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ENZYMATIC SYNTHESIS OF MALTODEXTRIN FATTY ACID ESTERS AND THEIR EMULSION STABILIZING AND MICROBIAL INHIBITORY PROPERTIES

by

Namhyeon Park

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved

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UTAH STATE UNIVERSITY
Logan, Utah
2021

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ABSTRACT

Enzymatic synthesis of maltodextrin fatty acid esters and
their emulsion stabilizing and microbial inhibitory properties

by

Namhyeon Park, Doctor of Philosophy

Utah State University, 2021

Major Professor: Dr. Marie K. Walsh
Department: Nutrition, Dietetics, and Food Sciences

Maltodextrin fatty acid esters are simply maltodextrins attached to fatty acids by esterification, and this group of compounds is within the category of carbohydrate fatty acid esters. They are non-toxic and biodegradable, satisfying the consumer's demand. Carbohydrate fatty acid esters are widely used in the food industry, especially sucrose esters. However, most of them exhibit poor water solubility, resulting in limited application.

To overcome this solubility issue, maltodextrin, consisting of several hydrophilic glucoses, was selected as a substrate. The reaction was facilitated using lipase and food-grade ethanol, and this produced six different maltodextrin fatty acid esters from two different average sizes of maltodextrin and three different types of fatty acids.

The ratio of fatty acids attached to glucose units of maltodextrin was low (0.026 to 0.016), and the resulting highly hydrophilic compounds showed high aqueous solubility (101% to 93%) at a 1% level (w/v).

In an emulsion system (20% oil-in-water), esterified maltodextrin caused highly increased stability of emulsions. Notably, maltodextrin fatty acid esters showed lowered emulsion forming abilities but indicated enhanced emulsion stabilizing properties compared to commercial emulsifiers.

In vitro microbial inhibitory assay using growth media (measuring minimum inhibitory concentrations) showed a broader inhibitory spectrum of maltodextrin fatty acid esters than commercial sucrose laurate against 11 food-related microorganisms. Maltodextrin (larger molecular size) attached to laurate, in particular, showed the highest inhibitory levels. This inhibition is generally accompanied by bacterial morphological damages and increased leakage of proteins from bacterial cells.

In various food models at different storage temperatures, maltodextrin laurate yielded a high reduction of *Bacillus subtilis* in milk at 4°C and *Lactobacillus plantarum* in apple juice at all temperatures. However, the inhibitory activity of maltodextrin laurate against *L. plantarum* was highly decreasesd in tomato ketchup and French dressing, where sucrose laurate was still inhibitive at 4°C. In addition, maltodextrin laurate showed decreased toxicity compared with commercial sucrose laurate against 2 different human colonic epithelial cells.

Maltodextrin fatty acid esters showed variable efficacy depending on the complex influence of the food matrix and storage temperatures, and this activity change suggests the varying applicability of compounds for specific food products. However, maltodextrin fatty acid esters generally showed high emulsion stabilizing and broad microbial inhibitory

properties with good water solubility and low cytotoxicity, suggesting the potential of the compounds as food additives.

(176 pages)

PUBLIC ABSTRACT

Enzymatic synthesis of maltodextrin fatty acid esters
and their emulsion stabilizing and microbial inhibitory properties

Namhyeon Park

Food additives have been one of the traditional methods for preserving foods and are still in everyday use for multiple reasons. As the market size of processed foods grows, the use of food additives is increasing. Simultaneously, consumers try to avoid specific food additives or demand less processed foods because of several potential health concerns, suggesting the need for safe food additives.

In this environment, one group of food additives consisting of carbohydrates and fatty acids have received attention because of its nontoxicity and biodegradability with diverse functions. Notably, carbohydrate fatty acid compounds made of sucrose have been approved and used in the food industry. However, because of a relatively large ratio of low water-soluble fatty acid to sucrose, most of these compounds have demonstrated poor water solubility, leading to limited application.

Maltodextrin is produced by the breakdown of starch and consists of many smaller units which are water-soluble. This product was selected to attach to fatty acids and led to good water solubility. In addition, maltodextrin attached to fatty acids stabilized the immiscible mixture of oil and water not to be separated and inhibited multiple food-spoilage or poisoning microorganisms in lab and food models. This compound also showed low intestinal toxicity.

Maltodextrin attached to fatty acids appears to be a food additive that can decrease the potential health risks of food products while maintaining the food quality by stabilizing the food system and/or preventing microbial contamination.

ACKNOWLEDGMENTS

I would like to express my deep gratitude for the academic and financial support from my primary supervisor, Dr. Marie K. Walsh. Among many things, especially, I could learn a lot from her scientific writing and logical comments. Then, I can still enjoy experiments and dream of a bigger goal, thanks to her support and encouragement.

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I would like to give a thanks to my family for the endless support to let me stand

here, and I am indebted to them for the proper role as a member of our family. Lastly, I would like to extend a special thanks to my wife, Jiyoun and my daughter, Jio.

Namhyeon Park

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LIST OF ABBREVIATION

ANOVA	Analysis of Variance
AGU	Anhydrous glucose unit
BCA	Bicinchoninic acid
BHI	Brain heart infusion
CFA	Carbohydrate fatty acid ester
CFU	Colony-forming unit
DE	Dextrose equivalent
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DS	Degree of substitution
DSC	Differential scanning calorimeter
DW	Distilled water
EDTA	Ethylenediamine tetraacetic acid
ELSD	Evaporative light scattering detector
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
FDA	Food and drug administration

FTIR	Fourier-transform infrared spectroscopy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IC_{50}	Half-maximal inhibitory concentration
LB	Luria-Bertani
MBC	Minimum bactericidal concentration
MFA	Maltodextrin fatty acid ester
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
ML	Maltodextrin laurate
MPC	Milk protein concentrate
MRS	De Man, Rogosa and Sharpe
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NB	Nutrient broth
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate Buffered Saline
PES	Polyethersulfone

ppm	Part per million
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute
SE	Sucrose ester
SL	Sucrose laurate
SEM	Scanning electron microscope
SFA	Sucrose fatty acid ester
TSB	Tryptic soy broth
T20	Tween 20
UV-VIS	Ultraviolet–visible
VL	Vinyl laurate
VP	Vinyl palmitate
YM	Yeast malt

CHAPTER Ⅰ

INTRODUCTION

Consumers require proper safety, quality, and shelf-life of food products, but at the same time, there is consumers' growing demand for products containing no food additives (Saltmarsh & Insall, 2013). People investigate the labeling to avoid specific food additives before purchasing products or selecting fresh, organic, or less processed food products. Those consumer behaviors pressure the food industry, resulting in a continuous need for alternatives to current food additives.

In this context, carbohydrate fatty acid esters (CFAs), carbohydrates esterified with fatty acids, receive attention as food additives. They are reported to be non-toxic and biodegradable (Nobmann, Bourke, Dunne, & Henehan, 2010) and synthesized from two different food sources, suggesting fewer potential health effects.

Sucrose has been a popular substrate for CFAs' synthesis because of its affordable price (Neta, Teixeira, & Rodrigues, 2015) with easy availability and a simple structure. Sucrose fatty acid esters (SFAs) have been actively studied and are allowed by the U.S. Food and Drug Administration (U.S. FDA) as food additives. Regardless of the many merits of SFAs, most of these compounds other than sucrose laurate (SL) could not exhibit good water solubility (Nagai et al., 2016), leading to limitations in the food industry.

High water solubility is vital for food applications because of the various functions such as emulsifiers and stabilizers in the aqueous system (Guo, Hu, Wang, & Ai, 2017), which is the typical food system. Besides, lower water solubility was suggested to correlate to reduced microbial inhibitory activity (Smith, Nobmann, Henehan, Bourke, & Dunne,

2008), which is one of the properties of interest.

In a desire to relieve this solubility issue, the present study selected maltodextrin. Maltodextrin is starch hydrolysate (glucose polymers) and is reported to be non-toxic and cheap, making it a common food ingredient (Udomrati & Gohtani, 2014) and the potential candidate for CFAs' synthesis. Notably, maltodextrin is highly soluble in water, and that property was mainly chosen to enhance the aqueous solubility of maltodextrin fatty acid esters (MFAs) synthesized. Many hydroxyl groups in maltodextrin were suggested for the high aqueous solubility because those multiple hydroxyl groups have strong interaction with water molecules (Guo et al., 2017).

The current study focused on synthesizing MFAs with a relatively low degree of substitution (DS) (the ratio of fatty acids attached), which was a contrary approach from previous studies maximizing DS (Sun, Yang, Wang, Liu, & Xu, 2013; Udomrati & Gohtani, 2014). Functional properties of CFAs are thought to result from an amphiphilicity by adding hydrophobic fatty acids, so MFAs' activities such as emulsifiers or antimicrobials could be related to DS. That might be the reason for optimizing reactions regarding DS in the previous studies (Sun et al., 2013; Udomrati et al., 2014).

Instead, this additional hydrophobicity is also likely related to reduced water solubility because increased hydrophobicity could reduce interactions between hydroxyl groups of maltodextrin and water (Rajan, Sudha, & Abraham, 2008; Udomrati & Gohtani, 2014). Therefore, one objective of this study was to impart MFAs relatively low DS for enhanced water solubility and to differentiate the hydrophobicity of MFAs using different types of fatty acids, which could diversify the properties of MFAs. By the suggested methods, the enhanced water solubility of MFAs is expected while maintaining acceptable

activities as food additives.

Then, the next aim of the current research was to analyze different MFAs synthesized regarding activities as emulsifiers or antimicrobials and cytotoxicity and to evaluate MFAs' applicability in the food industry compared to commercially approved SL.

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CHAPTER II

LITERATURE REVIEW

Food additives

The primary use of food additives is traditionally for the preservation of foods, but increasingly, the food industry utilizes them for various reasons such as flavors, texturizing, and other benefits.

Food additives are defined as "any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food." (U.S. Food and Drug Administration [U.S. FDA], 2020). Here direct food additives are added to the foods targeted for specific purposes, and indirect ones become part of the food during packaging, storage, or another handling.

According to the U.S. FDA, food additives are categorized into 6 groups, including preservatives, nutritional additives, coloring agents, flavoring agents, texturizing agents, and miscellaneous agents, with several subgroups of each group. Some additives may act with more than one function in foods. (Carocho, Barreiro, Morales, & Ferreira, 2014).

The transportation distance necessary for food products to reach consumers increases, emphasizing food additives to ensure the desired food qualities remain until consumed (Carocho et al., 2014). Also, the increase in processed food consumption worldwide results in more usage and development of food additives, and this current trend is expected to continue in the coming years (Laudisi, Stolfi, & Monteleone, 2019).

The safety of all food additives, whether of natural origin or synthetically produced, is rigorously tested and periodically re-assessed (Saltmarsh & Insall, 2013). Notably, U.S. FDA approves the usage level at a far lower concentration than that which begins to affect human health (Shukla, Akshay, & Ashok, 2017).

However, food additives' health effects are still controversial because several health problems have been reported. The safety concerns arise mainly from the lack of proper scientific information on the impact of sustained exposure to these compounds in humans (Shukla et al., 2017). Also, the antimicrobial activities of most natural products are considered not to satisfy practical needs (AlFindee et al., 2018). Complex food matrix including different organic molecules and environment components (e.g., temperature and pH), can be challenging factors affecting the proper efficacy of antimicrobials (Bastarrachea, Denis-Rohr, & Goddard, 2015). This dissatisfaction is why continuous research is needed for developing effective food additives with minimal human health risks.

Carbohydrate fatty acid esters

Carbohydrate fatty acid ester (CFA) is defined as a carbohydrate attached to fatty acids by esterification (Fig 2.1), and the carbohydrate part includes mono-, di-, or polysaccharides (Chang & Shaw, 2009). In the food industry, they are used as emulsifiers or stabilizers. Also, interest in antimicrobial properties as fatty acid derivatives against multiple food pathogens increases because of their diverse inhibitory action (Nobmann, Bourke, Dunne, & Henehan, 2010). According to which form of fatty acids is used, such as methyl or vinyl esters, the reaction can be transesterification. Their physicochemical properties, including solubility and melting temperatures, can be varied by type of fatty

acid or carbohydrate groups and the possible linkages between them (Neta, Teixeira, & Rodrigues, 2015).

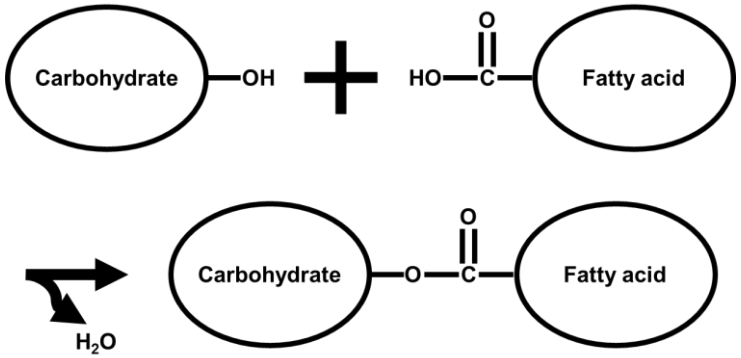

Fig. 2.1. The schematic synthesis of carbohydrate fatty acid esters.

Maltodextrin

Maltodextrin is a water-soluble white powder and a common food ingredient. It consists of mainly D-glucose polymers linked with a high number of α-(1,4) linkages (Fig. 2.2) (Dokic-Baucal, Dokic, & Jakovljevic, 2004). Maltodextrin is produced from the hydrolysis of starch.

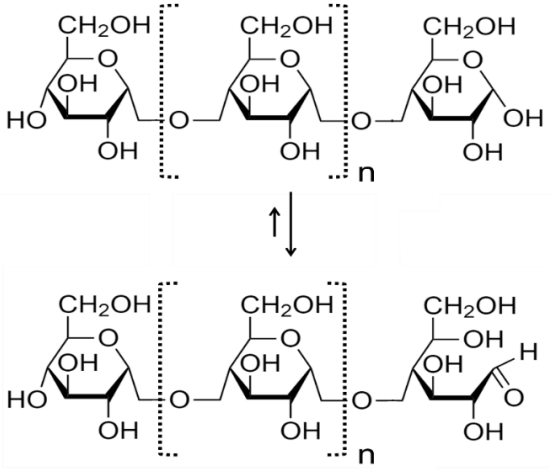

Fig. 2.2. The simplified structure of maltodextrin.

Starch, a base material for maltodextrin, is a natural polysaccharide in a granule form. This polysaccharide includes two types of molecules: amylose (linear and helical) and amylopectin (branched) (Fig. 2.3). The amylose has long chains with several hundred or thousand glucosyl units, and the amylopectin has comparatively short chains (Bertoft, 2017). The linkages between glucose in starch are susceptible to acid and enzyme hydrolysis. The enzyme method is relatively more specific for certain linkages than the acid hydrolysis, considered a more arbitrary process (Wang, & Wang, 2000).

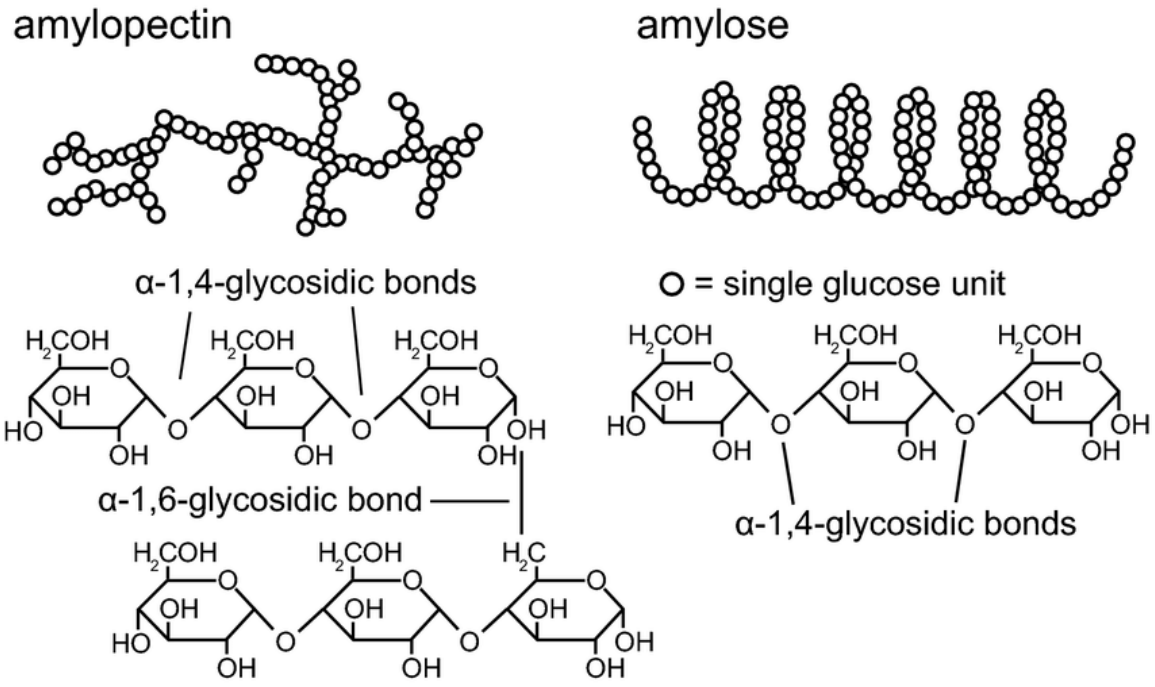

Fig. 2.3. The structure of starch constituting amylopectin and amylose (Willfahrt, Steiner, Hötzel, & Crispin, 2019).

Based on different hydrolysis methods and their degree, maltodextrins can have different ranges of molecular sizes. They can be classified by dextrose equivalent (DE) (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004), which is defined as percent reducing sugars (0 – 100%) calculated as glucose (D'hulst & Verbeke, 1992).

This value is used to characterize molecular weight (the number of DE is inversely proportional to the average molecular weight of maltodextrin) and is typically related to physical properties. Maltodextrins with different DE have shown different properties such as viscosity (Dokic, Jakovljevic, & Dokic-Baucal, 1998). However, maltodextrins with the same DE could exhibit different properties according to the hydrolysis procedure and source of native starch (Dokic-Baucal et al., 2004).

One end of the glucose polymers in maltodextrin can be converted from the cyclic form to an open-chain form with a free aldehyde group (Fig. 2.2), which acts as a reducing agent. This reducing group can react with amino groups (called Maillard reaction), affecting food quality and flavors (Van Boekel, 2006). However, sucrose, consisting of glucose and fructose, does not undergo this reaction due to its structural inability to convert to an open chain with an aldehyde group (Fig 2.4). In addition, longer glucose molecules of maltodextrin in a solution tend to reassociate over storage, called retrogradation (Dokic-Baucal et al., 2004). These structural differences between maltodextrin and sucrose, indicating different physicochemical properties, can dictate their different applications in the food industry.

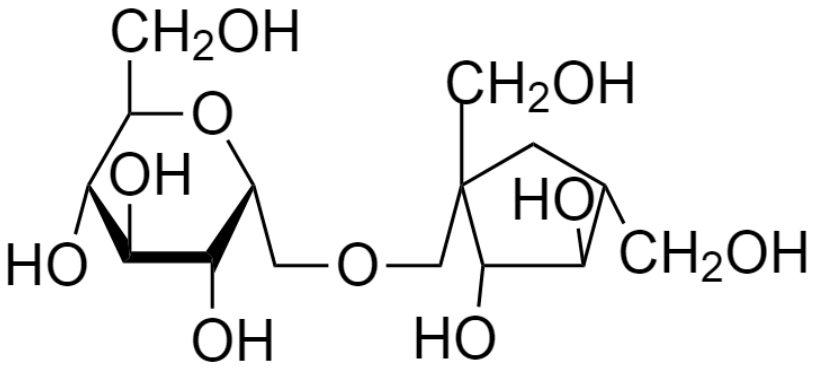

Fig. 2.4. The structure of sucrose.

Fatty acid

A fatty acid is a carboxylic acid with an aliphatic chain (Fig. 2.5), and the chain can be differentiated by the length, the existence of double bonds (saturated or unsaturated), the configuration of double bonds (*cis* or *trans*), and the presence of branches. The important dietary sources of fatty acids are vegetable oils, dairy products, meat products, grains, and fatty fish or fish oils, and fatty acids represent 30–35% of total energy intake in many industrial countries (Rustan & Drevon, 2001)

Fatty acids are poorly soluble in water because of their hydrophobic methyl chain, and generally, the increased chain length can decrease the aqueous solubility. Also, fatty acids with longer chains show a higher melting point, but double bonds or branches can decrease the melting point.

These compounds are strongly perceived as energy or nutritional source and are also known to be related to food rancidity. However, the additional functionalities of free fatty acids and their derivatives, including antimicrobials (Yoon, Jackman, Valle-González, & Cho, 2018) and emulsifiers (Robins & Wilde, 2003), receives increasing attention. Notably, the reaction of fatty acids with carbohydrates can produce various distinct compounds maintaining some of the fatty acid properties, making additional potential as food additives.

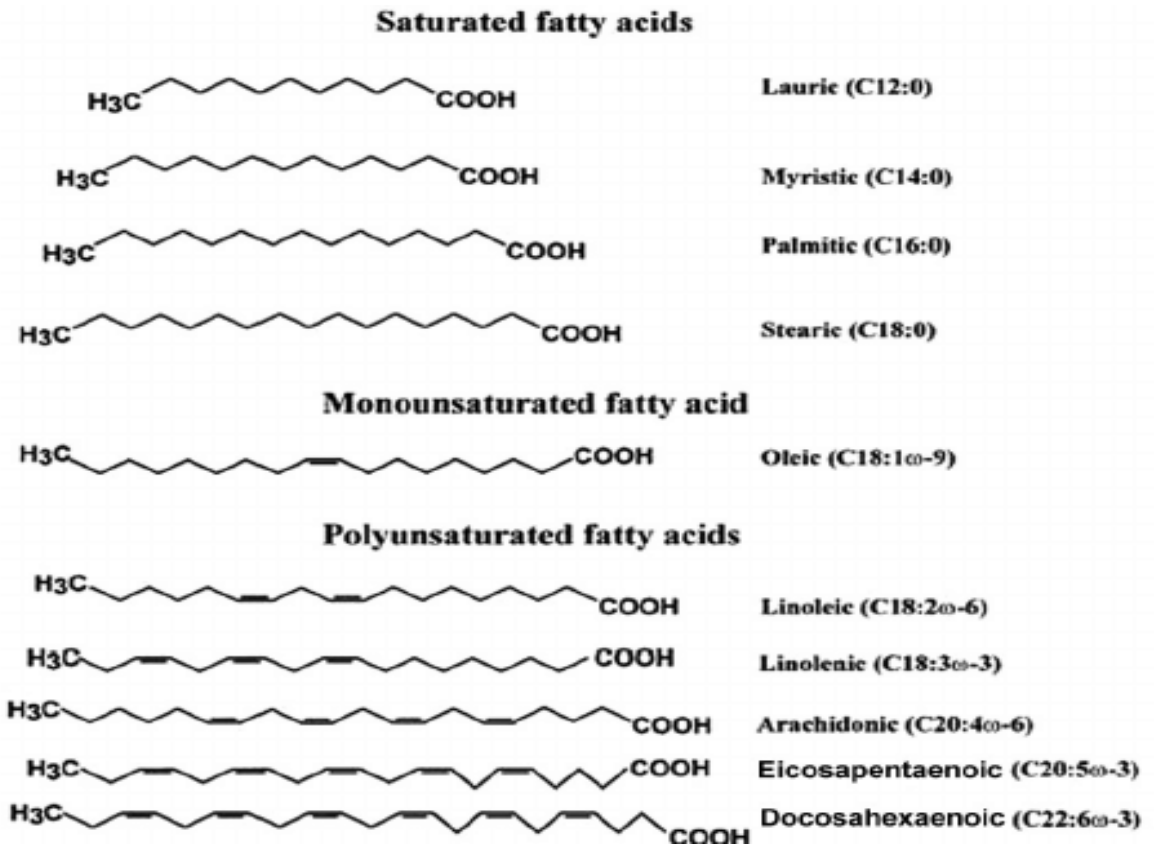

Fig. 2.5. The chemical structure of different fatty acids (Grenon, Hughes-Fulford, Rapp, & Conte, 2012).

Synthesis of CFAs

Traditionally synthesis of CFAs is carried out at elevated temperatures with strong acid or alkaline catalysts, which is reported to be relatively simple and easy to be controlled. However, final products are generated with many undesired products due to the non-selectivity of chemical reactions toward different hydroxyl groups. That leads to the need for subsequent purification steps (Karmee, 2008). In addition to toxic chemicals used, the relatively higher temperature (e.g., over 100ºC) can be associated with a high energy cost during the entire process (Neta et al., 2015). These issues have given rise to an alternative synthesis method.

Enzymatic synthesis uses biocatalysts such as lipases, and this method has gained growing interest because of selectivity of enzymes and mild reaction conditions such as relatively less toxic and lower temperatures (e.g., around 50ºC) (Chang & Shaw, 2009). Lipases catalyze the hydrolysis of lipids in aqueous media, but this equilibrium reaction is shifted toward synthesis or esterification in non-aqueous solvents with the presence of a small amount of water (Neta et al., 2015).

In addition to the previously mentioned selectivity of lipases, these enzymes also remain active in organic solvents, and quite stable under high temperature when immobilized or obtained from thermophilic bacteria (Hasan, Shah, & Hameed, 2006), which makes the enzymatic process more preferable to chemical synthesis when applied at an industrial level.

Synthesis and characterization of maltodextrin fatty acid esters (MFAs)

While lipase-catalyzed synthesis of CFA has gained popularity, most researchers and reviews have been focused mainly on simple sugar products, including esters of glucose, fructose, and sucrose (Adachi & Kobayashi, 2005; Chang & Shaw, 2009; Neta, Teixeira, & Rodrigues, 2015). However, there has been little research on the enzymatic synthesis of polymerized saccharides such as maltodextrins (Udomrati & Gohtani, 2014) which are glucose polymers.

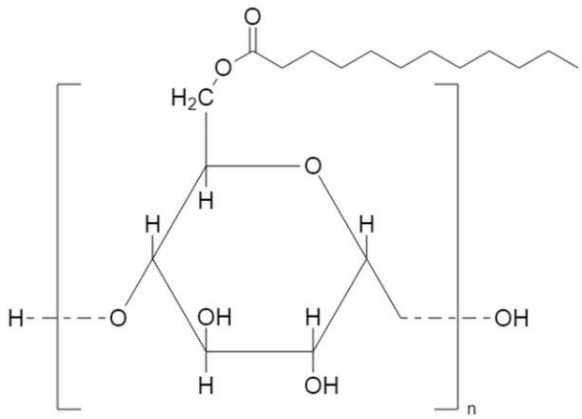

Fig. 2.6. The simplified structure of maltodextrin esterified to laurate.

