, USA
- *2013.* Certification of Korean History (Level 3), National Institute of Korean History, South Korea
- *2009.* Certification of Food Science Secondary Teaching, Pukyong National University, South Korea

Additional Recent Publication

- Growth Inhibitory Properties of Lactose Fatty Acid Esters. 2017. Seung-Min Lee, Guneev Sandhu and Marie K. Walsh. Saudi Journal of Biological Science. *Volume 24, Issue 7.*