The structure of MFAs is indicated in Fig. 2.6, and the compounds are maltodextrin attached to fatty acids by an ester bond. Several studies about the synthesis and characterization of MFAs were reviewed, including lipase-catalyzed synthesis of MFAs under relatively lower temperatures (Sun, Yang, Wang, Liu, & Xu, 2013; Udomrati et al., 2014; Udomrati & Gohtani, 2015; Udomrati et al., 2016; Pantoa, Shompoosang, Ploypetchara, Gohtani, & Udomrati, 2019; Udomrati, Cheetangdee, Gohtani, Surojanametakul, & Klongdee, 2020a; Udomrati et al., 2020b) with the ionic liquid-catalyzed method (Biswas, Shogren, & Willett, 2009; Shogren, Biswas, & Willett, 2010) and chemical reactions under alkaline conditions by using octenyl succinic anhydride (OSA) as a source of fatty acids (Bai & Shi, 2011).

There have been several studies to synthesize MFAs without the utilization of lipases. Biswas et al. (2009) prepared maltodextrin stearate with 2 different fatty acid types (vinyl stearate and stearic acid) by an ionic liquid-catalyzed reaction, and the temperature was optimized at 75°C with confirmation of the degree of substitution (DS) by ^{1}H NMR. The products from different fatty acid sources exhibited similar DS but different solubility, and the reason was suggested as a different substitution pattern or alteration of maltodextrin

backbone by acetaldehyde (Biswas et al., 2009).

Shogren et al. (2010) followed the previous research to prepare maltodextrin stearate with different DS values (0.069, 0.62, and 2.4), and characterization of synthesized products was studied by methods such as ^{1}H NMR, X-ray diffraction, differential scanning calorimeter (DSC), and others. However, ionic liquids are very expensive, and thus it is preferable to minimize their amounts in reaction processes for economic reasons (Karimi & Enders, 2006). While an alternative method was suggested for recycling ionic liquids by dialysis (Biswas et al., 2009), this additional step was long (4 days), which is still time-consuming.

In terms of chemical synthesis, Bai and Shi (2011) synthesized OSA-modified maltodextrin esters by using the ability of OSA to react with hydroxyl groups under alkaline conditions and reported higher reaction efficiency when maltodextrin was used instead of starches. However, this research used harsh chemicals like NaOH and anhydride for an esterification reaction (Xu et al., 2012),

Several researchers have studied the enzymatic synthesis of MFAs using lipases. Sun et al. (2013) synthesized maltodextrin stearate by using immobilized lipase (from *Candida antarctica*) and optimized molar ratios of maltodextrin to fatty acid (1:4), enzyme concentration (0.7% of solvent, w/v), reaction time (60 h), and temperature (65°C) to maximize DS. The synthesized products were analyzed by Fourier-transform infrared spectroscopy (FTIR), scanning electron microscope (SEM), and DSC (Sun et al., 2013).

Udomrati et al. (2014) conducted an enzymatic synthesis of MFAs in DMSO system by using lipase (from *Thermomyces lanuginosus*), tapioca maltodextrin (DE: 16),

and 3 different fatty acids (C10, C12, and C16). Optimum conditions for molar ratios of maltodextrin to fatty acid (1:0.5), temperature (60°C), time (4 h) to produce the highest DS were investigated with the additional analysis on physical properties such as solubility (Udomrati & Gohtani, 2014). In the following studies by this group, similar lipase-synthesized MFAs were analyzed for their different properties (Udomrati et al., 2015; Udomrati et al., 2016; Pantoa et al., 2019; Udomrati et al., 2020a; Udomrati et al., 2020b).

Analysis of MFAs in an emulsion system

An emulsion is defined as a dispersion of liquid droplets in another immiscible one (Fig. 2.7) and can be formed by shear and pressure (Garg, Martini, Britt, & Walsh, 2010). In general, an emulsion system is classified as an oil-in-water emulsion, such as milk, cream, dressings, mayonnaise, beverages, soups, and sauces, and as a water-in-oil, including margarine and butter (McClements, 2015).

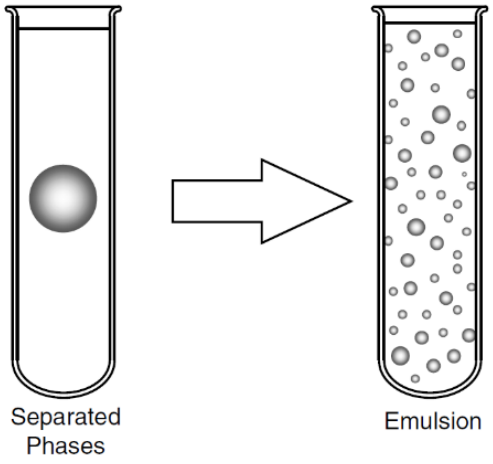

Fig. 2.7. The schematic structure of emulsion (McClements, 2015).

Emulsion stability is an important factor for food quality. The contact between oil and water molecules is thermodynamically unfavorable, making emulsions

thermodynamically unstable, but it is possible to form emulsions that are metastable for a certain period (McClements, 2015). The duration of stability required depends on product types. A stable emulsion is one with no discernible change in the size distribution of the droplets, or state of aggregation, or their spatial arrangement within the sample vessel, over storage (Dickinson., 2003). The most common physical mechanisms responsible for the instability of food emulsions are shown in Fig. 2.8 (McClements, 2015), including particle migration (creaming and sedimentation) and particle size variation or aggregation (coalescence and flocculation) (Mengual et al., 1999).

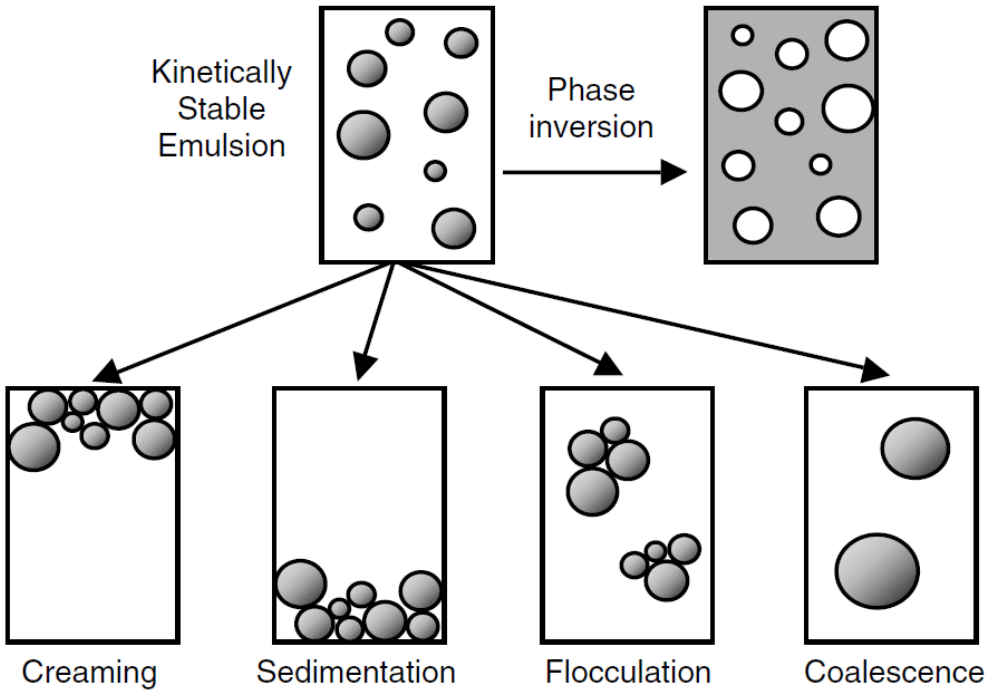

Fig. 2.8. The physical mechanisms to destabilize food emulsions (McClements, 2015).

Stable emulsions can be formulated with the help of two common ingredients, including emulsifying agents and stabilizers. The emulsifying agent (or emulsifier) promotes emulsion formation and short-term stability by lowering interfacial tension, and

the stabilizer confers long-term stability by an absorption mechanism or viscosity modification (Dickinson., 2003). Compounds may act as one of the ingredients or both.

Emulsifying agents or stabilizers from natural sources such as lecithin from egg yolk and various proteins, including casein, are used in the food industry, and more recently, synthetic compounds such sorbitan esters, their ethoxylates, and sucrose fatty acid esters (SFAs) have been used in food emulsions (Kralova & Sjöblom, 2009). As a type of CFAs, MFAs are surface-active molecules due to their amphiphilic property, making them different from maltodextrins which are not surface-active in an emulsion system because of strong hydrophilic properties (Udomrati et al., 2016).

Several techniques have been used to study MFAs in an emulsion system either with or without a commercial emulsifier such as tween 80, including optical or fluorescence microscopic assays, droplet size distribution analysis, interfacial tension measurement, and visual quality inspection (Udomrati et al., 2014; Udomrati et al., 2015; Udomrati et al., 2016; Pantoa et al., 2019; Udomrati et al., 2020a; Udomrati et al., 2020b). These studies have shown that MFAs are surface-active after esterification, increasing emulsion emulsifying and stabilizing properties.

Analysis of MFAs as a microbial inhibitory agent

Food spoilage makes foods unacceptable for human consumption due to altered sensory characteristics such as texture, smell, taste, or appearance, even though they may still be safe to eat because no pathogens or toxins exist (Rawat, 2015). Microbial spoilage may show several signs such as visible growth (slime and colonies), textural changes (degradation of polymers), or off-odors and off-flavors (Gram et al., 2002).

Besides food spoilage microorganisms, *Listeria monocytogenes* is of interest because it is a significant cause of foodborne disease, listeriosis, with approximately 20 to 30% of mortality (Milillo et al., 2012). Furthermore, this food pathogen is relatively resistant to various conditions in food and its environments, including survival at refrigeration temperatures. This resistance makes it colonize food plant environments, overcome hurdles during processing and storage, and proliferate in food products (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017).

The antimicrobial effects of CFAs have a long history, and most studies have focused on CFAs made of simple sugars, mainly sucrose. However, there has been only one study to report the antimicrobial ability of MFAs (up to 20% of concentrations, w/w) against 2 microbes (Pantoa et al., 2019). That study could suggest the potential of MFAs as an antimicrobial agent, but the number of microorganisms tested was too small and the concentrations treated were high. Also, further studies in the food system are needed to evaluate MFAs' applicability for food products.

The exact mode of antimicrobial activity of CFAs is unknown. However, there has been some evidence that bacterial cell membrane is the principal site of action for fatty acids and their esters (Kabara & Marshall, 2005). The attack of cell membranes by CFAs may cause altered membrane permeability, inhibited membrane-associated enzymes, and disrupted energy production (Fig. 2.9) (Yoon, Jackman, Valle-González, & Cho, 2018). The mechanism studies for CFAs were conducted with sucrose derivatives, proposing cell membrane damage or increased leakages (Nobmann et al., 2010; Zhao et al., 2015).

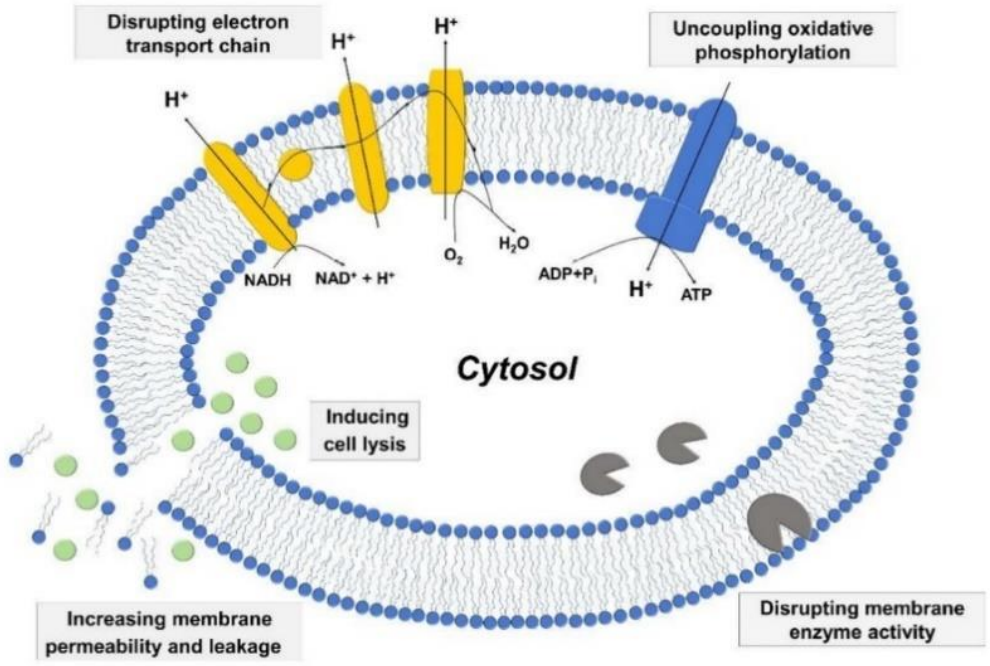

Fig. 2.9. The mechanisms of cell inhibition by fatty acids and their derivatives (Yoon et al., 2018).

Analysis of toxicity of MFAs

CFAs are made from food-based sources and are considered non-toxic. Among them, several toxicological studies have been reported using SFAs in vitro (AlFindee et al., 2018) and in vivo (EFSA Panel on Food Additives and Nutrient Sources added to Food [ANS], 2010). They were reported not to cause short- or long-term toxicity and to be highly hydrolyzed in the gastrointestinal tract into food constituents, resulting in small absorption of intact form and secretion of the incompletely hydrolyzed form (EFSA Panel on Food Additives and Nutrient Sources added to Food [ANS], 2010).

Among different toxicological assays, in vitro cell-based technique (Fig. 2.10) is relatively inexpensive and straightforward (Parboosing, Mzobe, Chonco, & Moodley, 2017) but still provides valuable information on the specific tissue and fundamental data for in

vivo studies.

MFAs as a type of CFAs are also believed to be non-toxic, but toxicological information is still necessary to support their proper application to the food industry. However, to the best of our knowledge, there has been no research studying the toxicity of MFAs either in vitro or in vivo.

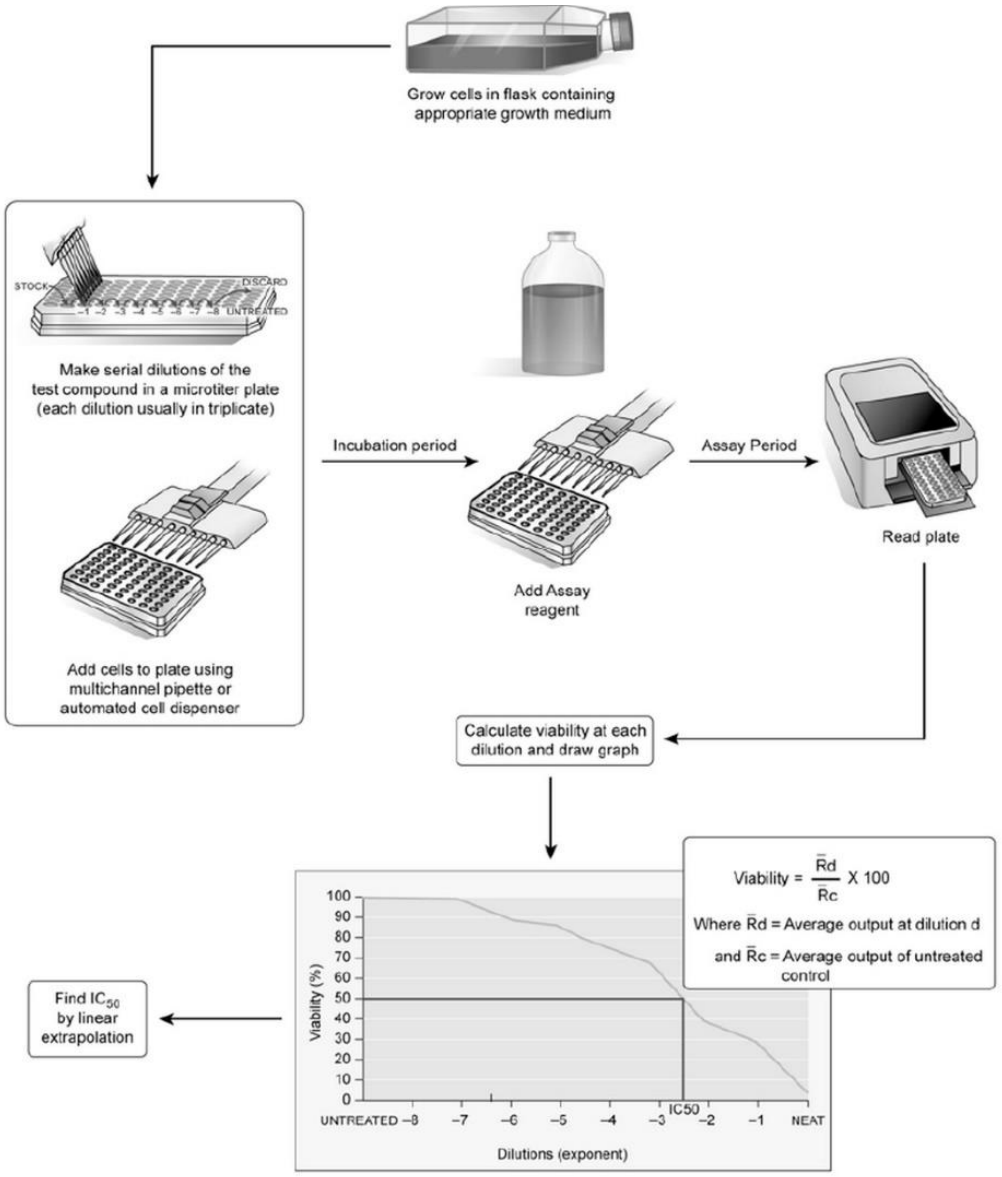

Fig. 2.10. The cell-based cytotoxicity procedures (Parboosing et al., 2017).

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HYPOTHESIS AND OBJECTIVES

Hypothesis

1. Fatty acid types esterified to maltodextrins will affect the properties of compounds synthesized, including water solubility and degree of substitution.

2. The stability of emulsions containing maltodextrin fatty acid esters (MFAs) will be affected by the fatty acid chain length, dextrose equivalent, degree of substitution, concentration, and/or temperature.

3. The antimicrobial ability of MFAs will vary based on the dextrose equivalent, the fatty acid esterified, and/or maltodextrin composition.

4. Food models and storage temperatures will influence the level of microbial inhibition by MFAs.

5. MFAs will show cytotoxicity comparable to commercial sucrose fatty acid ester due to their structural similarity.

Objectives

1. Development of methods to synthesize and purify 6 different MFAs.

2. Investigation of physical properties, including the yield, water solubility, and degree of substitution of synthesized MFAs.

3. Analysis of MFAs in 20:80 oil-in-water emulsions by observing the thickness of the clarification layer and droplet size at two different temperatures (4°C and 22°C).

4. Analysis of the microbial inhibitory ability against food-related microorganisms of MFAs in vitro using growth media and various food models.

5. Analysis of in vitro cytotoxicity of MFAs against human intestinal cells.

CHAPTER Ⅲ

PHYSICAL AND EMULSION STABILIZING PROPERTIES OF MALTODEXTRIN FATTY ACID POLYMERS PRODUCED BY LIPASE-CATALYZED REACTIONS IN ETHANOL[1]

Abstract

Enzymatic synthesis of maltodextrin (MD) fatty acid esters (MFAs) has gained growing interest. Here, MFAs were synthesized in a food-grade ethanol system, and the properties of the products were analyzed. Six different MFAs were produced with 2 different MD sources and 3 combinations of fatty acids (lauric, palmitic, and both) with yields ranging from 73 - 83%. With an increase in fatty acid carbon length, the degree of substitution (0.026 to 0.016) and solubility (101% to 93%) were significantly decreased. All emulsions formulated with MFAs were stable except those containing the lowest concentration (0.125%, w/w) of MFAs esterified with palmitate. Notably, MD esterified with laurate showed an enhanced emulsion stabilizing ability as compared to commercial emulsifiers. In conclusion, the emulsion stabilizing ability of MFAs may have application in the food industry.

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[1] Carbohydrate Polymers. Physical and emulsion stabilizing properties of maltodextrin fatty acid polymers produced by lipase-catalyzed reactions in ethanol. Volume 226, December 2019, Page 115309. Namhyeon Park, Marie K. Walsh. (Original copyright notice as given in the publication in which the material was originally published) “With permission of Elsevier”

Introduction

Maltodextrins are polysaccharide products made by partial hydrolysis of starch, consisting of D-glucose units linked with alpha 1,4 glycosidic bonds and alpha 1,6 glycosidic bonds (Udomrati, Ikeda, & Gohtani, 2011). Maltodextrins are non-toxic and relatively inexpensive, making these products widely used as thickening agents in foods and as a binding agent in pharmaceuticals (Biswas, Shogren, & Willett, 2009; Udomrati & Gohtani, 2014). These compounds have different molecular sizes and are generally classified by dextrose equivalent (DE) (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004), which is defined as percent reducing sugars calculated as glucose (D'hulst & Verbeke, 1992).

Because DE can characterize the molecular weight of maltodextrins, this is typically related to physical properties. Maltodextrins with different DE showed different properties such as viscosity (Dokic, Jakovljevic, & Dokic-Baucal, 1998), but maltodextrins with the same DE could exhibit dissimilar properties according to hydrolysis procedure and source of native starch (Dokic-Baucal, Dokic, & Jakovljevic, 2004).

Maltodextrins are strongly hydrophilic in nature, and they are not surface-active in an emulsion system (Udomrati et al., 2016). However, an emulsion, which is a dispersion of droplets of a liquid in another immiscible one, can be stabilized by maltodextrins through viscosity modification or gelation (Dickinson, 2003).

Maltodextrin fatty acid esters (MFAs) are non-ionic surface-active surfactants due to their amphiphilic property, which makes them different from maltodextrins. These MFAs can be produced by lipases via the esterification of fatty acids in non-aqueous solvents with the presence of a small amount of water (Neta, Teixeira, & Rodrigues, 2015), and this

enzymatic method has gained growing interest because of fewer by-products and mild reaction conditions such as relatively lower reaction temperatures (Chang & Shaw, 2009). So far, there have been a limited number of reports on the enzymatic esterification of maltodextrins (Udomrati & Gohtani, 2014).

Fatty acids enzymatically esterified to maltodextrins may have potential use in foods as emulsion stabilizing molecules. In this research, MFAs were synthesized in a food-grade ethanol system, different from previous solvents used (dimethyl sulfoxide and butanol). Additionally, individual fatty acids and a mixture of 2 different fatty acids were employed for the esterification of maltodextrins, followed by the analysis of the degree of substitution (DS) and solubility of MFAs. Emulsions containing MFAs were produced using two maltodextrin types, three fatty acid types, three concentrations, and two storage temperatures resulting in 36 treatment combinations, then analyzed for emulsion formation and stability using both a vertical-light scattering detector and a particle size analyzer and compared to native maltodextrins and commercial emulsifiers.

Materials and methods

Materials

Vinyl laurate (L) and vinyl palmitate (P) were purchased from TCI America (Portland, OR, USA). Immobilized lipase from *Thermomyces lanuginose* (TM2, Chiral Vision, Netherlands) was used. High-performance liquid chromatography (HPLC)-grade ethanol and acetonitrile were from Thermo Fisher (PA, USA), Whatman glass microfiber filters and molecular sieves (3Å) were from Sigma-Aldrich (St. Louis, MO, USA). Sucrose ester (Ryoto L-1695; sucrose laurate) was purchased from Mitsubishi-Kagaku (Tokyo, Japan).

Tween 20 (Polysorbate 20) was from MP Biomedicals (USA), and commercial corn oil was purchased at a local grocery store. Maltrin M100 with DE 9 – 12 and 30% of degree of polymerization (DP) 1-7, 35% of DP 6-25, 1% of DP 26-40, and 34% of DP over 40 and Maltrin M250 (DE 23 – 27 and 60% of DP 1-7, 35% of DP 6-25, and 5% of DP over 40) were purchased from Grain Processing Corporation (Muscatine, IA), HPLC (Beckman System Gold 125 Solvent Module, Ontario, Canada) was utilized with an evaporative light scattering detector (Agilent Technologies, Santa Clara, CA, USA) and SynChropak RP-4 column (Synchrom, Inc., Lafayette, IN). Nuclear magnetic resonance **(**NMR) (Bruker Avance III 500, Bruker, Billerica, MA, USA), LS Beckman Coulter droplet size analyzer (LS 230, Coulter Corporation, Miami, FL, USA), TurbiScan (MA2000, Toulouse, France), Ultra-turrax T25 (Janke and Kunkel, Staufen, Germany), incubator shaker (Eppendorf, Edison, NY, USA), microfluidizer (Microfluidics Corporation, Newton, MA, USA) and benchtop 5L freeze drier (Virthis Company, Inc., Gardiner, NY, USA) were also used in this research.

Synthesis and purification of maltodextrin fatty acid Esters

The enzymatic reaction was conducted by previously reported methods with several modifications (Udomrati & Gohtani, 2014; Walsh, Bombyk, Wagh, Bingham, & Berreau, 2009). Maltodextrin (2.0 g) (M100 or M250), 4.0 g of molecular sieves, and 160 ml of ethanol (dried with molecular sieves) were mixed in a 250 ml bottle. Then, vinyl fatty acid (280 mg of vinyl laurate [VL], 350 mg of vinyl palmitate [VP] or half VL [140 mg] and half VP [175 mg] respectively which are a 1:0.1 M ratio between anhydrous glucose [162 g] and vinyl fatty acid) was added to the reaction bottle with 2.4 g of TM2. The reactions were then incubated for 8 days in a shaker at 500 rpm and 55°C. An additional 2.4 g of

TM2 with 4.0 g of molecular sieves was added each day over the reaction time of 8 days. The reaction was finished at 8 days when the ratio of MFA in the mixture was over 95% by HPLC analysis. After filtration to remove the molecular sieves and enzyme, the final filtrate was dried in a fume hood with 100 feet/min face velocities at 22°C for 1 day. The dried sample was resuspended in distilled water (DW) and washed with hexane. This washed aqueous solution was pre-frozen at -80°C for 1 day and freeze-dried by a manifold type at 22°C and 50 mTorr for 1 day. The dried samples were weighed to calculate yield by measuring the final sample weight against starting weight of maltodextrin (2.0 g). A total of 6 different products were synthesized (M100L, M100P, M100LP, M250L, M250P, and M250LP), and their specific information is given in Table 3.1.

Table 3.1. The composition of maltodextrin fatty acid esters in this research.

Name (abbreviation)	Substrates	
	Carbohydrate group	Fatty acid group
M100L	Maltodextrin (Maltrin M100)[a]	Vinyl laurate (C12)
M100P	Maltodextrin (Maltrin M100)[a]	Vinyl palmitate (C16)
M100LP	Maltodextrin (Maltrin M100)[a]	Mixture of vinyl laurate and palmitate
M250L	Maltodextrin (Maltrin M250)[b]	Vinyl laurate (C12)
M250P	Maltodextrin (Maltrin M250)[b]	Vinyl palmitate (C16)
M250LP	Maltodextrin (Maltrin M250)[b]	Mixture of vinyl laurate and palmitate

[a]Maltrin M100 (Dextrose equivalent (DE) 9 – 12 and 30% of DP 1-7, 35% of DP 6-25, 1% of DP 26-40, and 34% of DP over 40).
[b]Maltrin M250 (DE 23 – 27 and 60% of DP 1-7, 35% of DP 6-25, and 5% of DP over 40).

The purity of MFA was analyzed by a previously reported method after several modifications (Walsh et al., 2009). A binary mobile system was utilized with 1.0 ml/min of flow rate including (A) 5% acetonitrile: water and (B) 95% acetonitrile: water, and the gradient was from 10% of (A) to 100% of (A) over 30 min. The compounds were detected by an evaporative light scattering detector (ELSD) at 40°C and 3.55 bar of nitrogen gas

Degree of Substitution (DS)

The DS was calculated according to a previously reported method (Kapusniak & Siemion, 2007). Samples (2 - 5 mg) were dissolved in 600 µl of D_2O, and the solution was transferred to NMR tubes. The ^{1}H-NMR spectra were recorded. The peaks between 3.24 and 5.60 ppm corresponded to the signals from the seven protons of the anhydrous glucose unit (AGU) structure (except the peak of residual solvent at 4.80 ppm). The terminal methyl group of the fatty acyl chain was observed as a triplet at 0.88 ppm. The DS was estimated according to the following equation (3.1).

$$\mathrm{DS} = \frac{\text{Area of Methyl/3 (moles of fatty acid)}}{\text{Area of AGU/7 ((moles of AGU))}} \quad (3.1)$$

Solubility

The analysis of solubility of produced MFAs was done according to Udomrati & Gohtani, (2014) with the following modifications. Samples (50 mg) were suspended in 5 ml of distilled water (DW). After stirring for 30 min, samples were centrifuged at 7810×g for 10 min. After filtration of supernatant by using 0.45 µm syringe filter, the filtrate was collected and dried at 80°C for 1 day. The solubility (%) was measured by the ratio of the weight of dried supernatant to the weight of the initial sample (50 mg).

Preparation of Emulsions

Commercial corn oil (20 g) was added to 80 g of DW, and each water phase contained a different type of MFAS at different concentrations (0, 0.125, 0.25, or 0.5% (w/w)). Emulsions without additives were used as blanks, and emulsions containing native maltodextrin (M100 and M250) as negative controls and those with sucrose ester (SE) or tween 20 (T20) as positive controls were used. The water and oil mixture was first mixed by a high-speed blender at 8,000 rpm for 5 min followed by 3 times of process using microfluidizer at 15,000 psi. The cooling coil of the microfluidizer was put into a cooling bath filled with ice. A total of 36 emulsions using MFAs were produced by the combination of factors (2-maltodextrins x 3-fatty acids × 3-concentrations × 2-storage temperatures (4˚C and 22°C)) with additional emulsions of blanks, negative, and positive controls. The fresh and stored emulsions were used for further studies.

Droplet size analysis of emulsions

The analysis of droplet size distribution was conducted using a light scattering particle size analyzer. Small volume module was used, and 2 - 3 droplets of the middle layer of emulsion samples were placed into the suspension fluid for measurement. After an assay at day 0 (fresh), emulsions were stored at 2 different temperatures (4°C or 22°C) and were analyzed again at day 1, 3, 5, and 7. The results of sizes are given per volume (%) to show droplet size distribution.

Stability analysis of emulsions

The stability of emulsions was determined as previously described (Garg, Martini, Britt, & Walsh, 2010) with modifications. Approximately 6 ml of emulsion samples were placed in

cylindrical glass tubes. Transmitted and backscattered light were monitored as a function of time and glass tube height using Turbiscan classic MA 2000. The clarification layer (0 - 35 mm at the bottom of the tube) was monitored on days 0 (fresh), 1, 3, 5, and 7 on samples stored at 4°C or 22°C. The slope of the scatter plot assigning time (day) to a horizontal axis and clarification layer height (mm) to a vertical axis was used to determine the destabilization rate of emulsions (mm/day).

Statistical Analysis

Experiments for yield, DS, and oil droplet size were repeated in duplicate, while those for solubility and destabilization were conducted in triplicate. All data were expressed as mean ± standard deviation. The results of solubility and DS were analyzed by two-way ANOVA (maltodextrin and fatty acid factors) with post hoc analysis (p=0.05) (SAS OnDemand for Academics, SAS Institute, Cary, NC, USA). After the destabilization rates of emulsions containing different MFAs were analyzed by four-way ANOVA (maltodextrin, fatty acid, concentration, and temperature as factors) with post hoc analysis (p=0.05), the rates of emulsions with selected M100L and commercial emulsifiers were studied by three-way ANOVA (product, concentration, and temperature as factors) with post hoc analysis (p=0.05) (SAS OnDemand for Academics, SAS Institute, Cary, NC, USA). Effect size estimates were studied by calculating eta squared, and interaction plots were applied to confirm the effect of interactions in ANOVA.

Results and Discussion

Production of Maltodextrin Fatty Acid Esters (MFAs)

Six different MFAs were produced (M100L, M100P, M100LP, M250L, M250P, and

M250LP) by lipase-catalyzed reactions in ethanol with a yield ranging from 73% to 83% (Table 3.1 and 3.2). There have been limited studies investigating the enzymatic synthesis of MFAs (Sun, Yang, Wang, Liu, & Xu, 2013; Udomrati & Gohtani, 2014, 2015; Udomrati et al., 2016). One research group (Udomrati & Gohtani, 2014) utilized tapioca maltodextrins (DE 9 and DE 16), free fatty acids (decanoic acid, lauric acid, and palmitic acid), and immobilized lipase from *T. lanuginosus* in dimethyl sulfoxide with an optimum 1:0.5 molar ratio (D-glucose/fatty acid) while the other research was conducted with maltodextrin (unknown DE), stearic acid (optimum 1:4 molar ratio), with an immobilized lipase from *Candida antarctica* in t-butyl alcohol (Sun et al., 2013). Compared to these previous studies, our research was first to exploit ethanol for the reaction system, which is inexpensive (Sheldon, 2005) and food-grade, and the use of a mixture of fatty acids. These different substrates were expected to affect the properties of produced MFAs (Ferrer et al., 2005) for DS, solubility, and emulsion-related properties.

DS

The analyzed DS of MFAs by the ^{1}H-NMR method is given in Table 3.2. While there were no statistically significant effects of interaction or type of maltodextrin ($p>0.05$), the DS of MFAs was significantly associated with the chain length of fatty acids ($p<0.05$). The shorter length of esterified fatty acid in MFAs produced higher DS values, and each DS value between MFAs containing different fatty acids was significantly different ($p<0.05$). Especially, MFAs with mixed fatty acid resulted in intermediate values between those associated with each fatty acid.

A similar result of lower esterification of longer chain fatty acids was reported previously, and it was proposed that reduced mobility of longer fatty acids in the reaction

system resulted in the slower rate of esterification (Udomrati & Gohtani, 2014). The DS has been postulated to be modulated by materials such as the biocatalysts, solvents, fatty acids, and sugars (Ferrer et al., 2005), and DS is important because of its effect on the properties of final compounds synthesized (Fang, Fowler, Tomkinson, & Hill, 2002; Ferrer et al., 2005).

Table 3.2. Solubility, degree of substitution (DS), and yield of maltodextrin fatty acid esters.

Name*	Yield (%)	DS (moles of fatty acids /moles of AGU**)	Solubility in water (%)
M100L	82 ± 12	0.026 ± 0.001[a]	101 ± 2[a]
M100LP	73 ± 11	0.021 ± 0.002[b]	94 ± 2[b]
M100P	78 ± 21	0.018 ± 0.000[c]	93 ± 2[b]
M250L	83 ± 15	0.026 ± 0.001[a]	101 ± 1[a]
M250LP	79 ± 10	0.022 ± 0.002[b]	94 ± 2[b]
M250P	81 ± 13	0.016 ± 0.000[c]	93 ± 1[b]

[a,b,c] means with the same letter were not significantly different within each column ($p<0.05$).
*M100 is for maltodextrin with dextrose equivalent (DE): 9 - 12, M250 is for maltodextrin with DE: 23 - 27, L is laurate, P is palmitate, and LP is for half L and half P.
**AGU is an anhydrous glucose unit.

Solubility

MFAs were mixed into water, and the solubilized portions were measured and compared to the total weight for relative solubilities (%) in water (Table 3.2). This result showed that the solubilities of MFAs were not significantly associated with the DE of the maltodextrin

or interaction effects ($p>0.05$) but with fatty acid chain length only ($p<0.05$). Particularly, MFAs with laurate were more soluble than those containing palmitate, suggesting the latter's larger hydrophobicity could hinder the solubilization of MFAs and that solubility depends on the hydrophobic fatty acid moiety. Previous research stated a similar postulation in that increasing hydrophobicity resulted in reduced hydrogen bonds between hydroxyl groups of maltodextrin and water (Rajan, Sudha, & Abraham, 2008; Udomrati & Gohtani, 2014). Additionally, the solubilities of MFAs using tapioca maltodextrins and fatty acids (C10, 12, and 16) in this previous research were from 84.8% to 87.2% (Udomrati & Gohtani, 2014), which were lower than those of our products. Our higher solubilities may be the result of lower DS of our MFAs (0.016 to 0.026) than that of previous products cited (0.015 to 0.084), and this tendency was shown in a previous study (Qiao, Gu, & Cheng, 2006). Previously, different maltodextrin sources, for example, corn and tapioca, were assumed to affect solubility because of the varied properties exhibited by different botanical types (Dokic-Baucal et al., 2004). In addition, according to the descriptive term for solubility in JSFA (Joint Expert Committee for Food Additives), M100L and M250L were only considered "sparingly soluble" in water which means from 30 to less than 100 parts of solvent was required for 1 part of solute (Nagai et al., 2017). The aqueous solubility of emulsifiers has important implications because it could be related to the stability of oil in water (O/W) emulsions (Bendjaballah, Canselier, & Oumeddour, 2010).

Table 3.3. Destabilization rate (clarification layer (mm)/day) of emulsions containing different maltodextrin fatty acid esters, blank, and negative controls (M100 and M250) over 7 days.

Maltodextrin	Fatty acid	Concentration (%)	Destabilization rate (mm/day)	
			4°C	22°C
N/A	N/A	N/A	3.90 ± 0.08^{p}	5.92 ± 0.02^{q}
M100	N/A -	0.5	6.65 ± 0.15^{r}	10.09 ± 0.18^{s}
		0.25	10.26 ± 0.07^{s}	11.37 ± 0.18^{u}
		0.125	28.60 ± 0.45^{vw}	29.86 ± 0.93^{wx}
	L	0.5	0.21 ± 0.01^{a}	0.28 ± 0.01^{ab}
		0.25	0.28 ± 0.01^{ab}	0.38 ± 0.01^{cdef}
		0.125	0.43 ± 0.00^{f}	0.61 ± 0.00^{i}
	LP	0.5	0.24 ± 0.04^{ab}	0.32 ± 0.01^{bcd}
		0.25	0.30 ± 0.02^{bc}	0.39 ± 0.01^{cdef}
		0.125	0.53 ± 0.02^{gh}	0.84 ± 0.02^{k}
	P	0.5	0.32 ± 0.01^{bcd}	0.42 ± 0.02^{f}
		0.25	0.57 ± 0.02^{hi}	0.89 ± 0.01^{k}
		0.125	1.20 ± 0.02^{l}	1.91 ± 0.01^{n}
M250	N/A	0.5	10.25 ± 0.08^{s}	11.65 ± 0.23^{u}
		0.25	10.93 ± 0.17^{t}	27.56 ± 0.28^{v}
		0.125	29.79 ± 0.22^{wx}	30.27 ± 0.23^{x}
	L	0.5	0.24 ± 0.02^{ab}	0.29 ± 0.01^{ab}
		0.25	0.29 ± 0.02^{ab}	0.45 ± 0.01^{fg}
		0.125	0.45 ± 0.01^{fg}	0.70 ± 0.01^{j}
	LP	0.5	0.26 ± 0.02^{ab}	0.33 ± 0.00^{bcde}
		0.25	0.39 ± 0.01^{def}	0.64 ± 0.02^{ij}
		0.125	0.90 ± 0.03^{k}	1.48 ± 0.05^{m}
	P	0.5	0.40 ± 0.00^{ef}	0.58 ± 0.01^{hi}
		0.25	0.55 ± 0.03^{hi}	0.90 ± 0.01^{k}
		0.125	1.44 ± 0.02^{m}	2.54 ± 0.07^{o}

Different superscript letters indicate significant differences among all columns and rows ($p<0.05$).
N/A means no sample.

Stability of Emulsions Containing MFAs

Destabilization rates of emulsions containing MFAs were determined by the increase in the clarification layer over time (7 days) (Table 3.3), and the effects of maltodextrins, fatty acids, concentrations, and temperatures were studied. The addition of native maltodextrins (M100 and M250) into emulsions significantly increased the destabilization rates at all concentrations from the blank ($p<0.05$). Similarly, decreased stability of emulsions containing native maltodextrins was reported indicating depletion flocculation due to non-absorbed maltodextrins (Dokic-Baucal et al., 2004). Also, a significant increase in destabilization rates was detected when higher DE maltodextrins were used at each temperature and concentration excepting 0.125% ($p<0.05$). This higher DE is related to the smaller molecular size of maltodextrin followed by the decreased viscosity, which is assumed to be a major reason for the greater instability, and this presumption was supported by previous research (Dokic-Baucal et al., 2004).

Even though polysaccharides, like maltodextrin, could be used as emulsion stabilizers by viscosity modification or gelation in the continuous phase (Dickinson, 2003), a sufficient concentration might be needed to achieve these effects (Klinkesorn et al., 2004). Previous research showed the enhanced stability of emulsions containing maltodextrins only in the case of high concentrations such as at 40% (Udomrati et al., 2011) or 25% (Dokic-Baucal et al., 2004), which were more elevated concentrations than reported here.

Even though the 4-way interaction effect (maltodextrin, fatty acid, concentration, and temperature) was statistically significant ($p<0.05$), this seemed less important than the significant main effects ($p<0.05$) because of the very small effect size of interaction factors compared to that of main effects by analyzing eta squared and the apparent general trends

for the main effects. Overall, emulsions containing MFAs with lower DE (DE10), a shorter chain length of fatty acid, higher concentrations, and at 4ºC produced lower destabilization rates compared to the alternatives (Table 3.3).

Regarding the effect of maltodextrin type (M100-DE: 9 - 12 and M250-DE: 23 - 27), the destabilization rates of emulsions with DE10 maltodextrin were significantly lower in 0.5% and 0.25% in negative controls, 0.25% and 0.125% of LP, 0.5% and 0.125% of P at each temperature with 0.125% of L at 22°C ($p<0.05$) while other emulsions indicated decreased rates by DE 10 of maltodextrin with no significance ($p>0.05$). Our results suggest that even after esterification, the effect of viscosity by different DE on the stability of emulsion may be valid, or the association between DE and glucose chains which indicate steric stabilization (Udomrati & Gohtani, 2015).

The destabilization rates at 4ºC were significantly lower than those at 22°C in the blank, 0.5% and 0.25% of M100 and M250, 0.25% and 0.125% of M100L, M250L, and M250LP, 0.125% of M100LP, and all concentrations of M100P and M250P ($p<0.05$) while other emulsions at 4ºC showed numerically decreased values that were not significant ($p>0.05$). Because the phase viscosity can be varied depending on temperatures (Rousseau, 2000), the increased stability at 4ºC (Table 3.3) was assumed to be from the greater viscosity at a lower temperature.

As the concentration of MFAs was increased, the destabilization rates of emulsions were significantly decreased in M100, M250, M100P, M250LP, and M250P at 4ºC and 22°C, and in M100L and M250L at 22°C ($p<0.05$) while others showed numerical decreases that were not significant between 0.5% and 0.25%. ($p>0.05$). This dose-dependence of emulsion stability seemed reasonable simply because emulsifiers could

cover more of the emulsion surface, as mentioned previously (Udomrati & Gohtani, 2014).

The effects of fatty acid type on destabilization rates were also studied (Table 3.3). The destabilization rates of 0.125% of MFAs and 0.25% of M250 fatty acid esters indicated significantly lowered values in the order of chain lengths (L, LP, and P) at each temperature ($p<0.05$) while those of 0.25% of M100 fatty acid esters and 0.5% of M250 fatty acid esters at each temperature with those of 0.5% of M100 fatty acid esters at 22°C indicated reduced destabilization rates with no significance between L and LP ($p>0.05$) which were significantly lower than P ($p<0.05$). The rates of 0.5% of M100 fatty acid esters at 4ºC were significantly different between L and P ($p<0.05$). The fatty acyl chain of MFAs is considered a driving force toward O/W interface to confer steric stabilization (Udomrati & Gohtani, 2015). According to our results, MFAs esterified by even shorter fatty acids with higher DS could show better stability, suggesting that the surface-active properties of MFAs may be varied in more complex patterns, and therefore the combination of DS and lengthy of fatty acid may be more important than each factor.

An emulsion is considered stable when the destabilization rate is less than 1.0 mm of clarification layer/day (Dickinson, 1992). Based on this standard, the pure and native maltodextrin emulsions were not stable at both temperatures, but after esterification, all MFA emulsions were stable excepting the lowest concentration of MFAs made of palmitate at both temperatures and M250LP at 22°C (Table 3.3). Our results showed that the esterification enhanced the emulsion stabilizing properties of maltodextrin and agree with those of a previous study (Udomrati & Gohtani, 2014). In addition, the emulsions with M100L, M100LP, and M250L maintained stability at all concentrations and temperatures, and notably, M100L was the most stable among MFAs.

Comparison of destabilization between emulsions containing M100L and commercial emulsifiers.

From the previous results (Table 3.3), M100L was selected as a representative of MFAs, and its emulsions were compared to those of 2 different commercial emulsifiers (sucrose ester (SE) and tween 20 (T20)) in terms of destabilization rates (clarification layer/day) (Fig. 3.1).

Similar patterns were detected for the emulsions containing SE and T20 in that the destabilization rates were decreased with higher concentration and lower temperature (Fig. 3.1). However, there was a significant two-way interaction between emulsifier types and concentrations ($p<0.05$). The destabilization rates of emulsions with M100L were higher than those of emulsions with commercial emulsifiers at 0.5% (non-significant at 4ºC and significant at 22ºC) ($p=0.05$) while those rates for M100L were significantly lowered at 0.25% and 0.125% excepting at 4ºC of 0.25% ($p=0.05$) (Fig. 3.1).

Using a standard of classification of less than 1.0 mm/day indicating a stable emulsion (Dickinson, 1992), only emulsions containing M100L were stable at all concentrations and temperatures, while those with SE and T20 were stable at 0.5% and 0.25%. To the best of our knowledge, the comparison between MFAs and commercial emulsifiers (SE and T20) is unique to this research.

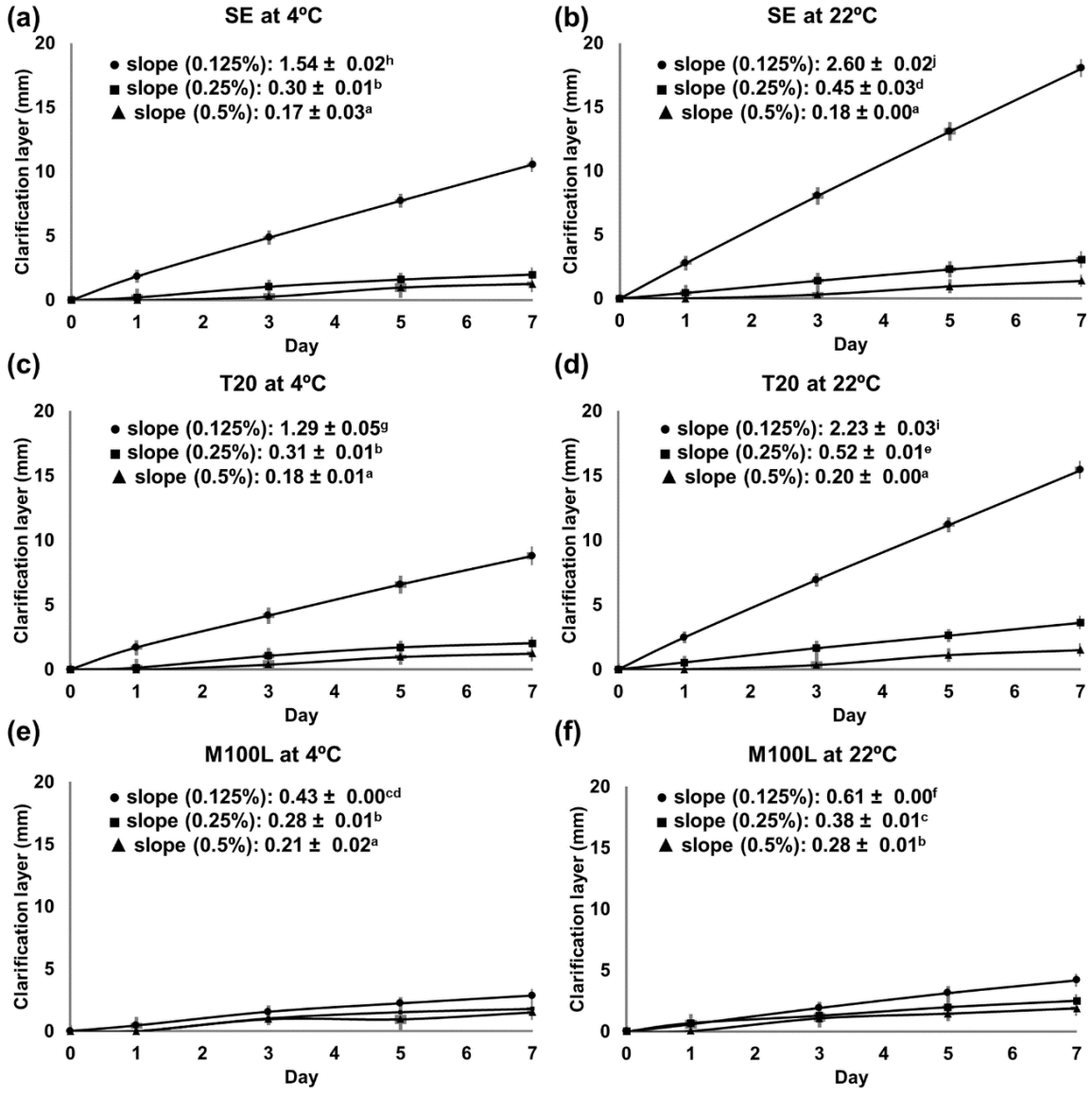

Fig. 3.1 Destabilization of emulsions containing M100L compared to commercial emulsifiers (sucrose ester (SE) and tween 20 (T20)) at 4ºC or 22ºC. Emulsions containing (a) SE at 4ºC, (b) SE at 22ºC, (c) T20 at 4ºC, (d) T20 at 22ºC, (e) M100L at 4ºC, and (f) M100L at 22ºC were studied. Different concentrations were used as follows: (●) 0.125%, (■) 0.25%, and (▲) 0.5%.

The increased stability of emulsions with M100L at lower concentrations would be due to an enhanced steric stabilizing layer from the non-adsorbed hydrophilic segments composed of glucose polymers. These segments may protrude away from the surface and produce a thick protective layer reducing oil droplet aggregation (Dickinson, 2003). The

increased stability of emulsions with the longer molecular size of MFAs demonstrated by this research could support the postulated stabilizing mechanism, which is size-related.

However, SE and T20, which have been reported to promote emulsification and minimal stabilization by interfacial action (Dickinson, 2003), might contribute to the stability of emulsions by the production of fine droplets or only surface coverage instead of the thick protective layers. This different stabilizing mechanism from MFAs might be associated with instability of emulsions with 0.125% of T20 and SE.

Droplet Size Distribution of Emulsions

The droplet size distributions (volume fraction by size) of emulsions containing different concentrations of M100L on day 0 and day 7 at different storage temperatures (4ºC and 22°C) are shown in Fig. 3.2. The size distribution of droplets with 0.5% M100L on day 0 was bimodal (Fig. 3.2a and d), and after 7 days the droplets were shifted to the left (smaller oil droplet size) (Fig. 3.2a and d) at both temperatures and the bimodal distribution was more pronounced. The distribution of droplets with 0.25% M100L on day 0 produced a bimodal shape with a shift to the left after 7 days at both temperatures (Fig. 3.2b and e). The distribution of emulsions with 0.125% M100L on day 0 was bimodal, and a left shift occurred after 7 days (Fig. 3.2c and f).

The bimodal droplet distributions of all fresh emulsions containing M100L were transferred to a similar shape with diameters lower than 1 μm after 7 days, and this pattern happened regardless of concentrations. This left shift was attributed to creaming-out of large droplets (Reddy & Fogler, 1981), suggesting again that the increased stability was from the inhibition of particle aggregation, as described earlier, instead of a complete block of creaming.

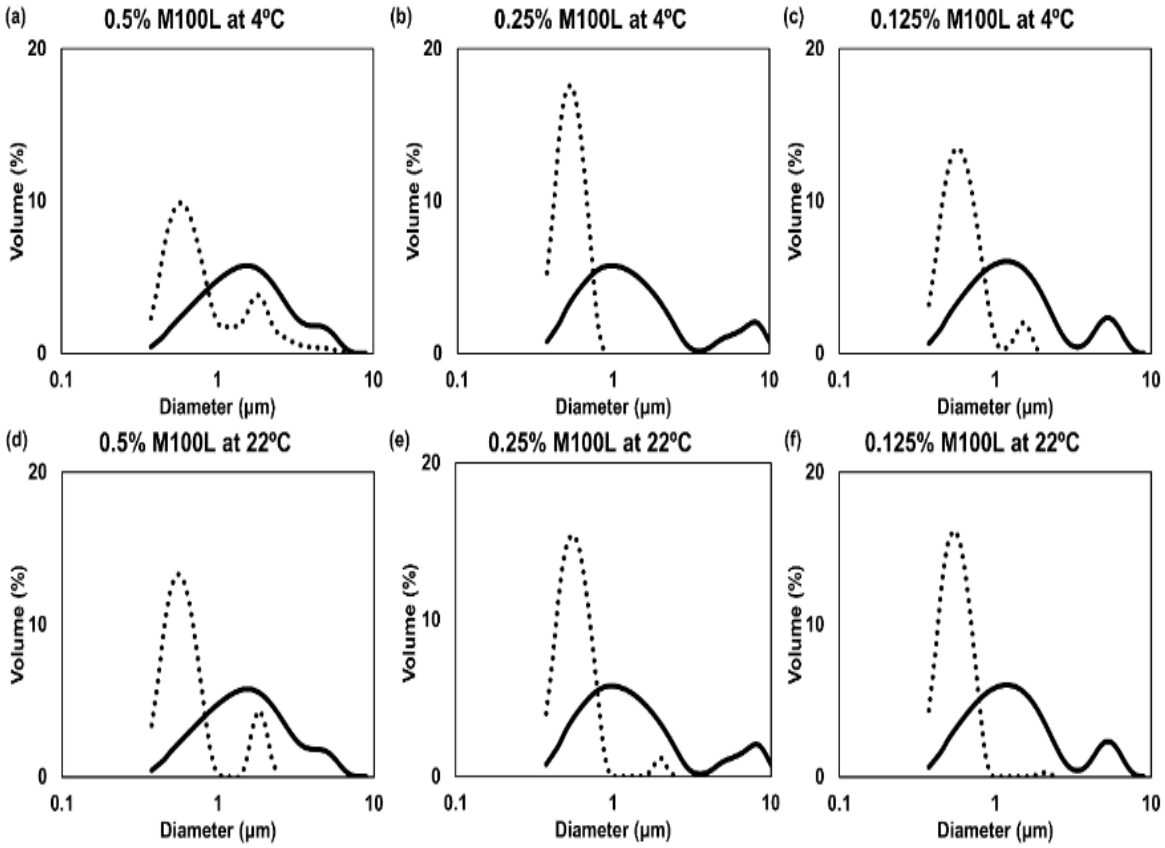

Fig. 3.2. Particle size distribution of emulsions stabilized by M100L at 4ºC or 22ºC. (a) 0.5% at 4ºC, (b) 0.25% at 4ºC, (c) 0.125% at 4ºC (d) 0.5% at 22ºC, (e) 0.25% at 22ºC, (f) 0.125% at 22ºC. Solid lines were at day 0 and dashed lines were at day 7.

Generally, stable emulsions show droplet sizes less than or around 1.0 μm (Binks & Lumsdon, 2001; Langevin, Poteau, Hénaut, & Argillier, 2004). An MFA-like compound containing octenyl succinate and starch had relatively low interfacial and emulsifying activity due to the high hydrophilicity and high molecular weight (Dickinson, 2003; McClements, 2015). This reduced emulsifying property was considered as a reason for the formation of broad bimodal droplet distributions at day 0 and 0.5% or less of MFAs, and the resultant relatively large droplets (> 1 μm) likely could lead to the shift of droplets over storages because only small droplets (around 1 μm diameter) contribute to the stability of emulsions (Binks & Lumsdon, 2001; Langevin et al., 2004).

In contrast to our results, previous research showed monomodal droplet distribution with 25% of MFAs (Udomrati & Gohtani, 2014), and this distribution difference might come from the higher concentration because a large excess of MFAs could compensate for the poor emulsifying ability by covering all the droplet surfaces properly (McClements, 2015). In addition, very similar droplet distributions and left-shifts were detected from the stable emulsions across all levels of M100L (Fig. 3.2), M250L (Fig. 3.4), and M100LP (Fig. 3.5) during storages at both temperatures (4ºC and 22ºC), indicating similar effectiveness of emulsifying abilities of these 3 MFAs at the concentrations of 0.5% or less.

The droplet distributions of emulsions with commercial emulsifiers were different from those containing MFAs. Both emulsions containing 0.5% SE or T20 showed narrow monomodal distribution on day 0 which stayed the same after 7 days of storage at 4ºC and 22°C (Fig. 3.3a, b, c, and d). The emulsions with 0.25% SE showed stationary narrow monomodal distribution on day 0 and 7 during storage at 4ºC (Fig. 3.3e), but the distribution was slightly shifted to smaller sizes during storage at 22°C (Fig. 3.3g). The emulsions with 0.25% T20 produced narrow monomodal distribution at day 0 with the left shift during storage at both temperatures (Fig. 3.3f and h). The emulsions with 0.125% SE or T20 showed relatively broad droplet distribution on day 0 and the number of smaller droplets were increased over 7 days of storage at both temperatures (Fig. 3.3i, j, k, and l).

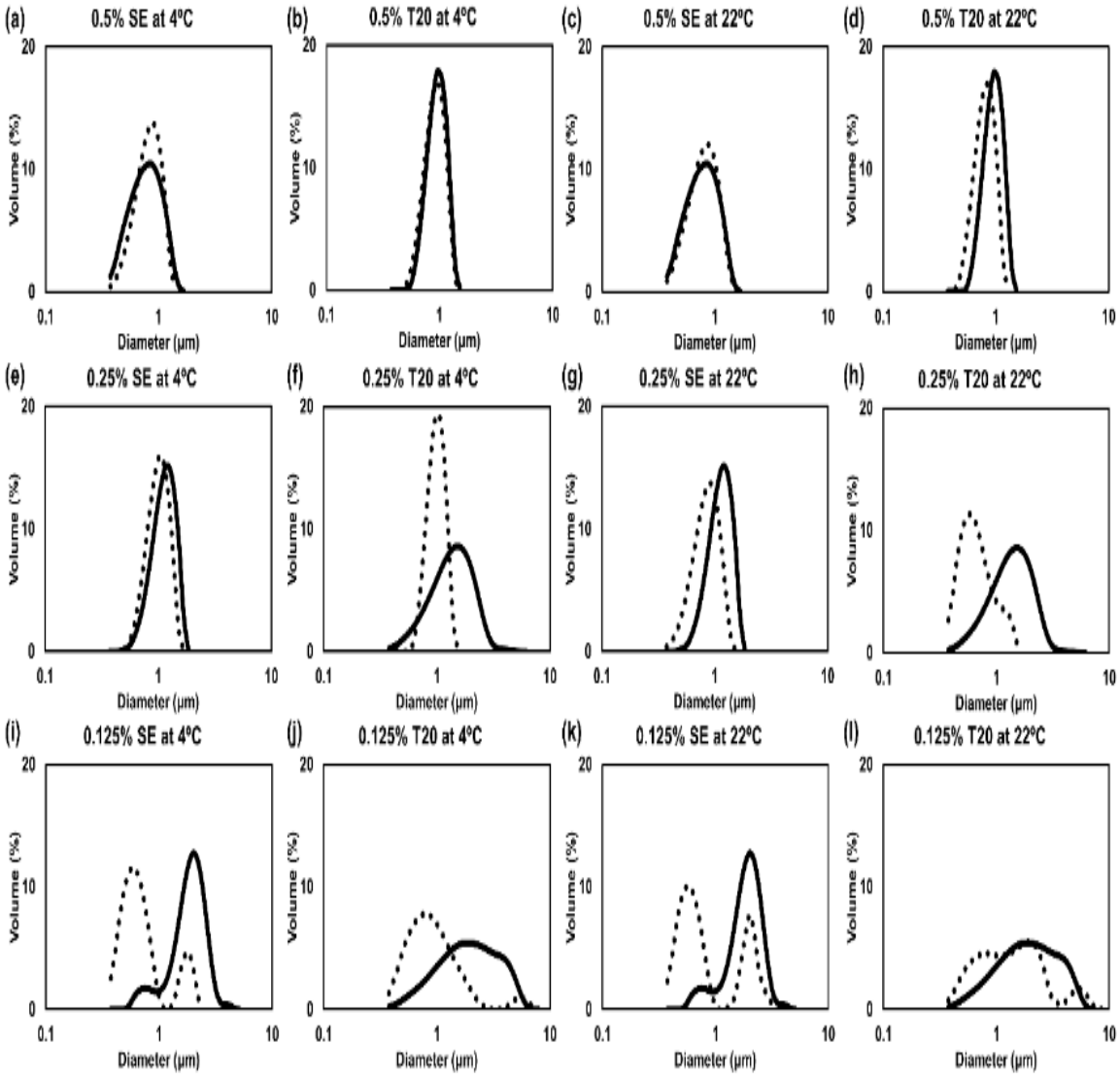

Fig. 3.3. Particle size distribution of emulsions stabilized by different concentration of sucrose ester (SE) or tween 20 (T20) at 4ºC or 22ºC. (a) 0.5% SE at 4ºC, (b) 0.5% T20 at 4ºC, (c) 0.5% SE at 22ºC (d) 0.5% T20 at 22ºC, (e) 0.25% SE at 4ºC, (f) 0.25% T20 at 4ºC, (g) 0.25% SE at 22ºC, (h) 0.25% T20 at 22ºC, (i) 0.125% SE at 4ºC, (j) 0.125% T20 at 4ºC, (k) 0.125% SE at 22ºC, and (l) 0.125% T20 at 22ºC. Solid lines were at day 0 and dashed lines were at day 7.

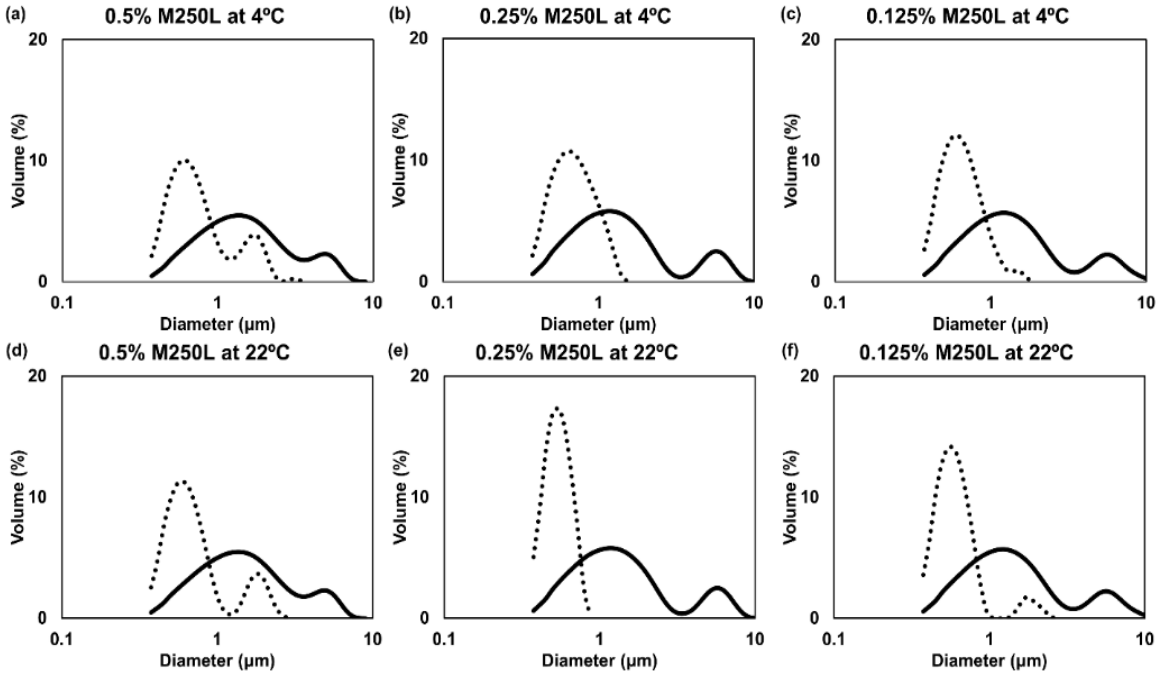

Fig. 3.4. Particle size distribution of emulsions stabilized by M250L at 4ºC or 22ºC. (a) 0.5% at 4ºC, (b) 0.25% at 4ºC, (c) 0.125% at 4ºC (d) 0.5% at 22ºC, (e) 0.25% at 22ºC, (f) 0.125% at 22ºC. Solid lines were at day 0 and dashed lines were at day 7.

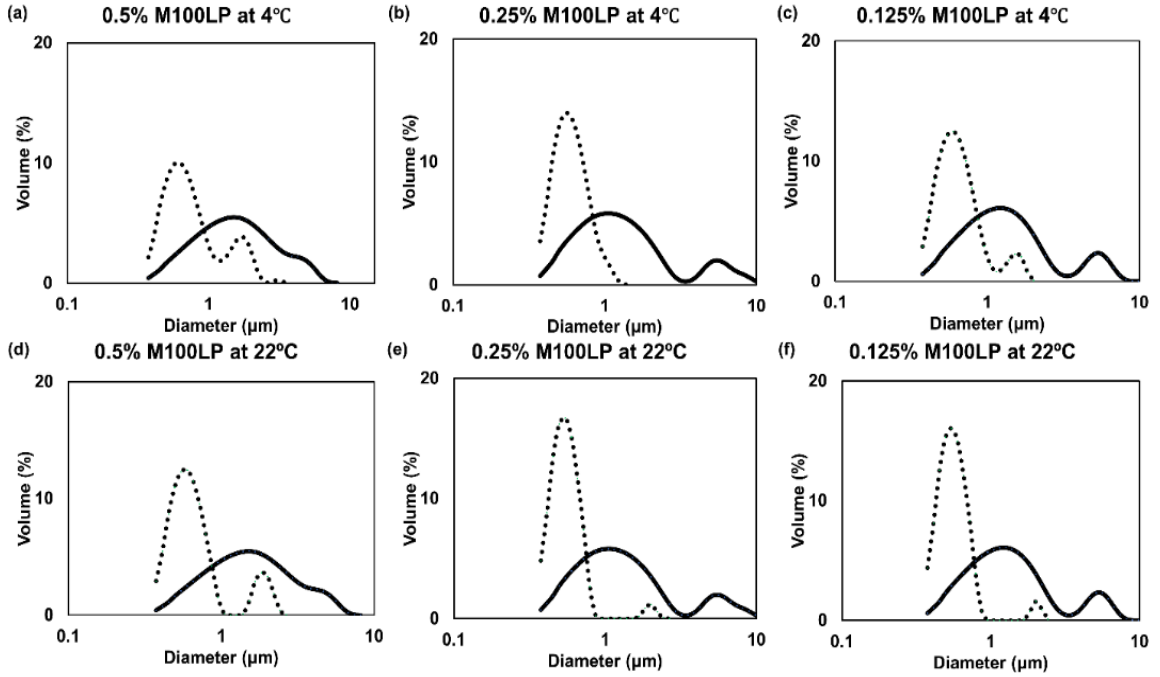

Fig. 3.5. Particle size distribution of emulsions stabilized by M100LP at 4ºC or 22ºC. (a) 0.5% at 4ºC, (b) 0.25% at 4ºC, (c) 0.125% at 4ºC (d) 0.5% at 22ºC, (e) 0.25% at 22ºC, (f) 0.125% at 22ºC. Solid lines were at day 0 and dashed lines were at day 7.

Compared to the emulsion distribution results of MFAs, the emulsions with SE and T20 produced narrow and stationary monomodal distribution depending on the concentration. Typically, only small molecule emulsifiers (SE and T20) can be rapidly adsorbed onto the interface during emulsification, producing relatively small droplets by decreasing interfacial tension (Anarjan & Tan, 2013; Dickinson, 2003; McClements, 2015). Taken together, the comparatively larger molecular size of MFAs, even though their amphiphilicity, is assumed to influence the lowered emulsification ability by slowing down the adsorption during emulsion formation.

In order to combine the emulsions results, the understanding of accuracy limitation by Turbiscan (a vertical-light scattering detector) was needed. In general, an emulsion tends to separate into three phases at the early stage (a lower serum layer, an intermediate emulsion layer, and a top cream layer) followed by only two phases toward later stages (serum and cream layers) (McClements, 2015). The 20% O/W system used in this research was the concentrated emulsion meaning the dispersed fraction is over 1% (Venkataramani & Aichele, 2015). At this level the vertical-light scattering method could monitor the separation by the position of the boundaries between the different layers rather than by the vertical droplet profile (McClements, 2015). This resulted in the emulsions indicating similar destabilization rates with the different droplet distributions by the undetected movement of droplets in the emulsion layer.

All emulsions containing MFAs showed stability (less than 1.0 mm/day of destabilization rate) with a clear left-shift of the bimodal distribution over 7 days at all concentrations. These results may be due to MFAs' high emulsion stabilizing by steric repulsion and reduced emulsifying abilities than commercial emulsifiers. Specifically, the

prevention of droplet aggregation by steric stabilization was postulated as a major mechanism of MFAs because of the reduced destabilization rates even with the shift of droplets.

For commercial emulsifiers, stability and droplet distribution were related. The emulsions containing commercial emulsifiers (SE and T20) at 0.5% and 0.25% were stable only when there was a narrow monomodal droplet distribution with none or minimal left-shift. These results may suggest that the formation of fine droplets by the enhanced emulsifying ability of SE and T20 is a main mechanism for the emulsion stability, or the complete and thick coverage of droplets may be needed to overcome the shallow surface repulsion due to the reduced stabilizing activities of SE and T20.

Conclusions

The six different types of MFAs were synthesized in an ethanol system (73 - 83% yields). Based on the substrates, the produced MFAs showed different properties. The fatty acid length significantly influenced the DS and solubility as shorter chain length resulted in higher DS and solubility. After the esterification reaction, all the MFAs showed enhanced stabilizing ability in 20% O/W emulsions compared to native maltodextrins. Emulsions containing M100L, M100LP, and M250L were stable at each concentration tested (0.5%, 0.25%, and 0.125%) during storage for 7 days at 4ºC and 22°C while those with commercial emulsifiers (SE and T20) showed stability only at the 0.5% and 0.25% concentrations. In addition, differences between droplet distribution patterns of stable emulsions containing MFAs (bimodality) or commercial emulsifiers (monomodal) were detected. In conclusion, the enhanced emulsion stabilization by the steric repulsion against

droplet aggregation and the reduced emulsifying abilities of MFAs compared to those of the commercial emulsifiers were observed. Therefore, MFAs may find an application in the food industry as emulsion stabilizers. Based on the combined data, emulsions formed with 0.5% M100L would be most suitable for use in food emulsions whose storage is between 4°C and 22°C.

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CHAPTER Ⅳ

MICROBIAL INHIBITORY PROPERTIES OF MALTODEXTRIN FATTY ACID ESTERS AGAINST FOOD-RELATED MICROORGANISMS[2]

Abstract

Carbohydrate fatty acid esters (CFAs) are increasingly used to improve food quality and safety but may have limited applications due to their low aqueous solubility. The use of hydrophilic maltodextrin for the synthesis of CFAs results in compounds with high aqueous solubility, but knowledge of their microbial inhibitory properties is lacking. The aim of this study was to evaluate the microbial inhibitory properties of maltodextrin fatty acid esters (MFAs) compared to commercial sucrose laurate (SL). Six different MFAs were synthesized from 2 different maltodextrins and 3 different vinyl fatty acids. Maltodextrin with a lower dextrose equivalent esterified with laurate had the highest inhibitory activity and a broader microbial inhibitory spectrum than SL, indicating a potential application in foods. Microbes susceptible to SL and MFAs showed morphological deformations observed by scanning electron microscopy and extracellular proteins released from cells, indicating compromised cell membranes.

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[2] LWT- Food Science and Technology. Microbial inhibitory properties of maltodextrin fatty acid esters against food-related microorganisms. Volume 147, July 2021, Page 111664. Namhyeon Park, Marie K. Walsh. (original copyright notice as given in the publication in which the material was originally published) “With permission of Elsevier”

Introduction

Carbohydrate fatty acid esters (CFAs) are chemically or enzymatically esterified simple sugars or polysaccharides (Chang & Shaw, 2009). Their properties depend on the types of fatty acids and carbohydrates and the degree of substitution (Neta, Teixeira, & Rodrigues, 2015). In addition to their nontoxicity and biodegradability, consumer demand for alternative antimicrobials to produce minimally processed foods with enhanced safety and quality has given attention to the microbial inhibitory properties of CFAs (Nobmann, Bourke, Dunne, & Henehan, 2010; Nobmann, Smith, Dunne, Henehan, & Bourke, 2009). One type of CFAs synthesized from simple sugars has been actively studied, and sucrose fatty acid esters (SFAs) are allowed in certain processed foods (U.S. Food and Drug Administration [FDA], 2018) with broad applications in commercial beverages in Japan (Zhao, Zhang, Hao, & Li, 2015). This usage can provide emulsion stability and bacterial inhibition (Habulin, Šabeder, & Knez, 2008).

Several studies showed microbial inhibitory properties of CFAs made from simple sugars against different microbial species (Ferrer et al., 2005; Habulin et al., 2008; Zhao et al., 2015), and in general, they were more active against Gram-positive than Gram-negative bacteria (Zhao et al., 2015). While the exact mechanism of antimicrobial activity of CFAs is unknown, the cell membrane has been believed to be the principal site of action of fatty acids and their derivatives (Nobmann et al., 2010). Interaction of CFAs with cell membranes can alter membrane permeability, inhibit membrane enzymatic activities, and disrupt energy production (Yoon, Jackman, Valle-González, & Cho, 2018). Previous research using sugar fatty acid esters suggested that cellular membrane damage resulted in altered permeability (Nobmann et al., 2010; Shao et al., 2018; Zhao et al., 2015).

The lower cost has incentivized sucrose as a common substrate for CFA synthesis (Neta et al., 2015). However, many commercial SFAs, except sucrose laurate (SL), may have insufficient solubility in aqueous environments (Nagai et al., 2016) and, therefore, can have limited applications in certain food products. The synthesis of commercial SFAs has also relied on the traditional chemical method using high temperatures and non-food grade solvents (Soultani, Ognier, Engasser, & Ghoul, 2003).

To overcome these solubility and chemical concerns, Park and Walsh (2019) enzymatically esterified maltodextrins in food-grade ethanol. Maltodextrins are inexpensive (Dong, Ouyang, Yu, & Xu, 2010), and their hydrophilicity renders them highly aqueous soluble even after esterification with fatty acids. Park et al. (2019) synthesized MFAs with a limited number of fatty acids attached, resulting in products with good aqueous solubility at a practical use level (1% w/v), which is comparable to that of commercial SL.

However, there are limited studies about the functional properties of MFAs, and only one previous study investigated the microbial inhibitory properties of MFAs against 2 microbes (Pantoa, Shompoosang, Ploypetchara, Gohtani, & Udomrati, 2019). That study synthesized 3 MFAs using decanoic, lauric, and palmitic acids, and MFAs containing laurate were the most inhibitory against *Escherichia coli* at the highest concentration (20%) used. However, further information on the microbial inhibitory properties of MFAs compared to commercial SFAs against a broad spectrum of microbes associated with food quality and safety is necessary.

This research aimed to investigate the microbial inhibitory properties of MFAs against diverse microorganisms compared to commercial SL. Six different MFAs were

enzymatically synthesized and tested against 11 food-related microorganisms by measuring minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentrations (MBC or MFC). Their inhibitory trends were compared to SL and analyzed in terms of structural features, including maltodextrin and fatty acyl groups with their degree of substitution (DS). The change in cellular morphology and the concentration of proteins released from bacterial cells upon treatment with MFAs or SL were also investigated. This study adds to the current knowledge of the microbial inhibitory properties of CFAs in general and expands the understanding of the microbial inhibitory properties of MFAs.

Materials and methods

Materials

Vinyl laurate (L0090 and purity: >99.0%) and vinyl palmitate (P0848 and purity: ≥ 96.0%) were obtained from TCI America (Portland, OR, USA). Lauric acid (W261416 and purity ≥ 98%) (Sigma-Aldrich, St. Louis, MO, USA) and palmitic acid (B20322 and purity ≥ 95% (Alfa Aesar, Ward Hill, MA, USA) were used. Immobilized lipase from *Thermomyces lanuginose* (IMMTLL-T2-150, activity: 10000 tributyrin unit (TBU)/g; 1 TBU = 1 μmol butyric acid released per minute/g immobilized enzyme at 40°C and pH 7.5) was purchased from Chiral Vision (Netherlands). Ethanol (HPLC grade, Pharmco Products Inc., Brookfield, CT, USA), acetonitrile (HPLC grade, Thermo Fisher, PA, USA), 250 mL size Pyrex glass bottles with polypropylene caps (Corning Glass Works, Corning, NY, USA), magnetic stir bar (38.1 × 9.5 mm) (Bel-Art Products, Pequannock, NJ, USA), 0.2 μm nylon membrane filter (47 mm) (Whatman International Ltd., Maidstone, UK) and 0.2 μm

polyvinylidene fluoride (PVDF) syringe filters (Foxx Life Sciences, Salem, NH, USA) were used. Whatman glass 1 μm microfiber filters and molecular sieves (3 Å) were from Sigma-Aldrich (St. Louis, MO, USA). Sucrose laurate (Ryoto L-1695, purity: 80% monoester and 20% diester and higher) was obtained from Mitsubishi-Kagaku (Tokyo, Japan). Maltrin M100 (dextrose equivalent (DE) 9–12 and degree of polymerization (DP) 1-7 30%, DP 6-25 35%, DP 26-40 1%, over DP40 34%) and Maltrin M250 (DE 23–27, DP 1-7 60%, DP 6-25 35%, DP over 40 5%) were obtained from Grain Processing Corporation (Muscatine, IA, USA). Microtiter well plates, 48 wells (Becton Dickinson, NJ, USA), polystyrene semi-micro cuvettes (VWR, Darmstadt, Germany), and Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) were used. Yeast malt (YM) broth was from HiMedia (India), and Luria-Bertani (LB) broth, glucose, and brain heart infusion (BHI) broth were from Sigma Aldrich (St. Louis, MO, USA). MRS broth (Merck, Darmstadt, Germany), tryptic soy broth (TSB), nutrient broth (NB) (Hardy Diagnostics, CA, USA), and agar (VWR, Darmstadt, Germany) were used.

Synthesis and purification of maltodextrin fatty acid esters (MFAs)

MFAs were prepared according to a previously reported method (Park et al., 2019). Two grams of maltodextrin (M100 or M250) and vinyl fatty acid (280 mg of vinyl laurate (VL), 350 mg of vinyl palmitate (VP) or half VL (140 mg) and half VP (175 mg) were added to 4.0 g of molecular sieves, 2.4 g of immobilized lipase, and 160 mL of ethanol in a 250 mL glass bottle. This reaction results in a 1:0.1 M ratio between maltodextrin and vinyl fatty acid (1 mol of anhydrous glucose (162 g)/mole of vinyl fatty acid). The reactions were conducted in a shaker (Eppendorf, Edison, NY, USA) at 500 rpm and 55°C for 8 days with an additional 2.4 g of immobilized lipase and 4.0 g of molecular sieves added each day.

Table 4.1. The characteristics of maltodextrin fatty acid esters used in this research.

Name (abbreviation)	Dextrose equivalent of carbohydrate group	Fatty acid group (Chain length)	Degree of substitution (mole/mole)
M100L	9 – 12	laurate (C12)	0.026 ± 0.001^{a}
M100P	9 – 12	palmitate (C16)	0.013 ± 0.002^{de}
M100LP	9 – 12	Mixture of laurate (C12) and palmitate (C16)	0.024 ± 0.000^{ab}
M250L	23 – 27	laurate (C12)	0.021 ± 0.000^{bc}
M250P	23 – 27	palmitate (C16)	0.009 ± 0.003^{e}
M250LP	23 – 27	Mixture of laurate and (C12) palmitate (C16)	0.017 ± 0.002^{cd}

^{a,b,c,d} means with the same letter were not significantly different within each column ($p<0.05$).

Following the reaction, the mixture was filtered with a glass microfiber filter to remove the immobilized lipase and molecular sieves. The filtrate was air-dried in a fume hood at 22°C. The dried filtrate was resuspended in 100 mL of distilled water followed by mixing with 100 mL of hexane to remove the unreacted fatty acids (solubility in water is 0.4868 mg/L for vinyl laurate and 4.983 µg/L for vinyl palmitate) (The Good Scents Company, 2018). After complete separation of the two phases, the final aqueous phase was freeze-dried in a benchtop 5 L freeze drier (manifold type, Virtis Company, Inc., Gardiner, NY, USA) at 6.7 Pa for 1 day. Six different products were synthesized (M100L, M100P, M100LP, M250L, M250P, and M250LP), and their information is given in Table 4.1. Stock

solutions (50 mg/mL of each compound) were prepared with ethanol/water (19:1, v/v) and kept at 20 °C. The purity of each MFA was determined by HPLC as described below.

Analysis of MFAs

HPLC analysis

The synthesized MFAs were analyzed by HPLC for purity according to Park et al. (2019) with modifications. An HPLC (Beckman System Gold 125 Solvent Mobile, Ontario, Canada) was equipped with an evaporative light scattering detector (ELSD) (Agilent Technologies Group, Hesperia, CA, USA).

Samples of MFAs (500 mg) and 10 mL of filtered (0.2 μm nylon membrane filter) ethanol/water (19:1, v/v) were placed into a glass bottle containing a magnetic stir bar and stirred on a heating stir plate (VWR, Darmstadt, Germany) at 55°C and 200 rpm for 10 min to achieve a transparent solution. An aliquot (2 mL) of the dissolved solution was diluted with 18 mL of ethanol/water (9:1, v/v), and this solution was filtered using a 0.2 μm PVDF syringe filter. This filter showed high recovery as determined by measuring the filtered MFA sample's dry weight after air-drying in a hood for 24 h at 21°C. Then, an aliquot (100 μL) of the filtered solution was diluted 1 to 5 with filtered ethanol/water (9:1, v/v), resulting in 1 mg/mL of MFAs, and 20 μL was injected into the HPLC system. Fatty acid and maltodextrin standards were prepared as described above as 1 mg/mL in HPLC grade ethanol or water, and 20 μL was injected into the HPLC.

The separation was conducted using a SynChropak RP-4 column (4.6 × 250 mm) (Synchrom, Inc., Lafayette, IN) at 50°C with two mobile phases (Hutchinson et al., 2012). Eluent A, water/acetonitrile (19:1, v/v) and eluent B, acetonitrile/water (19:1, v/v) were

filtered (0.2 μm nylon membrane filter) followed by degassing for 30 min using a sonicator (FS 60, Fisher Scientific, Pittsburgh, PA, USA) and they were used at a 1.0 mL/min flow rate. An isocratic step consisting of 0% of B was employed for 3 min followed by a linear gradient from 0% to 70% of B for 1 min, then 70% of B was maintained for 6 min. Another linear gradient from 70% to 100% of B for 1 min was done, and this 100% of B was maintained for 5 min. The final linear gradient was 100%–0% of B for 1 min, and this 0% of B was maintained for 3 min. The detector was set at 40°C and 35 kPa of nitrogen gas.

The area of each peak in the chromatogram obtained was measured by LP-chrom software (Lipopharm, Poland) (Fig. 4.1). The concentration of unesterified maltodextrin was calculated using a calibration curve (R^2 = 0.99) prepared with 7 different concentrations of maltodextrin standards (0.02, 0.03, 0.06, 0.13, 0.2, 0.25, and 0.5 mg/mL) that were graphed vs the peak area of each standard. The calibration curve was used to calculate the amount of unesterified maltodextrin in the purified samples shown in Fig. 4.1. The following equation was used to calculate the purity of MFAs: purity = (the injected concentration of MFA - the calculated concentration of unesterified maltodextrin)/the injected concentration of MFA.

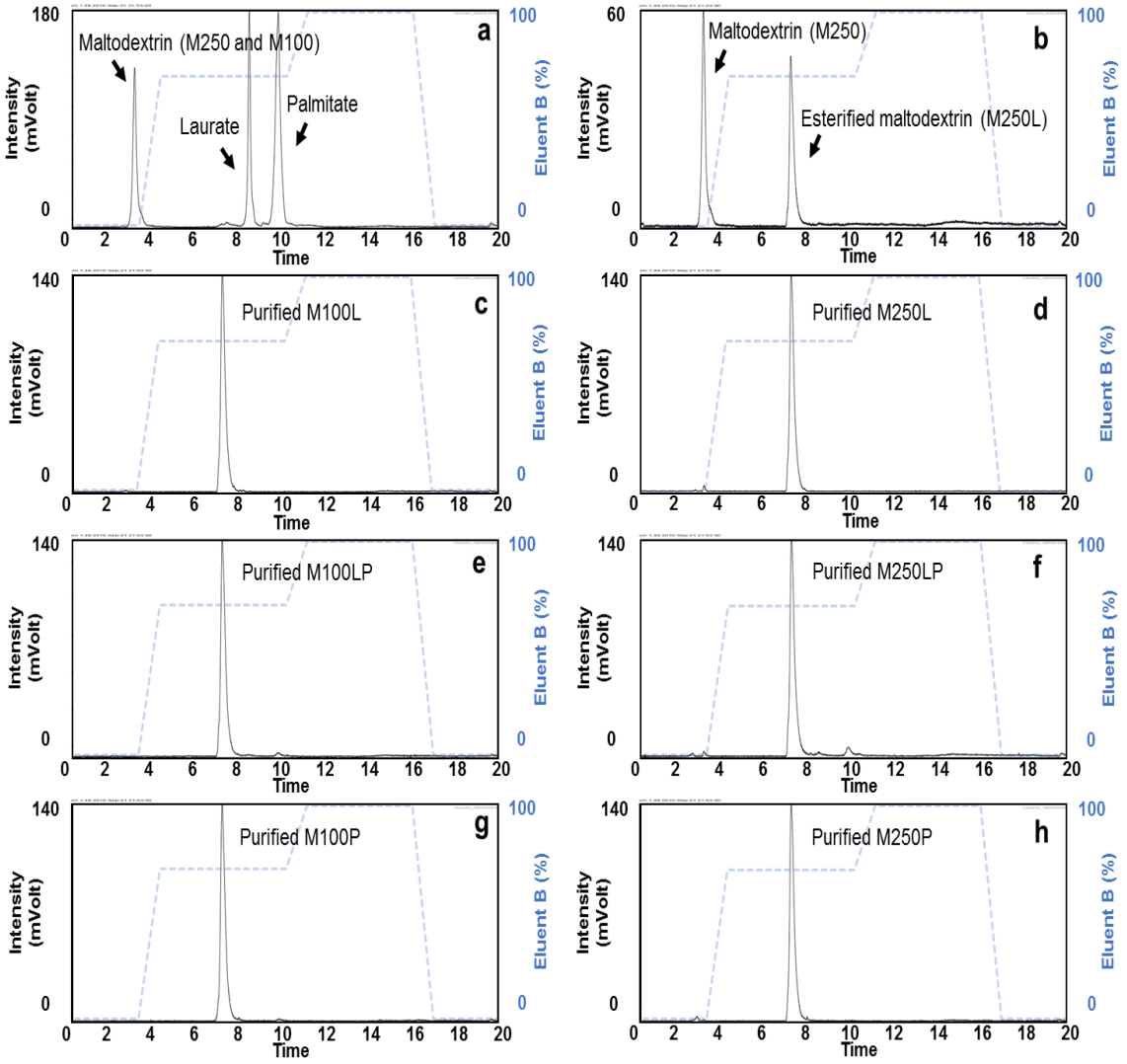

Fig. 4.1. HPLC chromatograms of maltodextrin fatty acid esters and substrates with the gradient profile of eluent B (%) in dashed lines. (a) Standards (1 mg/ml) and (b) sample reaction after 2 days (1:8 diluted with 50% ethanol in water). After 8 days reaction and purification, 1 mg/ml of the final samples; (c) M100L, (d) M250L, (e) M100LP, (f) M250LP, (g) M100P, and (h) M250P.

NMR analysis

The degree of substitution (DS) was determined by NMR analysis collected on Bruker Avance III-HD (Bruker, Billerica, MA, USA) with a proton operating frequency of 500.13 MHz. Samples were prepared by completely dissolving 5 mg in 1 mL of D_2O, and the

solutions were moved into NMR tubes. The 1H-NMR spectra were collected at 25°C with 32 scans, a 30° pulse (Bruker pulse program: zg30), and a relaxation delay of 5.0 s. The chemical shift (δ) was expressed in parts per million (ppm), and the residual solvent signal was used as a reference (D_2O at 4.79 ppm). Spectra were processed with Topspin 3.5pl7 (Bruker, Billerica, MA, USA), including manual phase correction and integration of the peak of interest.

The degree of substitution (DS) was calculated according to Kapusniak and Siemion (2007) with modifications. Maltodextrin contains glucose mainly linked by α-1.4 with a small number of differently branched α- or β-glucose (Lämmerhardt, Hashemi, & Mischnick, 2020). According to previous literature, the peaks for seven protons of the anhydrous glucose unit (AGU) of maltodextrin were specified (Cheng & Neiss, 2012; Dobruchowska et al., 2012). They were obtained between 3.20 and 5.50 ppm, excluding the residual solvent peak (4.79 ppm) (Fig. 4.2). Then, the signals for esterified fatty acid were recognized according to previous literature (Su et al., 2017; Yoko, Seong, & Adschiri, 2020) (Fig. 4.2). The terminal methyl group of the fatty acyl chain was the triplet peak at 0.87 ppm. The DS (mole/mole) was calculated according to the following equation (4.1):

$$\mathrm{DS} = \frac{\text{Area of Methyl/3 (moles of fatty acid}}{\text{Area of AGU/7 (moles of AGU)}} \quad (4.1)$$

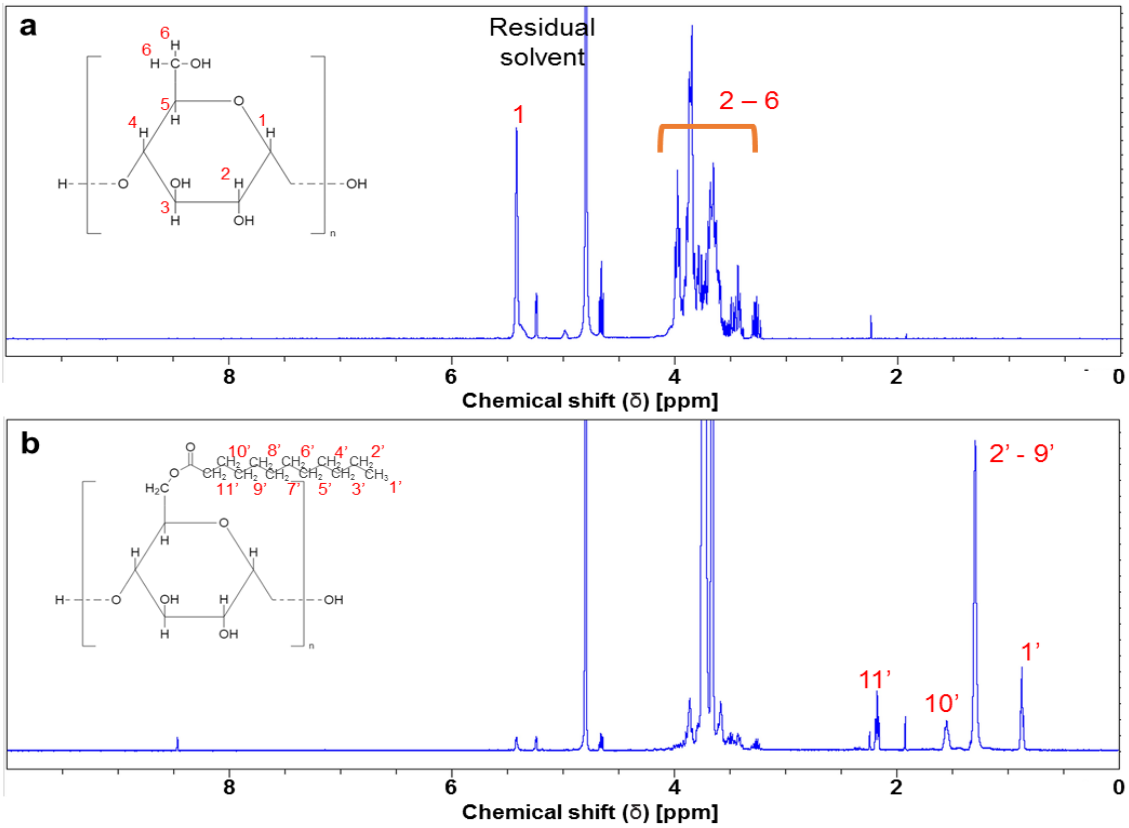

Fig. 4.2. Representative ^{1}H NMR spectra of maltodextrin and maltodextrin fatty acid ester. (a) maltodextrin (M250) and (b) its ester with laurate (M250L); δ 4.64, 4.98, 5.23, and 5.41 ppm: H-1 of α- or β-glucose with different linkages, δ 3.20 – 4.10 ppm: H-2 – H-6 of α- or β-glucose with different linkages, and δ 0.87 ppm: protons at the terminal methyl group of esterified fatty acid.

Preparation of microorganisms for microbial inhibitory analysis

Eleven food-related microorganisms were used, including spoilage yeasts and bacteria (Gram-positive and negative) with pathogenic Gram-positive bacteria. *Zygosaccharomyces bailii* (NRRL Y-7257) and *Z. rouxii* (NRRL Y-1793) were obtained from the Agricultural Research Service Culture Collection (nrrl.ncaur.usda.gov). *Escherichia coli* (ATCC 47009) was from Dr. Bastarrachea at Utah State University. Dr. Broadbent at Utah State University provided *Bacillus subtilis* (8G5) (Bron & Venema, 1972), *B. cereus* (ATCC 14579), *Streptococcus thermophilus* (ATCC 19258), *Lactococcus*

lactis (ATCC 7962), *Lactobacillus plantarum* (ATCC 4008), *Geobacillus stearothermophilus* (ATCC 7953), *Pseudomonas fluorescens* (ATCC 17467), and *Listeria monocytogenes* (FSL C1-056 from a human sporadic case, FSL J1-177 from a human sporadic case, FSL N3-013 from a food epidemic, FSL R2-499 from a human sporadic case, and FSL N1-227 from a food epidemic) (Fugett, Fortes, Nnoka, & Wiedmann, 2006). The detailed information about the microbes from the Agricultural Research Service Culture Collection and the American Type Culture Collection can be acquired from each institute's database. The optimum grown media and temperatures of all of the tested microbes are given in Table 4.2.

Frozen glycerol/media culture stocks (1:4, v/v) of each microorganism were prepared and maintained at -80°C before use. In the case of *Listeria monocytogenes*, after growth of each *Listeria* strain in TSB at 37°C for 24 h, a 5-strain cocktail was prepared by mixing the diluted cultures (optical density at 600 nm (OD_{600}) of 0.2) using a UV–vis spectrophotometer (Biospec 1601, Shimadzu, Japan). This cocktail was used to prepare a frozen glycerol stock of *L. monocytogenes*, as described by Lee, Sandhu, and Walsh (2017).

An aliquot (200 µL) of each frozen stock was individually moved into 10 mL of species-specific culture media and grown at the optimum temperature (Table 4.2) with 400 rpm of agitation for 24 h (12 h for *G. stearothermophilus* and 48 h for yeasts). One mL of the grown culture was added to 10 mL of fresh media and was then grown for 4 h (6 h for yeasts) at optimal temperatures (Table 4.2) with agitation. This secondary culture was measured at 600 nm and diluted with the same media used to reach OD_{600} of 0.2, which corresponds to 5×10^5–10^6 CFU/mL as determined by plate counts. These individual diluted secondary cultures were used for microbial inhibitory assays.

Microbial inhibitory analysis of MFAs in liquid media

Antimicrobial analysis for minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC) were determined by a broth dilution method in a microplate according to Zhao et al. (2015) with several modifications. The MFA solutions were prepared by serial dilutions (two-fold) in different liquid media specific for each microorganism, according to Table 4.2. Different concentrations of MFA solutions (250 μL) and the same volume of individual secondary microbial culture were moved into each well of a microtiter well plate (due to the solubility, 5 mg/mL was selected as the highest final concentration, and the test continued to the lowest concentration until no inhibition was detected). The plate was incubated at optimum temperatures (Table 4.2). Each microorganism was tested in a separate experiment. Pure media and SL added to media were used as controls. Colony-forming units (CFUs) were enumerated by plate count using species-specific agar media with serial dilutions (ten-fold) in 0.1 M phosphate buffer solution (PBS). MIC was defined as the lowest concentration showing a statistically significant logarithmic reduction of CFUs by t-test against control, and MBC or MFC were the lowest concentration where at least a 3 logarithmic reduction in CFUs was obtained.

Table 4.2. The microbial strains used in this research with their growth conditions and related foods.

Name	Strain	Type	Media	Temperature (°C)	Related foods
Bacillus subtilis	8G5	Spoilage - Gram +	LB	37	Mayonnaise (Kučerová et al., 2007)
Bacillus cereus	ATCC 14579	Pathogen - Gram +	NB	30	Dairy products (Lücking, Stoeckel, Atamer, Hinrichs & Ehling-Schulz, 2013)
Streptococcus thermophilus	ATCC 19258	Spoilage - Gram +	BHIG	37	Wine, cider, and beer (Kelly et al., 2012)
Lactococcus lactis	ATCC 7962	Spoilage - Gram +	MRS	30	Raw milk (Júnior et al., 2018)
Lactobacillus plantarum	ATCC 4008	Spoilage - Gram +	MRS	37	Orange juice, mayonnaise, and salad dressings (Li, Zhang, Jin, Turek, & Lau, 2005)
Geobacillus stearothermophilus	ATCC 7953	Spoilage - Gram +	TSB	55	Milk powder and canned foods (André, Vallaeys, & Planchon, 2017)
Listeria monocytogenes	FSL C1-056 FSL J1-177 FSL N3-013 FSL R2-499 FSL N1-227	Pathogen - Gram +	BHI	37	Dairy products (Harakeh et al., 2009)
Escherichia coli	ATCC 47009	Spoilage - Gram -	TSB	37	Apple juices (Ferrario, Alzamora, & Guerrero, 2015)
Pseudomonas fluorescens	ATCC 17467	Spoilage - Gram -	LB	30	Dairy products (Arslan, Eyi, & Özdemir, 2011)
Zygosaccharomyces bailii	NRRL Y-7257	Spoilage - yeast	YM	25	Fruit juices, sauces, and soft drinks (Martorell, Stratford, Steels, Fernández-Espinar, & Querol, 2007).
Zygosaccharomyces rouxii	NRRL Y-1793	Spoilage - yeast	YM	25	Same as above

Scanning electron microscopic (SEM) analysis

M100L, which showed the most significant inhibitory properties among the tested MFAs (Table 4.3), was used as a representative MFA with SL for comparison for SEM analysis. The microorganisms for SEM analysis were selected based on the inhibitory results to study the association between microbial inhibition and morphological alterations. Microbes susceptible to both M100L and SL or only to M100L based on MIC results were chosen. The final 4 microorganisms selected were *B. subtilis* and *G. stearothermophilus* because they were susceptible to both M100L and SL and *L. plantarum* and *Z. bailii*, both of which were susceptible only to M100L.

Previous research treated different bacteria with sucrose monocaprate and observed the resulting morphological changes in the bacteria by SEM (Zhao et al., 2015). This technique was applied with modifications to study M100L or SL's effect on bacterial morphology. The secondary cultures of tested microorganisms (OD_{600} = 0.2) were treated with M100L or SL at MIC levels (the concentration was at MIC level or 5 mg/mL if there were no MICs) with media only as controls. The samples were then incubated for 24 h at species-specific temperatures. Cells were harvested by centrifugation at 3500×g for 10 min (IEC Clinical centrifuge, Danon/IEC Division, Needham Heights, MA, USA), and the pellets were resuspended and fixed with glutaraldehyde/0.1 M HEPES buffer solution (1:49, v/v) at 4°C for 12 h.

The cells were transferred to a glass slide and were serially dehydrated using a series of ethanol/water (1:1, 3:1, 19:1, and 1:0, v/v) solutions followed by critical point drying (Tousimis Autosamdri 931, Tousimis, Rockville, MD, USA). The cells were mounted onto aluminum stubs and were sputter-coated with gold and palladium (10 nm)

using a rotary sputter coater (EMS150R ES, Electron Microscopy Sciences, Hatfield, PA, USA). The cells were analyzed under high vacuum mode by field emission scanning electron microscope (FE-SEM) (FEI Quanta FEG 650, Field Electron and Ion Company, Hillsboro, OR, USA). The resulting SEM micrographs show the microbial surface and the morphological changes associated with M100L or SL, which were analyzed by comparing the micrograph of cells treated with M100L or SL to that of untreated cells.

Extracellular protein concentration assay

Following cell treatment with M100L or SL, the concentration of extracellular proteins was measured as an indicator of cellular membranes' integrity according to previous methods (Lv, Liang, Yuan, & Li, 2011; Zhao et al., 2015) with modifications. Identical microorganisms used for SEM analysis (B. *subtilis, G. stearothermophilus, L. plantarum*, and *Z. bailii*) were selected to analyze extracellular protein concentration for evaluating the relationship between the results of SEM and extracellular protein concentrations.

Ten mL of secondary culture (OD_{600} = 1.0) of microorganisms were centrifuged for 10 min at 3500×g to harvest the microbial cells, followed by resuspending in 10 mL of 0.1 M PBS. This sample was centrifuged again, as stated above, and the process was repeated 2 more times. The washed cells were resuspended in 10 mL of PBS. The cell suspensions were incubated at a specific temperature (Table 4.2) with agitation (200 rpm) for 4 h in the presence of M100L, SL, or nothing added at MIC concentrations or 5 mg/mL if no MIC was obtained. Each cell suspension was then centrifuged at 10,000×g for 5 min (Marathon Micro A centrifuge, Fisher Scientific, Pittsburgh, PA, USA), and the supernatant was collected. For measuring extracellular proteins' concentration, 100 µL of supernatant was reacted with 2 mL of BCA protein kit working reagent and incubated at 60°C for 30

min. After cooling, the reacted solution's absorbance was measured at 562 nm, and the protein concentration (μg/mL) was calculated using a calibration curve prepared with bovine serum albumin standards (5 concentrations: 5, 25, 50, 125, and 250 μg/mL). A correction to offset the concentration of pre-existing extracellular proteins was made by the protein concentration of the supernatants containing the same concentration of M100L or SL after 5 min of contact with microbial cells.

Statistical analysis

All experiments were replicated at least two times, and results were expressed as mean ± standard deviation. The results for DS were analyzed by two-way ANOVA (the types of maltodextrins and fatty acids were factors) followed by Tukey's post hoc analysis (the significant difference was set at $p < 0.05$) (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). The concentrations of extracellular proteins were analyzed by one-way ANOVA with Tukey's post hoc analysis (the significant difference was set at $p < 0.05$). In the case of MIC results, the CFUs of control and each treatment were compared by a two-sample t-test (the significant difference was set at $p < 0.05$) (Microsoft Excel 2004, Microsoft Corp, Redmond, WA, USA).

Results and discussion

Synthesis of MFAs

Six different MFAs were synthesized using 2 different maltodextrins (M100 and M250) and 3 different fatty acids (laurate, palmitate, and the mixture of laurate and palmitate) (Table 4.1). The reaction ratio was 1 mol of maltodextrin to 0.1 mol of vinyl fatty acid, and

the DS of synthesized MFAs ranged between 0.009 and 0.026 (Table 4.1). These values are similar to the DS of MFAs (0.016–0.026 DS) synthesized in a previous study with the same substrate ratios and method (Park et al., 2019).

Chromatograms for substrates and MFAs, along with the gradient profile, are shown in Fig. 4.1. The separation of analytes was based on the hydrophobic interaction with the reverse phase column, and this interaction was decreased by increasing the percentage of acetonitrile in the mobile phase (Boone & Adamec, 2016). Maltodextrins did not interact with the column and eluted in the void volume, and the MFAs and free fatty acids eluted with an increase in acetonitrile concentration. The elution order was maltodextrin, esterified maltodextrin, followed by free fatty acid (Figs. 4.2a and b). After 2 days of reaction, the peak for M250L appeared between M250 and laurate (Fig. 4.1b). After 8 days of reaction and purification, a high level of a significant peak for the esterified maltodextrins was obtained (Figs. 4.2c, d, e, f, g, and h). The purity of the final MFAs after purification ranged from 91.5 to 99.8%. The substrates and the DS for esterification are considered important because these attributes affect the compounds' properties (Fang, Fowler, Tomkinson, & Hill, 2002; Park et al., 2019). In this research, the microbial inhibitory properties of MFAs were analyzed in terms of the types of maltodextrins and fatty acids and the DS.

Microbial inhibition by MFAs

MFAs and commercial SL's microbial inhibitory properties were evaluated by analyzing MIC and MBC (or MFC) values against food-related microorganisms (Table 4.3). MIC was the lowest concentration showing a statistically significant reduction of CFUs ($p <$

0.05), and MBC (or MFC) was the lowest concentration with at least a 3 logarithmic reduction in CFUs.

The MIC values of M100L (0.08–5 mg/mL), M100LP (0.08–5 mg/mL), and M250L (0.16–5 mg/mL) were obtained against all tested microorganisms, while M100L produced MBC values against 5 bacteria (*B. subtilis, B. cereus, S. thermophilus, L. lactis*, and *G. stearothermophilus*) and M100LP and M250L showed MBC values against 4 bacteria (*B. subtilis, B. cereus, S. thermophilus*, and *G. stearothermophilus*). M250LP showed MIC values ranging from 0.31 to 5 mg/mL) against all tested organisms except *L. plantarum* and *Z. rouxii* and MBC values were obtained against 4 bacteria (*B. subtilis, B. cereus, S. thermophilus*, and *G. stearothermophilus*). M100P showed MIC values (2.5–5 mg/mL) against *B. subtilis, B. cereus, S. thermophilus*, and *G. stearothermophilus*, and *L. monocytogenes* and M250P showed MIC values (5 mg/mL) against only *G. stearothermophilus* while both compounds showed no MBC (or MFC) values against any tested organisms. The MIC values (0.01–0.31 mg/mL) with SL were obtained against all tested microbes except *L. plantarum, Z. bailii*, and *Z. rouxii* and this compound showed MBC values against 3 bacteria (*S. thermophilus, B. cereus*, and *G. stearothermophilus*).

Previous studies have demonstrated the antimicrobial properties of other CFAs comprised of lactose (0.05–5 mg/mL for MICs and 1–5 mg/mL for MBCs) (Lee et al., 2017) and glucose (0.008–0.083 mg/mL for MICs and 0.033–0.1 mg/mL for MBCs) (El-Baz et al., 2021). These reported inhibitory ranges are similar to the current results, but the direct comparison may be difficult due to the different assay methods and microbial species tested. Also, the antibacterial activity of CFAs can be varied depending on carbohydrate and fatty acid components and their combination (Zhao et al., 2015).

As shown in Table 4.3, the microbial inhibitory properties of M100L showed the broadest inhibitory spectrum with low MIC/MBC values against the tested microorganisms. M100P and M250P showed some MIC values but no MBC/MFC values.

In general, lower DE (DE: 9–12) of the maltodextrin (M100) and shorter fatty acid (laurate) esterified showed better or at least the same inhibition as other MFAs against the tested microorganisms. The effect of the fatty acyl group of CFAs on microbial inhibition has previously been reported. There was a negative relationship between length and inhibitory properties of CFAs (Karlova, Polakova, Šmidrkal, & Filip, 2010; Lee et al., 2017; Zhao et al., 2015) with shorter chain fatty acids being more inhibitory, which is similar to the results presented here. Specifically, MFAs esterified with decanoate (C10) and laurate (C12) were more inhibitory than those esterified with palmitate (C16) (Pantoa et al., 2019).

This is the first report that evaluated the microbial inhibitory abilities of MFAs according to DE, which is related to molecular size. Previous research comparing mono- and disaccharide esters showed higher inhibition properties of disaccharide esters than monosaccharide esters (Ferrer et al., 2005; Zhao et al., 2015). The lower solubility of monosaccharide fatty acid esters in an aqueous system than disaccharide esters was assumed as the main reason for the lower inhibitory activity of monosaccharides (Ferrer et al., 2005). However, the same MFAs previously synthesized in Park et al. (2019) showed solubilities ranging from 93 to 100% at 10 mg/mL regardless of DE. The higher antimicrobial activity of MFAs with lower DE is not due to solubility, so an additional explanation is necessary.

Based on an existing theory about the microbial membrane as the primary target of fatty acids and their derivatives (Nobmann et al., 2010), we suggest the higher inhibition

with lower DE (M100) is associated with the size-dependent mode of action on the cell membranes. Due to weak or no interaction of the carbohydrate with lipid membranes (Kawakami, Nishihara, & Hirano, 2001), it is hypothesized that MFAs are inserted into membranes by the hydrophobic fatty acyl group and may cause more membrane disruption than di- or monosaccharides. This disruption of the highly ordered packing of fatty acids in the membrane may cause loss of vital functions for the microbes.

When considering DS, this was not directly associated with the degree of microbial inhibition by MFAs. A similar result was shown in previous research using MFAs with decanoate, laurate, and palmitate (Pantoa et al., 2019), which exhibited the highest microbial inhibition with maltodextrin esterified with laurate with a DS of 0.03. A combination of DS and chain length of fatty acids esterified may be important because it determines the final hydrophobicity of MFAs (Pantoa et al., 2019).

M100L, which generally showed the broadest inhibitory spectrum with the lowest MIC and MBC values, was compared to commercial SL. Overall, the MIC and MBC values of M100L were greater (meaning higher concentrations needed) than those of SL against the susceptible microorganisms. However, these higher concentrations of MFAs are still less than the amount of commercial sucrose fatty acid esters used in foods (Ferrer et al., 2005) and the U.S. FDA-approved use level (FDA, 2018).

Additionally, M100L showed MIC values against all tested microorganisms while SL did not inhibit *L. plantarum, Z. bailii*, and *Z. rouxii*, and MBC values against *B. subtilis* and *L. lactis* were not obtained by SL but were obtained by M100L. This broader inhibitory activity of M100L against Gram-positive bacteria and yeast may support the hypothesis of the increased steric hindrance resulting in enhanced disruption of membranes by the larger

molecular size of maltodextrin than that of sucrose. The higher MIC and MBC values of M100L compared to SL may be due to other inhibitory actions independent of size. It is also possible that SL may form many small pores since it has a higher DS causing considerable disruption in the cell membrane. This suggestion may be supported by the lower MICs of SL than those of M100L in Gram-positive bacteria whose cell walls are generally highly permeable. The similarly lower MICs of SL in Gram-negative bacteria (*P. fluorescence* and *E. coli*) may be due to porin-facilitated diffusion of SL whose exclusion limitation by size is around 600 g/mol (Zgurskaya, Lopez, & Gnanakaran, 2015).

Table 4.3. Minimum inhibitory concentrations (MICs) and minimum bactericidal (or fungicidal) concentrations (MBCs or MFCs) of sucrose laurate (SL) and maltodextrin fatty acid esters against food-related microorganisms.

Microorganism	Type*	Antimicrobial activity (mg/mL)						
		SL	M100L	M100LP	M100P	M250L	M250LP	M250P
Bacillus subtilis	MIC	0.04	1.25	1.25	5	1.25	2.5	>5
	MBC	>5	1.25	2.5	>5	2.5	2.5	>5
Bacillus cereus	MIC	0.08	0.31	0.63	2.5	0.31	0.63	>5
	MBC	0.31	1.25	1.25	>5	1.25	1.25	>5
Streptococcus thermophilus	MIC	0.04	0.31	0.63	5	0.31	0.63	>5
	MBC	0.16	0.63	2.5	>5	0.63	2.5	>5
Lactococcus lactis	MIC	0.31	0.63	0.63	>5	0.63	0.63	>5
	MBC	>5	5	>5	>5	>5	>5	>5
Lactobacillus plantarum	MIC	>5	2.5	2.5	>5	2.5	>5	>5
	MBC	>5	>5	>5	>5	>5	>5	>5
Geobacillus stearothermophilus	MIC	0.01	0.08	0.08	2.5	0.16	0.31	5
	MBC	0.04	0.31	0.31	>5	0.63	0.63	>5
Listeria monocytogenes	MIC	0.08	0.16	0.31	2.5	0.31	0.31	>5
	MBC	>5	>5	>5	>5	>5	>5	>5
Escherichia coli	MIC	0.16	0.32	0.32	>5	0.63	0.63	>5
	MBC	>5	>5	>5	>5	>5	>5	>5
Pseudomonas fluorescens	MIC	0.16	5	5	>5	5	5	>5
	MBC	>5	>5	>5	>5	>5	>5	>5
Zygosaccharomyces bailii	MIC	>5	0.63	1.25	>5	0.63	1.25	>5
	MFC	>5	>5	>5	>5	>5	>5	>5
Zygosaccharomyces rouxii	MIC	>5	5	5	>5	5	>5	>5
	MFC	>5	>5	>5	>5	>5	>5	>5

*MIC was defined as the lowest concentration showing a statistically significant logarithmic reduction of CFUs by t-test against control and MBC (or MFC) were the lowest concentrations where at least a 3 logarithmic reduction in CFUs was obtained.
>5 means no MICs and MBC (or MFC) was obtained in the range of lower than 5 mg/mL.

The microbial inhibitory properties of MFAs and SL showed a trend regarding microbial type. In general, MFAs and SL showed MBC values against most Gram-positive bacteria with no MBCs against Gram-negative bacteria and yeast. Therefore, MFAs may be more effective against Gram-positive than Gram-negative bacteria and yeast. Previous research using different CFAs (Habulin et al., 2008; Shao et al., 2018; Zhao et al., 2015) reported that Gram-positive bacteria were generally more susceptible than Gram-negative bacteria. This may be due to the difference in cell wall permeability against inhibitory compounds reaching the sensitive inner membrane (Gram-positive bacteria are more permeable than yeast and Gram-negative bacteria) (Anarjan & Tan, 2013). The different vulnerability between Gram-positive bacteria was explained by the different cell wall structures and other mechanisms dependent on species or strain type, including membrane stabilization (Desbois et al., 2010).

Morphological analysis of microbial cells by SEM

The visual demonstration of membrane damage was conducted by analyzing the electron micrographs of cells. The cellular morphologies of *B. subtilis, G. stearothermophilus, L. plantarum*, and *Z. bailii* untreated or treated with M100L or SL are shown in Fig. 4.3. Smooth rod-shaped cells were detected in untreated B. cereus (Fig. 4.3a), but cell damage, including cell rupture, is shown in *B. cereus* treated with SL (Fig. 4.3b) and M100L (Fig. 4.3c). The morphologies of *G. stearothermophilus* were intact rod-shaped (Fig. 4.3d) but showed distorted and cracked surfaces after treatment with SL (Fig. 4.3e) or M100L (Fig. 4.3f).

Long and smooth rod cells of *L. plantarum* were detected in untreated cells (Fig. 4.3g) and treated with SL (Fig. 4.3h). However, membrane wrinkling and deformation with

splits were in the *L. plantarum* cells treated with M100L (Fig. 4.3i). The smooth oval shape cells of *Z. bailii* were observed in the cells untreated (Fig. 4.3j) and treated with SL (Fig. 4.3k). However, uneven broken cells with rough surfaces were detected after being treated with M100L (Fig. 4.3l).

Even though cell viability is difficult to discern via SEM (Bogosian & Bourneuf, 2001), SEM provides essential information, including microbial morphology, cell numbers, and cell distribution (Kenzaka & Tani, 2012). The changes in the bacterial surface can be from either bacteriostatic (growth inhibition) or bactericidal activity (cell death) from CFAs (Zhao et al., 2015), suggesting the damaged cells may be dead or only compromised. The visual information by SEM can be helpful to study the effect of treatments on cell morphology.

M100L and SL produced morphological changes such as irregular surfaces and cavities against all susceptible microbial cells tested. In other words, the morphological changes happened only in the cases of growth inhibition. From the results and discussion above, it is proposed that the inhibitory action of MFAs is via attacking the cell membrane leading to a change in cellular morphologies.

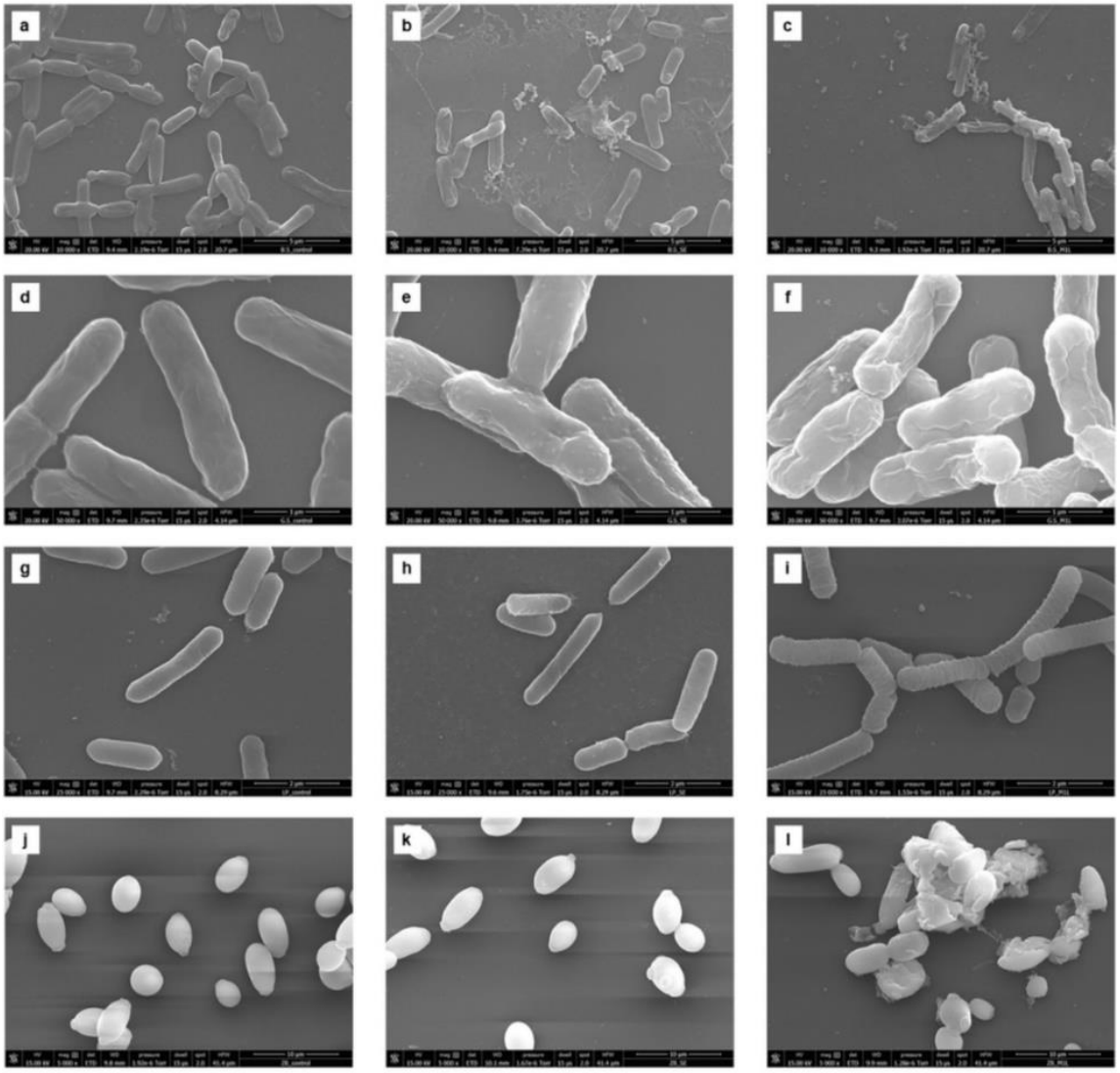

Fig. 4.3. Scanning electron microscope (SEM) images of *Bacillus subtilis*: (a) untreated, (b) treated with sucrose laurate at 0.04 mg/mL (minimum inhibitory concentration (MIC)), and (c) treated with M100L at 1.25 mg/mL (MIC), of *Geobacillus stearothermophilus*: (d) untreated, (e) treated with sucrose laurate at 0.01 mg/mL (MIC), and (f) treated with M100L at 0.08 mg/mL (MIC), of *Lactobacillus plantarum*: (g) untreated, (h) treated with sucrose laurate at 5 mg/mL (no MIC), and (i) treated with M100L at 2.5 mg/mL (MIC), and of *Zygosaccharomyces bailii*: (j) untreated, (k) treated with sucrose laurate at 5 mg/mL (no MIC), and (l) treated with M100L at 1.25 mg/mL (MIC).

Analysis of extracellular proteins released

Proteins are important bacterial cell constituents, and their presence in a suspension may be an indicator of cell membrane damage. Thus, the cell membrane's integrity was determined by measuring the extracellular proteins in suspension from the microorganisms treated with M100L or SL (the concentrations treated were at MIC level or 5 mg/mL if no MICs were obtained), and the values are shown in Table 4.4.

We expected to find an increase in extracellular proteins from *B. subtilis, G. stearothermophilus, L. plantarum*, and *Z. bailii* treated with M100L and from *B. subtilis* and *G. stearothermophilus* treated with SL. This was the case except for *G. stearothermophilus*, which showed no increase in extracellular protein with either treatment.

To date, various techniques have been used to study microbial cell membrane permeability, including a protein leakage assay (Zhao, Zhang, & Yang, 2017), epifluorescence microscopy (Zhao, Zhao, Phey, & Yang, 2019), and a potassium ion leakage assay (Duan et al., 2020). These previous studies using antimicrobial compounds showed an increased cell leakage which corresponded to morphological changes in microbes detected by microscopic techniques, which is similar to the results in this research

These current and previous results suggest that the specific inhibitory mode of action may be microbe-specific, but primary inhibition may occur via attacking the microbial cell membrane by the fatty acyl moiety, leading to increased cellular component leakage due to the loss of membrane integrity (Zhao et al., 2015) with other possible mechanisms, including inhibition of membrane enzymatic activities and disruption of energy production (Desbois et al., 2010; Yoon et al., 2018). Additionally, Zhao et al. (2019)

suggested the effect of low pH and high oxidation-reduction potential on fatty acid oxidation in the membrane leading to enhanced cell leakage. However, this explanation may not apply to our results since MFAs cannot significantly change the pH and oxidation-reduction potential.

Table 4.4. Effect of maltodextrin fatty acid esters and sucrose laurate (SL) on the concentrations of extracellular proteins.

Bacillus subtilis		*Geobacillus stearothermophilus*		*Lactobacillus plantarum*		*Zygosaccharomyces bailii*	
Treatment*	Protein (μg/mL)	Treatment*	Protein (μg/mL)	Treatment*	Protein (μg/mL)	Treatment*	Protein (μg/mL)
Control	22.6 ± 0.3[c]	Control	53.0 ± 0.5[a]	Control	34.4 ± 5.2[b]	Control	14.5 ± 1.0[b]
SL (0.04 mg/mL - MIC)	27.1 ± 0.2[b]	SL (0.01 mg/mL-MIC)	49.3 ± 0.3[b]	SL (5 mg/mL)	25.1 ± 2.6[c]	SL (5 mg/mL)	14.5 ± 0.1[b]
M100L (1.25 mg/mL - MIC)	29.4 ± 0.3[a]	M100L (0.08 mg/mL-MIC)	53.7 ± 0.3[a]	M100L (2.5 mg/mL-MIC)	45.9 ± 1.0[a]	M100L (0.63 mg/mL-MIC)	18.3 ± 0.8[a]

[a,b,c,] means with the same letter were not significantly different within each column ($p<0.05$).
* Concentrations at MIC levels or 5 mg/mL was used in the case of no MICs.

Combining our current results of growth inhibition, SEM, and concentration of extracellular proteins with previous studies, we suggested that the antimicrobial activities of MFAs may arise from the general assault on their cell membranes, but the precise mechanisms resulting in altered cellular morphologies may be diverse and depend on the specific microorganisms.

Conclusions

This research investigated the microbial inhibitory properties of 6 different MFAs synthesized by lipase-catalyzed esterification against 11 food-related microorganisms, including spoilage bacteria (Gram-positive and Gram-negative) and yeast. In terms of structure and inhibition relationship, lower DE (larger size) of maltodextrin (M100) and shorter fatty acid (laurate) esterified showed the highest microbial inhibitory activity among the MFAs. This is the first study investigating the microbial inhibitory abilities of MFAs in terms of the DE of the carbohydrate moiety. M100L showed broader inhibition than SL by inhibiting all microbes tested, while SL did not inhibit 3 microorganisms (*L. plantarum, Z. bailii*, and *Z. rouxii*). M100L also exhibited 2 additional MBC values than SL (against *B. subtilis* and *L. lactis*). Gram-positive bacteria were more susceptible to MFAs than Gram-negative bacteria and yeasts, and similar results were shared with SL. The morphological alterations detected by SEM occurred in the cells inhibited by M100L and SL, but concentrations of extracellular proteins released from cells did not correspond directly to microbial inhibition. Lower DE maltodextrin esterified with laurate can be used as microbial inhibitory agents with broader inhibitory properties compared to commercial SL for decreasing spoilage in foods that are vulnerable to *B. subtilis, L. plantarum, Z. bailii*, and *Z. rouxii.*

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CHAPTER V

INFLUENCE OF DIFFERENT FOOD MODELS AND STORAGE TEMPERATURES ON THE BACTERIAL INHIBITION BY MALTODEXTRIN LAURATE AND SUCROSE LAURATE AND INVESTIGATION OF THEIR CYTOTOXICITY

Abstract

Maltodextrin esterified to laurate (ML) was synthesized enzymatically and compared to commercial sucrose laurate (SL) for microbial inhibitory properties in food systems. There was broad microbial inhibition by ML in growth media, but practical information about various foods and storage temperatures is limited with no toxicological information. This research investigated the antimicrobial property of ML against two spoilage bacteria (*Bacillus subtilis* and *Lactobacillus plantarum*) in growth media and related food models stored at different temperatures (4°C, 22°C, and 35°C). Its inhibitory property was evaluated compared to commercial SL. Additionally, in vitro cytotoxicity of ML was investigated against two colonic cells using the MTT assay. ML was highly inhibitive against *L. plantarum* in apple juice at all temperatures tested. SL could inhibit both bacteria in the food models tested (milk, apple juice, tomato ketchup, French dressing) at 4°C. In cytotoxicity assay, ML (up to 500 µg/mL) did not show toxicity against two human colonic cells, while SL was toxic to both cells. In conclusion, ML can be an alternative to commercial SL as a food additive in certain products.

Introduction

Food spoilage indicates consumers' undesirable or unacceptable food status by any sensory property changes (Abdel-Aziz, Asker, Keera, & Mahmoud, 2016). Because foods are susceptible to many different changes, preservatives such as antimicrobial agents in food processing have become one of the current preserving techniques (Bensid, El Abed, Houicher, Regenstein, & Özogul, 2020). Two Gram-positive bacteria closely related to food spoilage were selected for this research

Bacillus subtilis can initiate the sporulation process under nutrient restriction conditions, and these endospores are more resistant than vegetative cells to common inactivation and disinfection treatments (Cho & Chung, 2020). Previous research frequently identified *B. subtilis* group with the *B. cereus* from the dairy processing environments and products (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013). Lactic acid bacteria have been widely studied for their probiotic properties, but they are also considered challenging spoilage microorganisms in food industries (Ramírez, Smid, Abee, & Groot, 2015). *L. plantarum*, in particular, can cause the spoilage of acidified products vulnerable to contamination by the repeated use despite low pH (Sanders, Oomes, Membré, Wegkamp, & Wels, 2015).

An antimicrobial agent is a natural or synthetic substance that kills or inhibits bacteria, fungi, and/or algae (McCarthy, 2011). Various chemically synthesized compounds, including salts and organic acids, have officially been recognized and used as food antimicrobials (Kalpana & Rajeswari, 2019). In the meantime, natural products have gained popularity, but most of their antimicrobial activities are considered not sufficient for practical use (AlFindee et al., 2018). As a result, there is still a need for effective and

safe antimicrobial food additives

Carbohydrate fatty acid esters (CFAs) are simple- or polysaccharides attached to fatty acids by the esterification reaction. They are known to be biodegradable and non-toxic with antimicrobial activity, which is of interest (AlFindee et al., 2018; Nobmann, Bourke, Dunne, & Henehan, 2010). Chemical or enzymatical methods can synthesize these compounds, and there is growing interest in the application of enzymatic synthesis due to its advantages, such as the regioselectivity and mild reaction conditions (Chang & Shaw, 2009).

Sucrose has been a common substrate for CFA synthesis because of its advantage in the industry (e.g., price) (Neta et al., 2015). Sucrose fatty acid esters (SFAs) have been well studied and are allowed by the U.S. Food and Drug Administration to be added to certain processed foods (Zhang, Song, Taxipalati, Wei, & Feng, 2014) with broad applications in commercial beverages in Japan (Zhao, Zhang, Hao, & Li, 2015).

They are known to be extensively hydrolyzed in the gastrointestinal tract, and most of the intact form is excreted, resulting in a small amount of absorption with no toxicological effect (Additives & Food, 2010). However, many commercial SFAs, except sucrose laurate (SL), may have insufficient solubility in aqueous environments (Nagai et al., 2016), resulting in limited applications.

In previous research, maltodextrins enzymatically synthesized showed highly aqueous solubility due to their large hydrophilicity even after esterification with fatty acids (Park & Walsh, 2019). This study indicated that MFAs with a limited number of fatty acids attached resulted in good aqueous solubility at a practical use level (1% w/v) (Park & Walsh, 2019), comparable to commercial SL.

To date, there has been a limited number of studies investigating the enzymatic esterification of maltodextrin (Udomrati & Gohtani, 2014), and among them, very few analyzed their microbial inhibitory properties (Pantoa, Shompoosang, Ploypetchara, Gohtani, & Udomrati, 2019; Park & Walsh, 2021). Investigating the microbial inhibitory activities of maltodextrin fatty acid esters (MFAs) regarding different food models and storage temperatures has never been reported to the best of our knowledge. Also, the toxicological information about these compounds is lacking, even though they are considered non-toxic.

Here, the microbial inhibitory property of maltodextrin laurate (ML), a type of MFAs, was tested in various food models and storage temperatures compared to commercial SL, and their inhibition was evaluated according to the different conditions. Additionally, the in vitro cytotoxicity of ML was investigated using the intestinal cells assessing ML as a food ingredient in comparison with commercial SL.

Materials and methods

Materials

Immobilized lipase from *Thermomyces lanuginose* (10000 tributyrin unit (TBU)/g; 1 TBU = 1 μmol butyric acid released per minute/g immobilized enzyme at 40ºC and pH 7.5) was obtained from Chiral Vision (Netherlands). Molecular sieves (3Å) (Sigma-Aldrich, St. Louis, MO) and vinyl laurate (TCI America, Portland, OR) were used. Pyrex glass bottles with polypropylene caps (Corning Glass Works, Corning, NY) and magnetic stir bars (Bel-Art Products, Pequannock, NJ) were used. Ethanol (HPLC grade, Pharmco Products Inc., Brookfield, CT), hydrophilic polyvinylidene fluoride (PVDF) syringe filters (0.2 μm)

(Foxx Life Sciences, Salem, NH), and microtiter well plates (48 wells) (Becton Dickinson, NJ) were used. Glass microfiber filters (1 μm) and nylon membrane filter (47 mm and 0.2 μm) were from Whatman International Ltd., Maidstone, UK. Sucrose laurate (Ryoto L-1695) was from Mitsubishi-Kagaku (Tokyo, Japan). Maltrin M100 (dextrose equivalent (DE) 9 - 12) was obtained from Grain Processing Corporation (Muscatine, IA). Polypropylene centrifuge tubes (15 mL and 50 mL) and polystyrene semi-micro cuvettes (VWR, Darmstadt, Germany) were used. Luria-Bertani (LB) broth and glucose were obtained from Sigma Aldrich (St. Louis, MO). De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) and agar (VWR, Darmstadt, Germany) were used. Shelf-stable low-fat (1% fat) and whole milk (3.5% fat) (Gossner Foods Inc. Logan, UT), tomato puree (Conagra Brands, Chicago, IL), salt (Morton Salt Inc, Chicago, IL), and vegetable oil (Western Family Foods, Tigard, OR) were purchased from a local grocery market. Commercial distilled white vinegar, syrup, and granulated sugar of Great value (Wal-Mart Stores, Inc., Bentonville, AR) were also from a local grocery market. Starch (Alfa Aesar, Haverhill, MA) and milk protein concentrate (MPC) 70 (Darigold, Seattle, Washington) were used. McCoy’s 5A medium, fetal bovine serum (FBS), Eagle's Minimum Essential Medium (EMEM), Trypsin-EDTA Solution, Dulbecco's Phosphate Buffered Saline (D-PBS) were obtained from ATCC (Manassas, VA). DMSO, cell culture flask with vent cap (75.0 cm2), and 0.2 μm syringe filter (PES membrane) were from VWR (Radnor, PA). 96-well cell culture-treated plates were obtained from Fisher Scientific (Waltham, MA).

Preparation of ML

ML was prepared according to a previously reported method (Park et al., 2019). Two grams of maltodextrin and 280 mg of vinyl laurate were mixed with 160 mL of ethanol in a 250

mL glass bottle, followed by adding 4.0 g of molecular sieves and 2.4 g of immobilized lipase. The molar ratio between maltodextrin and vinyl laurate was 1:0.1 (mole of anhydrous glucose (162 g)/mole of vinyl laurate). The reactions were conducted in a shaker (Eppendorf, Edison, NY, USA) at 500 rpm and 55˚C for 8 days with daily addition of 2.4 g of immobilized lipase and 4.0 g of molecular sieves on day 2 to 7. In total, 5 reactions in different bottles were conducted at once.

After the reaction, each bottle's mixture was filtered by a glass microfiber filter, and the whole filtrate was combined. The removed lipase and molecular sieves in each bottle were washed and filtered using 100 mL of ethanol with the same filter twice, and the resulting solution was added to the original filtrate. This final filtrate from whole reactions was dried in a fume hood at 22°C. The dried filtrate was resuspended in 1 L of distilled water followed by mixing with 1 L of hexane to remove the unreacted fatty acids (aqueous solubility of vinyl laurate is 0.4868 mg/L (The Good Scents Company, 2018). After complete phase separation, the final aqueous phase was freeze-dried in a benchtop 5 L freeze drier (manifold type, Virtis Company, Inc., Gardiner, NY) at 6.7 Pa for 2 days. Stock solution (50 mg/mL) was prepared with 95% ethanol in water and kept at -20°C. The final ML was characterized for the degree of substitution (DS) (moles of fatty acid/moles of glucose unit) and purity (%) via NMR and HPLC methods (Park & Walsh, 2021), and the information is given in table 5.1.

Table 5.1. The characteristics of maltodextrin laurate used in this research.

Name	Carbohydrate group (Dextrose equivalent)	Fatty acyl group (Chain length)	Degree of substitution (moles of fatty acid / moles of glucose unit)	Purity by HPLC (%)
ML	9 – 12	laurate (C12)	0.026 ± 0.001 (Park & Walsh, 2021)	99.6 ± 0.3 (Park & Walsh, 2021)

Bacterial preparation for inhibitory analysis

Two spoilage gram-positive bacteria were used, including *Bacillus subtilis* and *Lactobacillus plantarum*. The growth media and temperatures for the tested bacteria are given in Table 5.2.

Table 5.2. The bacterial strains in this study and their information related.

Name	Strain	Type	Growth condition		Related foods
			Media	Temperature	
Bacillus subtilis	8G5	Gram-positive	LB*	37°C	Dairy foods (Lücking et al., 2013)
Lactobacillus plantarum	ATCC 4008	Gram-positive	MRS*	37°C	Acidified foods (Sanders et al., 2015)

*LB is for Luria-Bertani, and MRS indicates De Man, Rogosa, and Sharpe.

Frozen glycerol stocks (20%, v/v) of each bacterium were prepared and maintained at -80°C before the inhibitory assay. Each frozen stock aliquot (200 µL) was moved into 10 mL of species-specific media (Table 5.2) and grown at 37°C and 400 rpm for 24 h. One mL of the grown culture was sub-cultured in 10 mL of each fresh media and was then grown for 4 h at 37°C and 400 rpm. This secondary culture was measured at 600 nm using a UV-vis spectrophotometer (Biospec 1601, Shimadzu, Japan) and diluted with the

same media used to reach OD_{600} of 0.2, which corresponds to around 6 logs colony-forming unit (CFU)/mL as determined by plate counts. This diluted secondary culture was used for inhibitory assays.

Preparation of food models

Milk model

Two different types of milk (whole [3.5%) and low fat [1%]) were select. Before bacterial inhibitory analysis, milk's sterility was checked according to previous methods, including the aerobic and psychrotrophic plate counts (Chen & Godwin, 2006) with modifications. Undiluted milk (50 μL) was spread on standard method agar (known as plate count agar) containing 5 g/L of tryptone, 2.5 g/L of yeast extract, 1 g/L of glucose, and 1.5 g/L of agar, and plates were incubated at 7°C for 2 weeks for the psychrotrophic assay or at 35°C for 2 days for total aerobic assay. After the incubation, no microbial contamination was confirmed, and milk was used for the bacterial assay.

Apple juice model

Apple juice was prepared by the previous method (Raybaudi-Massilia, Mosqueda-Melgar, & Martín Belloso, 2009) with modifications. Each apple was washed, peeled, and cut into small pieces. These apples were blended using a blender on the pulse setting (Totalbelnder, Blendtec, Orem, UT) for 10 min. After centrifugation at 24336 × g and 4ºC for 30 min using Sorvall RC 5B centrifuge (Dupont Instruments, Wilmington, DE) with a rotor (SLA-3000, Thermo Scientific, Rockford, IL), the supernatant was filtered using a glass microfiber filter and autoclaved at 121ºC for 20 min. The pH of the juice was 3.91±0.01. T The Brix (%) of final juice was 13.1 ± 0.1 by an optical Brix refractometer (Model

300001, Sper Scientific, Scottsdale, AZ). The final juice was stored at 4°C for further as say.

Tomato ketchup model

According to previously reported methods, Tomato ketchup was prepared (Belović et al., 2018; Cai, Du, Zhu, Cai, & Cao, 2020) with modifications. Tomato puree (500 g) was put into a glass bottle with an addition of 1 L of distilled water, 240 g of sugar, 120 g of syrup, 20 g of salt, and 50 g of starch. The mixture was stirred at 400 rpm and heated at 100°C until its Brix (%) reached 23. After autoclaving at 121°C for 20 min and cooling to ro om temperature, 20 g of vinegar pre-sterilized using 0.2 µm PVDF syringe filter was aseptically added to the mixture. The final tomato ketchup's pH and Brix (%) were measured using aliquots as 3.80 ± 0.01 and 23.1 ± 0.1. The final tomato ketchup was stored at 4°C for further assay.

French dressing model

French dressing was prepared according to the previous method (Moghaddam, Mizani, Salehifar, & Gerami, 2013) with modification. An aqueous part in a glass bottle was made from 180 g of distilled water and 16 g of MPC 70, followed by autoclaving at 121°C for 20 min. After cooling down, 64 g of pre-sterilized vinegar using a 0.2 µm hydrophilic PVDF syringe filter was added. While the aqueous part was prepared, vegetable oil was sterilized by dry heat at 180°C for 1 hour according to a previous method (Kupiec, Matthews, & Ahmad, 2000). The aqueous part and 140 g of vegetable oil were homogenized at 20,000 rpm for 3 min by a homogenizer (Ultra-Turrax T25, Janke and Kunkel, Staufen, Germany) equipped with a sterilized dispersion tool by an autoclave at

121°C for 15 min.

The pH of the final French dressing was measured as 3.99 ± 0.01. The addition of vinegar and oil and the homogenization were aseptically conducted. The final French dressing was stored at 4°C and stirred at 400 rpm and an ambient temperature for 30 min before the inhibitory analysis.

Bacterial inhibitory assay

Inhibitory analysis in growth media

The bacterial inhibitory analysis was conducted according to ((Zhao et al., 2015) with modifications. The sterile ML and SL were prepared by filtration of diluted stock solution (25 mg/ml) in 90% ethanol using 0.2 μm hydrophilic PVDF syringe filter, followed by dry using a laminar flow cabinet (Class II A/B3 Biological Safety Cabinet, Forma Scientific, Inc., Waltham, MA). Dried samples were aseptically added to different growth media whose pH was adjusted to 6.4 for *B. subtilis* and 4.0 for *L. plantarum*, and 10 mg/ml of ML or SL were prepared. Pure growth media were used as controls.

An aliquot (250 μL) of solutions with the same volume of secondary bacterial culture was placed into each well of a microtiter well plate, making the final concentration 5 mg/ml. After incubation for 1 day at 3 different storage temperatures (4, 22, and 35°C), serial dilutions were conducted using 1 M phosphate buffer solution (PBS), and 100 μl of the solution was spread on agar plates and incubated at 37°C (1 – 2 days for *B. subtilis* and 2 – 3 days for *L. plantarum*), followed by the enumeration of CFUs. All of the steps were aseptically conducted.

Inhibitory analysis in food models

A bacterial assay using different food models was conducted according to the previous method (Monu, Techathuvanan, Wallis, Critzer, & Davidson, 2016) with modifications. Sterile ML and SL were prepared using the same method above and used to make food models containing 10 mg/ml of ML or SL, except 10 mg/g for tomato ketchup due to its high viscosity. Then, each food model containing bacterial cells (around 6 logs CFU/mL or g) was prepared by adding the cells of the secondary culture, which were centrifuged at 3500 × g for 10 min (IEC Clinical centrifuge, Danon/IEC Division, Needham Heights, MA, USA) and washed using 1 M PBS twice.

Five mL (or g) of each food containing treatment was placed in a sterile 50 mL tube. Each food model, excluding ML or SL, was used as the control. The same aliquot (5 mL) of each food containing bacteria was mixed with each food model containing treatments in a 50 mL tube, making the final concentration 5 mg/ml or /g. The mixtures in tubes were stored for 7 days at different temperatures (4°C, 22°C, and 35°C).

An aliquot (100 μL) of each sample (or 500 mg for tomato ketchup) was collected at day 0, 1, 3, 5, and 7, and they were spread on agar plates after serial dilutions with 1 M PBS. After the incubation at 37°C (1 – 2 days for *B. subtilis* and 2 – 3 days for *L. plantarum*), the CFUs were calculated. All of the steps for inhibitory analysis were aseptically conducted.

pH measurement

The pH of different growth media and food models prepared was determined before the inhibitory assay using a pH meter (Orion 3-star pH meter, Thermo Fisher Scientific, Waltham, MA, USA). After inoculation of *L. plantarum*, 2 mL of each acidified food

containing 5 mg/ml of ML or SL and control was collected on day 0, 1, and 7 over storage at different temperatures (4°C, 22°C, and 35°C), and the pH was measured.

Cell culture growth and preparation

HT-29 (ATCC HTB-38) and CCD 841 CoN (ATCC CRL-1790) were used. Each cell was cultured at 37°C with 5% CO_2 in McCoy's 5A media or EMEM with 10% (v/v) FBS supplementation for both. The cells were fed every 3 days with fresh media and subcultured in culture flasks. For the cytotoxicity assay, cells were detached by adding 3 mL of Trypsin−EDTA solution for 5 min and then neutralized with 8 mL of fresh media. The detached cells were counted using an automated cell counter (Countess, Invitrogen, Carlsbad, CA) and used for cytotoxicity assay after proper dilution with fresh media.

Cytotoxicity assay

Cytotoxicity was analyzed by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as previously described (Popova, Lazarus, Benninghoff, & Berreau, 2020) with modifications. The fresh cells (190 µL) were seeded in 96-well plates at around 10^4 cells/well and allowed to settle to the bottom for 20 h. Different concentrations of ML or SL were prepared in cell-specific media with 0.2 µm syringe filter sterilization, and they (10 µL) were added to the wells. The final concentrations of ML or SL range from 1 to 500 µg/m. The media and DMSO were used as controls. The plates, including cells and treatments, were incubated for 24 h.

Fresh MTT solution was prepared at 5 mg/mL in sterile PBS and filtered through a 0.2 µm syringe filter. This solution (20 µL) was added to each well and incubated for an additional 3 h. After removing the media of each well, the metabolized formazan pellets

were solubilized by adding 200 μL of DMSO, and the absorption was measured by a Modulus Microplate reader (Turner Biosystems, Sunnyvale, CA) at 560 nm with 750 nm for background. Results are expressed as percentage viability compared with the media control. IC_{50} values were calculated using a nonlinear regression where at least a 50% reduction of cell viability was observed. For each cell line, three independent biological experiments with three technical repetitions of each were performed.

Statistical analysis

All experiments were replicated at least two times, but the inhibitory experiments were repeated twice for each bacterial culture, where two different cultures were used for each bacterium. Results were expressed as mean ± standard deviation. Each food's pH data were analyzed by three-way ANOVA using PROC GLM (fixed factors: treatment, temperature, and day) followed by post hoc analysis using Simulation method (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). Inhibitory results in media were analyzed by two-way ANOVA using PROC Mixed (random factor: bacterial culture and fixed factors: treatment and temperature) followed by post hoc analysis using the Simulation method. Food model data were analyzed by three-way ANOVA using PROC Mixed (random factor: bacterial culture and fixed factors: treatment, temperature, and day) followed by post hoc analysis using the Simulation method. The significant difference was calculated according to $p<0.05$.

Results and discussion

ML was enzymatically synthesized from maltodextrin and vinyl laurate, and after purification, the final ML showed 0.026 of DS and 99.6% purity (Park & Walsh, 2021)

(Table 5.1). The other compound in this research was commercial SL consisting of 80% monoester with 20% di, tri, and polyesters. (Husband, Sarney, Barnard, & Wilde, 1998), suggesting approximately 0.5 of DS.

A compound's characteristics, such as DS and composition, can influence the synthesized compound's physical and antimicrobial properties (Fang, Fowler, Tomkinson, & Hill, 2002; Ferrer et al., 2005). The primary difference between ML and SL is their different carbohydrate moieties and the extent of DS, and these differences can be used to evaluate different inhibitory properties of ML or SL according to different foods and storage temperatures.

ML's or SL's inhibition against *B. subtilis* in media for 1 day is shown in Fig. 5.1a. In control, bacterial numbers increased at 22°C and 35°C but decreased at 4°C, while these differences were not significant ($p>0.05$) (Fig. 5.1a). The growth temperature of *B. subtilis* ranges from 11°C to 52°C (Budde, Steil, Scharf, Völker, & Bremer, 2006) corresponding to the current results. Even though *Bacillus* spores could survive at low temperatures based on the previous information (Deng, Plaza-Garrido, Torres, & Paredes-Sabja, 2015), *B. subtilis* cells were suppressed at 4°C in a previous study (Satapute et al., 2012), supporting the reduction of *B. subtilis* in media at 4°C.

Inhibition of *B. subtilis* in media by ML was significant at all temperatures compared to the initial inoculation and controls at 22°C and 35°C ($p<0.05$) (Fig. 5.1a). However, SL showed significant inhibition at only 4°C and 22°C, considering the initial inoculation and controls at each temperature ($p<0.05$) (Fig. 5.1a). ML's inhibition was higher at 22°C and 35°C than 4°C, but SL showed the highest inhibition at 4°C with gradually reduced inhibition at higher temperatures.

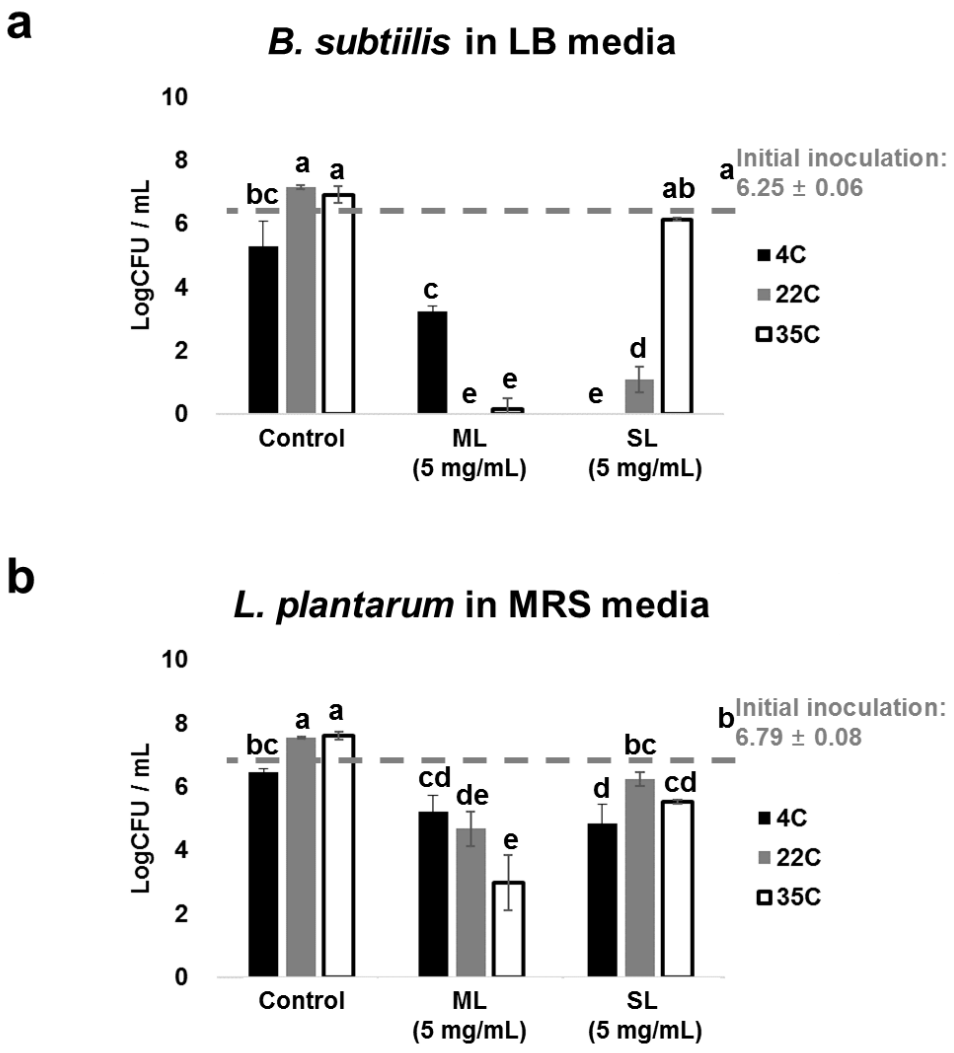

Fig. 5.1. The inhibition of (a) *Bacillus subtilis* and (b) *Lactobacillus plantarum* in growth media by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) after incubation (1 day) at 3 different temperatures. Different letters indicate the significant difference within each bacterium ($p<0.05$).

The microbial inhibition by both ML and SL as fatty acid derivatives is considered to target cell membranes. This attacking may induce various specific activities, including (1) the destabilization of the membrane, (2) the disruption of energy production, and (3) the inhibition of membrane-associated enzymes, leading to cell inhibition or death (B. K.

Yoon, Jackman, Valle-González, & Cho, 2018).

For understanding ML's and SL's temperature dependence, the temperature effect was investigated. The environmental temperature may affect bacteria's susceptibility by changing the membrane physical stability between a rigid gel structure by closely packed phospholipids at lower temperatures and a disordered fluid phase at higher temperatures. (Aronsson & Rönner, 2001).

Considering this temperature-dependent membrane transition, ML's and SL's distinct temperature-dependent inhibition may suggest different specific inhibitory mechanisms. ML's primary action may destabilize the membrane because of the enhanced inhibitory levels at higher temperatures where membranes are fluid.

In contrast, SL's larger inhibition at lower temperatures where membranes are closely packed may suggest that this compound mainly interacts not with cell membranes but with the cell membrane-related components, leading to the loss of vital functions and cell inhibition. The smaller carbohydrate moiety of SL than ML may be beneficial when fatty acyl moiety interacts with cell membrane-associated components. This interaction may be supported by the fact that even at low temperatures, the lipids surrounding the membrane's proteins are reported to remain liquid-crystalline (fluid) state (Denich, Beaudette, Lee, & Trevors, 2003).

Secondly, the temperature may directly affect the status of compounds. Unlike SL's sucrose moiety, ML's maltodextrin moiety consists of glucose polymers and tends to retrograde, (Dokic, Jakovljevic, & Dokic-Baucal, 1998). That process is induced at low temperatures, meaning reassociation of molecules, which can inhibit molecular mobility, indicating a less inhibitory activity towards cell membranes.

The increased association between ML may happen at lower temperatures, interfering with ML's interaction with cell membranes. These multiple hypotheses for the different temperature-dependence of ML and SL were suggested based on the possible temperature effects and must be further studied with the proper experiments.

The number of *B. subtilis* cells in low-fat milk containing ML or SL at different storage temperatures over 7 days is given in Fig. 5.2. At 22°C and 35°C, the cells in the low-fat milk maintained a high level without significant difference ($p>0.05$) (Fig. 5.2b and c). However, at 4°C the cells in the milk control gradually decreased, becoming significant at day 3 ($p<0.05$) (Fig. 5.2a). This gradual reduction of *B. subtilis* cells in low-fat milk at 4°C is assumed due to the inhibitory effect by low temperature, similar to the result using growth media (Fig. 5.1a).

ML in low-fat milk showed significant inhibition at 4°C and 22°C, while SL significantly inhibited *B. subtilis* cells at all temperatures ($p<0.05$) (Fig. 5.2), suggesting SL's higher inhibitory activity than ML in this model. The reduction levels by both compounds were high at 4°C (Fig. 5.2). Low temperature can retard microbial growth by affecting biochemical reactions and internal cellular changes (Sperber, 2009). These alterations may be related to ML's and SL's higher inhibition in cold storage.

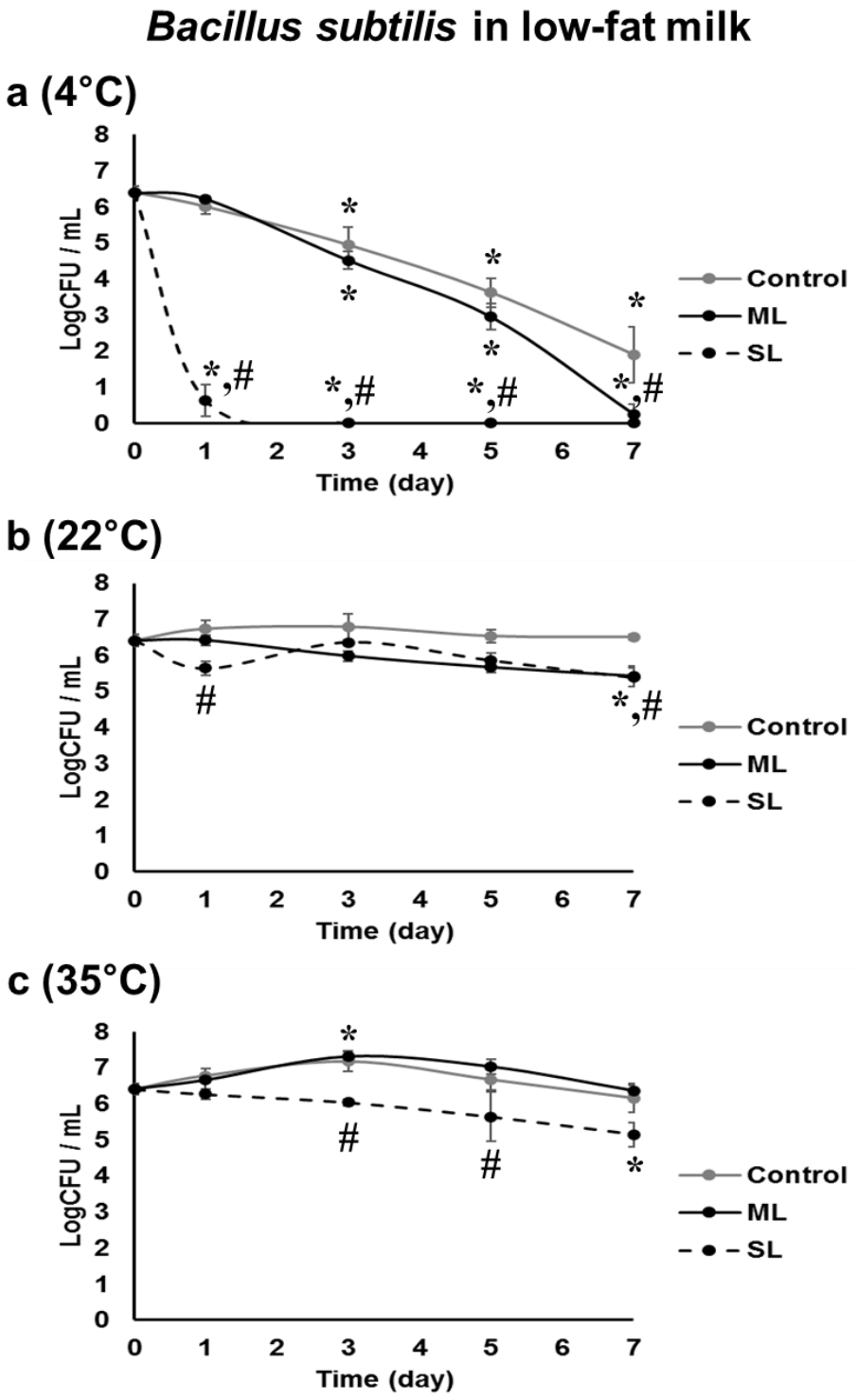

Fig. 5.2. The inhibition of *Bacillus subtilis* in low-fat milk by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) during storage at 3 different temperatures (a: 4°C, b: 22°C, and c: 35°C). * indicates the significant difference from the initial inoculum ($p<0.05$). # indicates the significant difference from the control on the same day ($p<0.05$).

Compared to *B. subtilis* cells in media, cell inhibition by ML or SL decreased in low-fat milk. Fat globules in low-fat milk may interact with ML's or SL's hydrophobic fatty acyl moiety (Y. Chen, Nummer, & Walsh, 2014; Lee, Nummer, Nummer, Wagh, & Walsh, 2019). That may partition the compounds into the fat phase in food and sort them out from cell membranes where the proposed primary target site is. Notably, ML's inhibitory activity decreased more than SL against *B. subtilis* in low-fat milk. The number of inhibitory fatty acyl moieties is related to DS. The DS (0.026) for ML is smaller than for SL (approximately 0.5), proposing much fewer ML residues than SL, which were not partitioned into the fat phase.

Even though the inhibition by compounds against *B. subtilis* was reduced in low-fat milk compared to in LB media, ML in low-fat milk lost its inhibitory activity during storage at 35°C. The one end of the maltodextrin moiety of ML contains a reducing carbohydrate that can participate in the Maillard reaction with an amino protein group contrary to sucrose (non-reducing sugar). The reaction can cause different functional properties according to reaction conditions (Martinez-Alvarenga et al., 2014), suggesting the possible negative effect on inhibitory activity.

In whole milk, *B. subtilis* maintained high cell numbers at 22°C and 35°C with a gradual reduction at 4°C (Fig. 5.3). Bacterial inhibition by ML or SL was higher at a lower temperature (Fig. 5.3). These general growth and inhibitory patterns for *B. subtilis* cells were similar to the data in low-fat milk. Instead, both compounds' overall inhibition in whole milk decreased than in low-fat milk, supporting the suggested interference by fat.

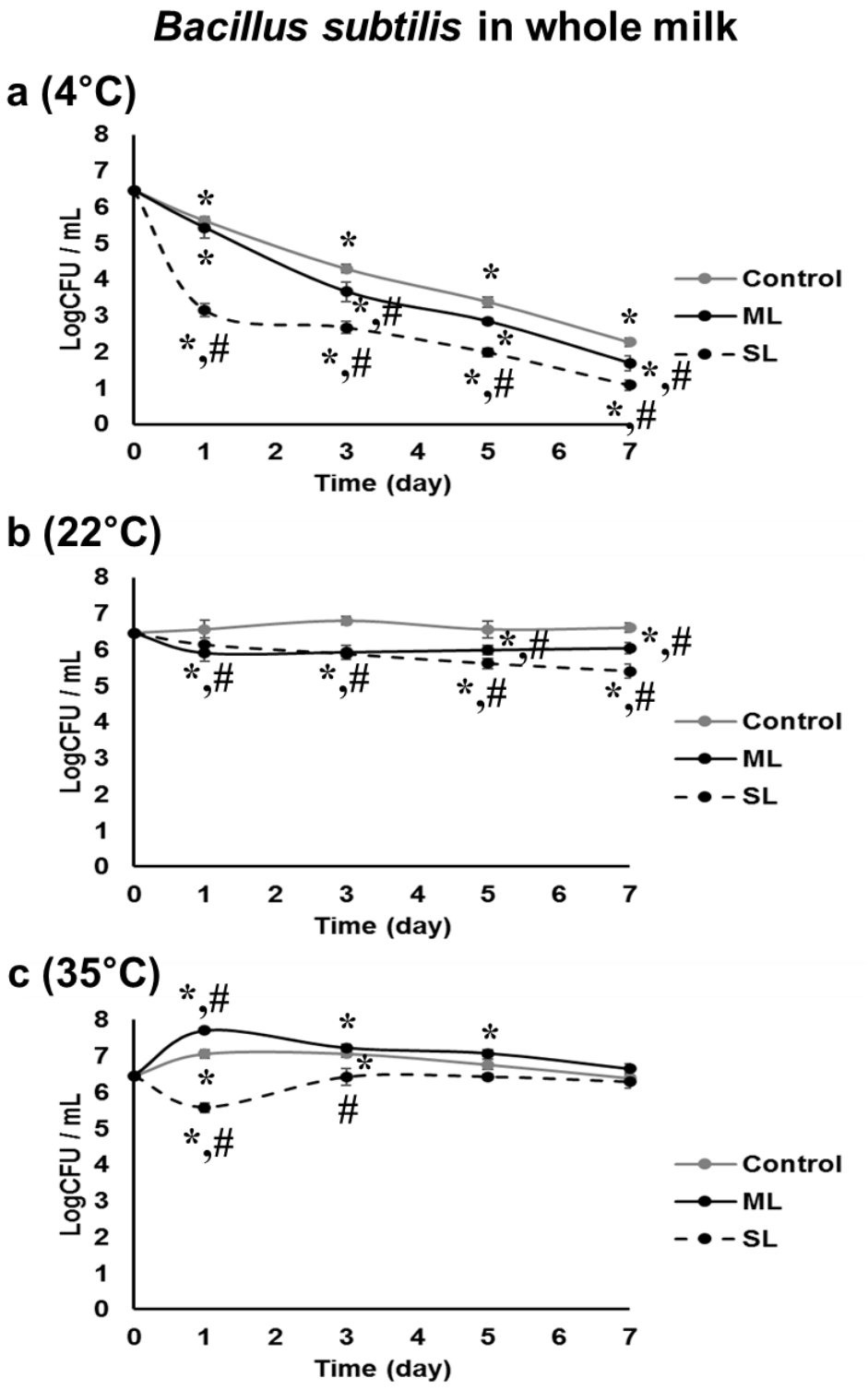

Fig. 5.3. The inhibition of *Bacillus subtilis* in whole milk by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) during storage at 3 different temperatures (a: 4°C, b: 22°C, and c: 35°C). * indicates the significant difference from the initial inoculum ($p<0.05$). # indicates the significant difference from the control on the same day ($p<0.05$).

ML's or SL's effect on *L. plantarum* in MRS media was determined, and the result was shown (Fig. 5.1b). *L. plantarum* in media without treatment grew significantly at 22°C and 35°C ($p<0.05$) despite a non-significant reduction at 4°C ($p>0.05$) (Fig. 5.1b). *L. plantarum*'s growth corresponded to previous research showing its ability to grow below pH 4 (Aryani, Den Besten, & Zwietering, 2016) and growth temperatures (15°C to 45°C) (Taylor & Vaisman, 2010). Notably, *L. plantarum* is reported to survive even at 4°C under acidic pH showing a slight reduction in cell numbers (Vivek, Mishra, Pradhan, & Jayabalan, 2019; K. Y. Yoon, Woodams, & Hang, 2006).

In MRS media, ML showed inhibition below the initial inoculation at all temperatures (Fig. 5.1b). However, SL showed significant inhibition below the initial inoculation at only 4°C and at 35°C ($p<0.05$) (Fig. 5.1b). Unlike ML, SL's highest inhibition occurred at 4°C.

Comparing the inhibitory result of *L. plantarum* cell*s* to *B. subtilis* cells*,* ML's and SL's inhibition showed a similar opposite temperature-dependence, explained by the hypotheses above. Besides, the reduction of *L. plantarum* was less than that of *B. subtilis*. This inhibitory gap may be from different vulnerabilities between these bacteria due to the distinct cell wall structures between species or strains and other mechanisms such as the different membrane stabilization (Desbois & Smith, 2010).

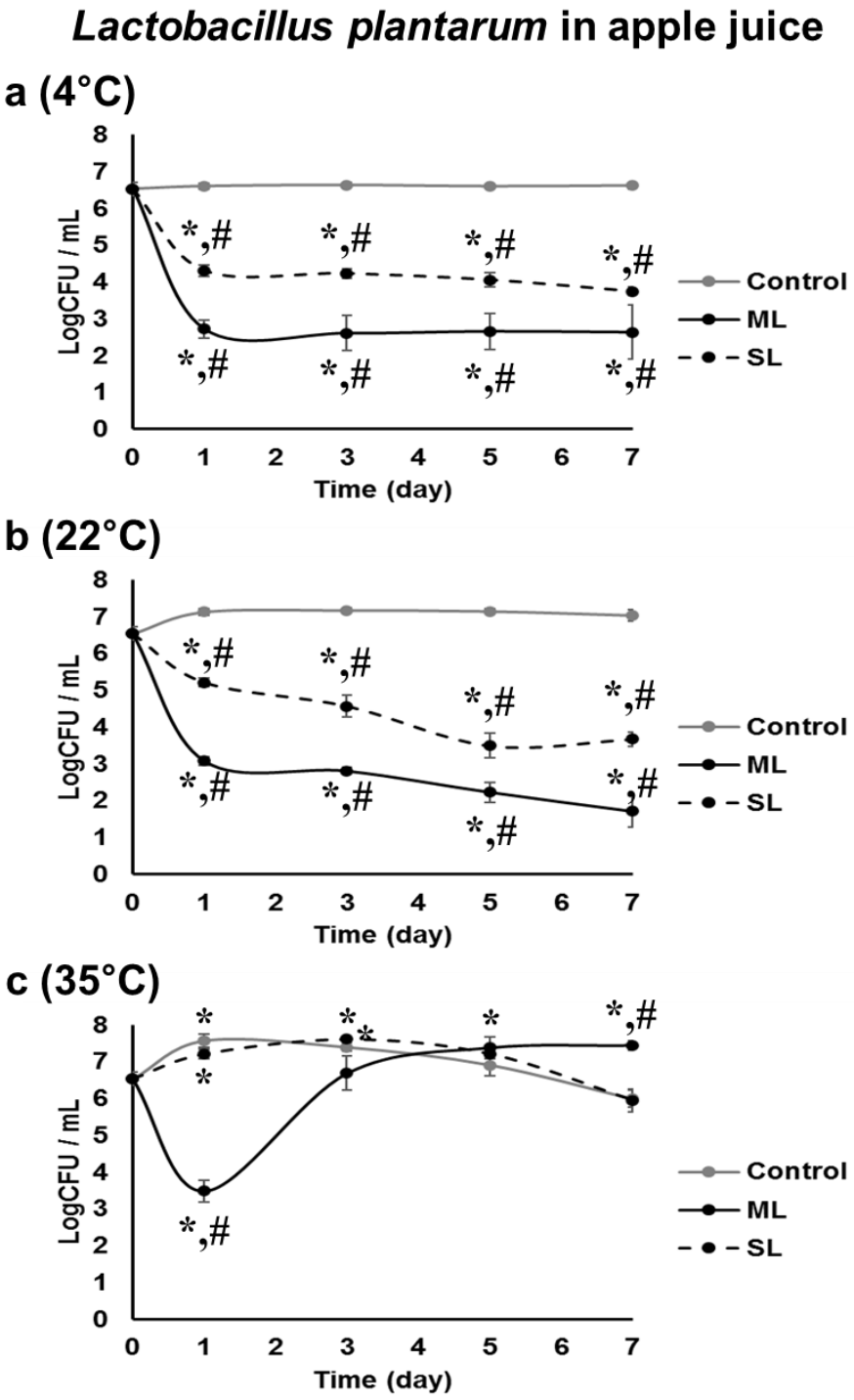

Fig. 5.4. The inhibition of *Lactobacillus plantarum* in apple juice by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) during storage at 3 different temperatures (a: 4°C, b: 22°C, and c: 35°C). * indicates the significant difference from the initial inoculum ($p<0.05$). # indicates the significant difference from the control on the same day ($p<0.05$).

The inhibition of *L. plantarum* in apple juice containing ML or SL over storage was obtained (Fig. 5.4). In the apple juice control, the cell counts at 4°C were similar to the initial inoculation over 7 days (Fig. 5.4a), but at 22°C, cells maintained insignificant growth ($p>0.05$) over storage (Fig. 5.4b). Cells at 35°C increased initially, followed by a gradual reduction over storage (Fig. 5.4c).

The bacterial decrease in apple juice control over storage at 35°C is assumed due to the inhibitory metabolites produced from growth, mainly including lactic acid (the primary organic acids by the sugar fermentation) (Mousavi et al., 2013). This acid's inhibitory effect may be from the lowered pH or the undissociated form (De Keersmaecker et al., 2006), which can transfer into the cytoplasm through the membrane, causing the inhibition (Mani-López, García, & López-Malo, 2012). The lower pH in apple juice control during storage at 35°C, where the highest growth occurred than at others (Table 5.3), may indicate the higher acid production, supporting the enhanced inhibition. The acid production of *L. plantarum* is closely related to the growth rate (Demir, BAHÇECİ, & Acar, 2006).

Both ML or SL in apple juice showed high inhibition over storage at 4°C and 22°C (Fig. 5.4a and b). At 35°C, there was no significant cell reduction from initial inoculation and controls on the same day ($p>0.05$) (Fig. 5.4c) except for the initial inhibition by ML (Fig. 5.4c).

Table 5.3. The pH of different foods containing 5 mg/mL of maltodextrin laurate (ML) or sucrose laurate (SL) over storage at different temperatures after inoculated with *Lactobacillus plantarum*.

Food	Treatment	Temperature	Time (day)		
			0	1	7
Apple juice	Control	4°C	3.91 ± 0.01^{gh}	3.90 ± 0.01^{h}	4.11 ± 0.00^{cd}
		22°C	3.91 ± 0.01^{gh}	4.01 ± 0.01^{e}	4.14 ± 0.00^{c}
		35°C	3.91 ± 0.01^{gh}	4.23 ± 0.02^{b}	3.76 ± 0.01^{j}
	ML	4°C	3.91 ± 0.01^{gh}	3.92 ± 0.00^{gh}	4.11 ± 0.01^{cd}
		22°C	3.91 ± 0.01^{gh}	3.94 ± 0.00^{fg}	4.03 ± 0.00^{e}
		35°C	3.91 ± 0.01^{gh}	3.97 ± 0.02^{f}	4.24 ± 0.01^{b}
	SL	4°C	3.91 ± 0.01^{gh}	3.86 ± 0.01^{i}	4.01 ± 0.00^{e}
		22°C	3.91 ± 0.01^{gh}	3.86 ± 0.00^{i}	4.29 ± 0.01^{a}
		35°C	3.91 ± 0.01^{gh}	3.91 ± 0.01^{gh}	4.09 ± 0.01^{d}
Tomato ketchup	Control	4°C	3.80 ± 0.01^{e}	3.84 ± 0.00^{de}	3.86 ± 0.01^{cd}
		22°C	3.80 ± 0.01^{e}	3.75 ± 0.01^{f}	3.24 ± 0.01^{j}
		35°C	3.80 ± 0.01^{e}	3.40 ± 0.00^{h}	3.05 ± 0.01^{l}
	ML	4°C	3.80 ± 0.01^{e}	3.93 ± 0.02^{b}	3.98 ± 0.02^{a}
		22°C	3.80 ± 0.01^{e}	3.80 ± 0.01^{e}	3.31 ± 0.01^{i}
		35°C	3.80 ± 0.01^{e}	3.44 ± 0.00^{gh}	3.11 ± 0.00^{k}
	SL	4°C	3.80 ± 0.01^{e}	3.91 ± 0.00^{b}	3.99 ± 0.01^{a}
		22°C	3.80 ± 0.01^{e}	3.90 ± 0.01^{bc}	3.47 ± 0.01^{g}
		35°C	3.80 ± 0.01^{e}	3.71 ± 0.00^{f}	3.41 ± 0.01^{h}
French dressing	Control	4°C	3.99 ± 0.01^{g}	4.00 ± 0.01^{g}	4.17 ± 0.01^{b}
		22°C	3.99 ± 0.01^{g}	4.01 ± 0.01^{g}	4.11 ± 0.01^{cd}
		35°C	3.99 ± 0.01^{g}	3.99 ± 0.00^{g}	4.09 ± 0.01^{d}
	ML	4°C	3.99 ± 0.01^{g}	4.05 ± 0.01^{e}	4.18 ± 0.01^{ab}
		22°C	3.99 ± 0.01^{g}	4.04 ± 0.00^{e}	4.12 ± 0.00^{c}
		35°C	3.99 ± 0.01^{g}	4.04 ± 0.01^{ef}	4.11 ± 0.00^{cd}
	SL	4°C	3.99 ± 0.01^{g}	4.01 ± 0.00^{fg}	4.20 ± 0.01^{a}
		22°C	3.99 ± 0.01^{g}	4.01 ± 0.01^{g}	4.17 ± 0.01^{b}
		35°C	3.99 ± 0.01^{g}	4.00 ± 0.00^{g}	4.15 ± 0.00^{b}

Different letters indicate significant difference within each food ($p < 0.05$).

Compared to the data in media, in general, ML's or SL's inhibition against *L. plantarum* in apple juice increased at lower temperatures. That situation suggests that a component in apple juice synergistically acts with ML or SL, especially at lower temperatures. Malic acid is a major acid in apple juice (Eisele & Drake, 2005) and would be a prime candidate because its undissociated form inhibited bacteria from entering into the cells (Raybaudi-Massilia et al., 2009). However, this acid was non-inhibitive in apple juice controls. That may be due to the higher pH of apple juice (around 4) than the first pK_a of malic acid ($pK_{a\,1}$: 3.40 and pK_{a2}: 5.13) (Uslu & Kırbaşlar, 2010), resulting in not enough amount of the undissociated form, the potential inhibitor.

Instead, the entry of malic acid is probably supported by ML's or SL's membrane destabilization, which may be more enhanced by ML due to the different primary actions suggested. Also, the lower inhibition at the higher temperature may be related to the possible degradation of malic acid to pyruvate by the malic enzyme (Landete, Ferrer, Monedero, & Zúñiga, 2013), whose presence was reported in *L. plantarum* (Tsuji, Kajikawa, Okada, & Satoh, 2013). This degradation may result in a reduced level of organic acids, and the higher pH on the first day in apple juice control as temperature increases (Table 5.3) may support the higher degradation of malic acid at 35°C. Also, the recovery of *L. plantarum* in apple juice after inhibition by ML at 35°C suggests insufficient malic acid to maintain the inhibitory effect. A similar synergistic effect was previously reported using fatty acids and organic acids (Kim & Rhee, 2013).

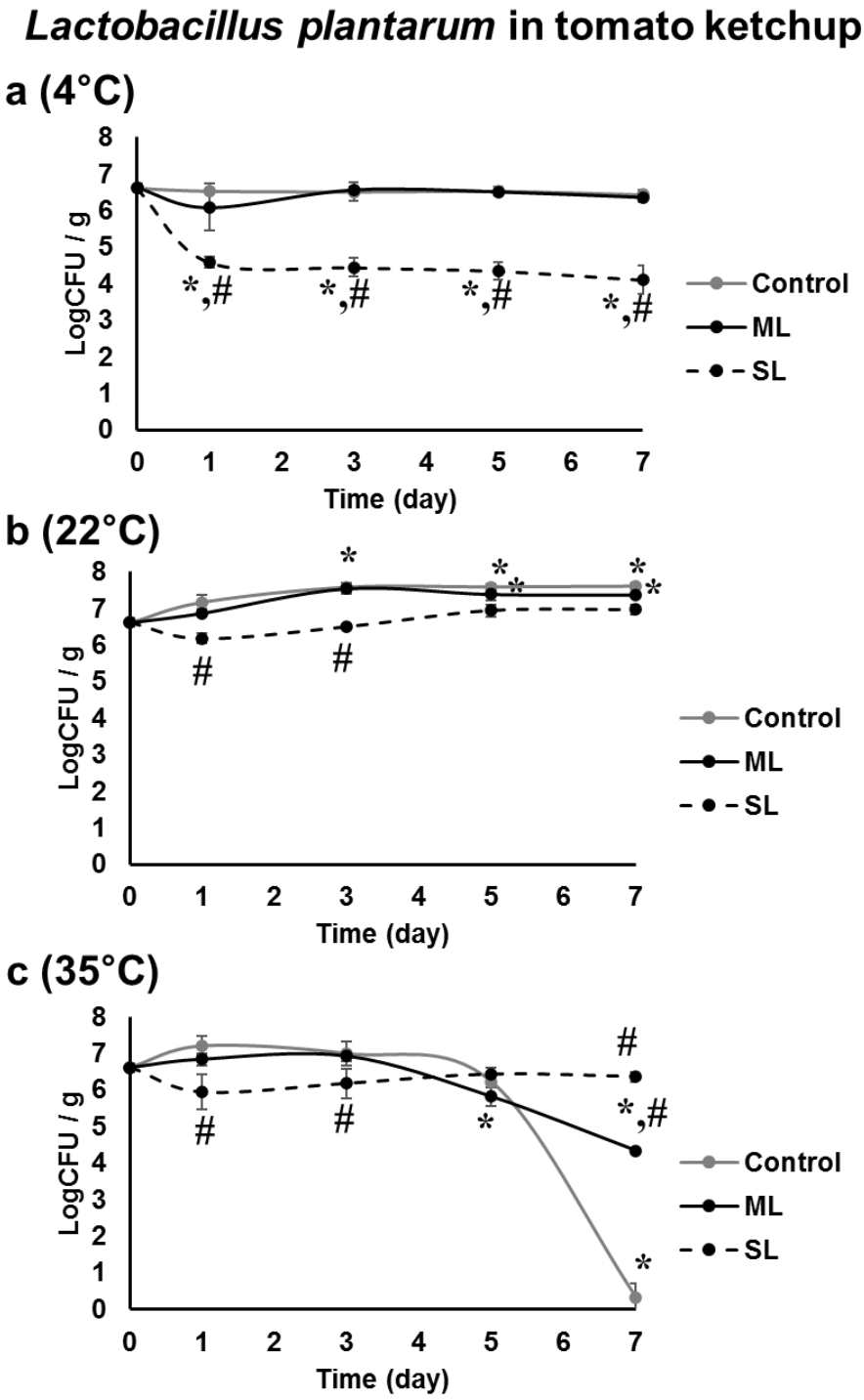

Fig. 5.5. The inhibition of *Lactobacillus plantarum* in tomato ketchup by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) during storage at 3 different temperatures (a: 4°C, b: 22°C, and c: 35°C). * indicates the significant difference from the initial inoculum ($p<0.05$). # indicates the significant difference from the control on the same day ($p<0.05$).

The effect of tomato ketchup containing ML or SL against *L. plantarum* at different storage temperatures was obtained (Fig. 5.5). The cell numbers in tomato ketchup control showed a consistent level at 4°C over storage (Fig. 5.5), increase lasting over storage at 22°C (Fig. 5.5b), and initial growth followed by a reduction up to the significant level at 35°C ($p<0.05$) (Fig. 5.5c). This growth pattern in tomato ketchup control was similar to that in apple juice control, while the reduction at 35°C was much higher in tomato ketchup control than in apple juice control.

This increased reduction at 35°C was assumed due to the possible excessive organic acids resulting from the high levels of sugars added to tomato ketchup. These sugars are the substrates fermentable by *L. plantarum* to produce organic acids, lowering the pH (Hedberg, Hasslöf, Sjöström, Twetman, & Stecksén-Blicks, 2008). The higher pH reduction from the initial level was obtained in tomato ketchup than in apple juice (Table 5.3). The addition of sugars could enhance acid production (De Keersmaecker et al., 2006).

However, the pH of tomato ketchup control at 22°C was lower than apple juice control at 35°C (Table 5.3), but the bacterial reduction was only obtained from apple juice. Added sugars in tomato ketchup available for *L. plantarum* could enhance the survival of *lactobacillu*s in the acidic condition by providing ATP to pump out intracellular protons (Corcoran, Stanton, Fitzgerald, & Ross, 2005), maintaining intracellular pH.

Generally, ML was not inhibitive against *L. plantarum* in tomato ketchup (Fig. 5.5). SL produced some bacterial inhibition, and especially this bacterial reduction lasted over storage at 4°C ($p<0.05$). This decline in ML's inhibitory activity in tomato ketchup than in media despite the constant inhibition of SL suggests the interference of components in tomato ketchup, especially with ML. A large amount of starch added to ketchup as a

thickening agent may be the primary candidate by its hydrophilic interactions with maltodextrin moiety leading to limited cell membrane approaches. Previous research showed reduced inhibitory activity of compounds targeting cell membranes due to the binding with food components (Veldhuizen, Creutzberg, Burt, & Haagsman, 2007).

Both ML and SL in tomato ketchup significantly maintained a high *L. plantarum* at 35°C (Fig. 5.5c). That is thought to be due to the slight inhibitory activities by compounds, resulting in less organic acids produced. The higher pH of tomato ketchup containing ML or SL than the control on day 7 (Table 5.3) may support this postulation. The highest pH on day 7 was mainly obtained in tomato ketchup with SL where the highest cell numbers remained.

The inhibition of *L. plantarum* cells in French dressing containing ML or SL is shown (Fig. 5.6). Bacterial cells in French dressing control showed gradual reduction at 4°C over storage (Fig. 5.6a), maintained initial inoculation level at 22°C (Fig. 5.6b), and steadily decreased at 35°C up to no bacterial cells at day 7 (Fig. 5.6c). Compared to *L. plantarum* in media, the bacterial numbers in French dressing after 1 day showed a similar reduction at 4°C despite a larger reduction at higher temperatures.

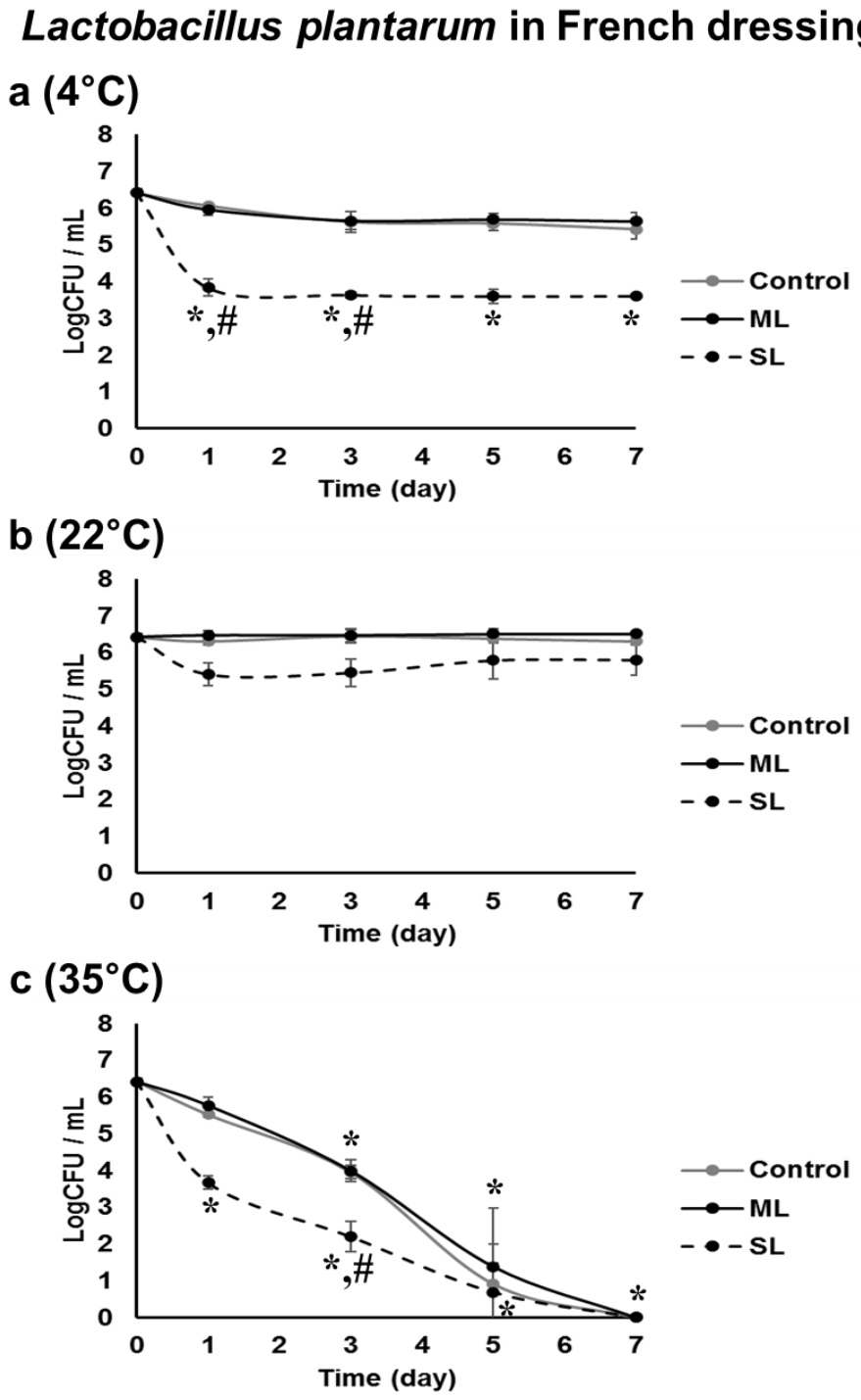

Fig. 5.6. The inhibition of *Lactobacillus plantarum* in French dressing by 5 mg/ml of maltodextrin laurate (ML) or sucrose laurate (SL) during storage at 3 different temperatures (a: 4°C, b: 22°C, and c: 35°C). * indicates the significant difference from the initial inoculum ($p<0.05$). # indicates the significant difference from the control on the same day ($p<0.05$).

In this case, acid production by *L. plantarum* may not be a reason due to the stable pH of French dressing (Table 5.3), meaning low bacterial metabolism. Instead, French dressing contained high levels (16%, w/w) of vinegar, and the acetic acid might cause a bacterial reduction. Acetic acid is considered a natural antimicrobial agent by lowering pH or the undissociated molecules (Smittle, 1977). Notably, the pK_a of acetic acid is 4.76 (Rhee, Lee, Dougherty, & Kang, 2003), and so the undissociated form would be prominent in the pH (around 4) in French dressing. As mentioned above, acetic acid's temperature-dependent inhibition may be due to the variable membrane status depending on temperatures.

This high amount of oil (35%, w/w) in French dressing might be the primary reason for the reduced ML's inhibitory activities by partitioning them from cell membranes by the hydrophobic interaction as in the results using different fat types of milk. SL only showed inhibition in French dressing (Fig. 5.6). That suggests several SLs are still left in the aqueous phase after partitioning in oil due to higher DS of SL, and these residues showed the synergistic effect with acetic acids, like ML and malic acid in apple juice.

CFAs made of sucrose are known to be extensively hydrolyzed in the gastrointestinal, and the residuals were excreted through a colon after the absorption of only small amounts (Additives & Food, 2010). In this research, two human intestinal cells, including HT-29 (cancer colonic epithelium) and CCD 841 CoN (normal colonic epithelium), were selected, and ML's or SL's cytotoxicity was evaluated (Fig. 5.7). SL showed approximately no viability over 200 μg/mL, and the IC_{50} values for SL were determined (93 ± 4 and 84 ± 14 for HT-29 and CCD 841 CoN, respectively). However, ML

maintained high viability within the ranges (1 – 500 µg/mL) (Fig. 5.7), indicating lower intestinal toxicity of ML than SL.

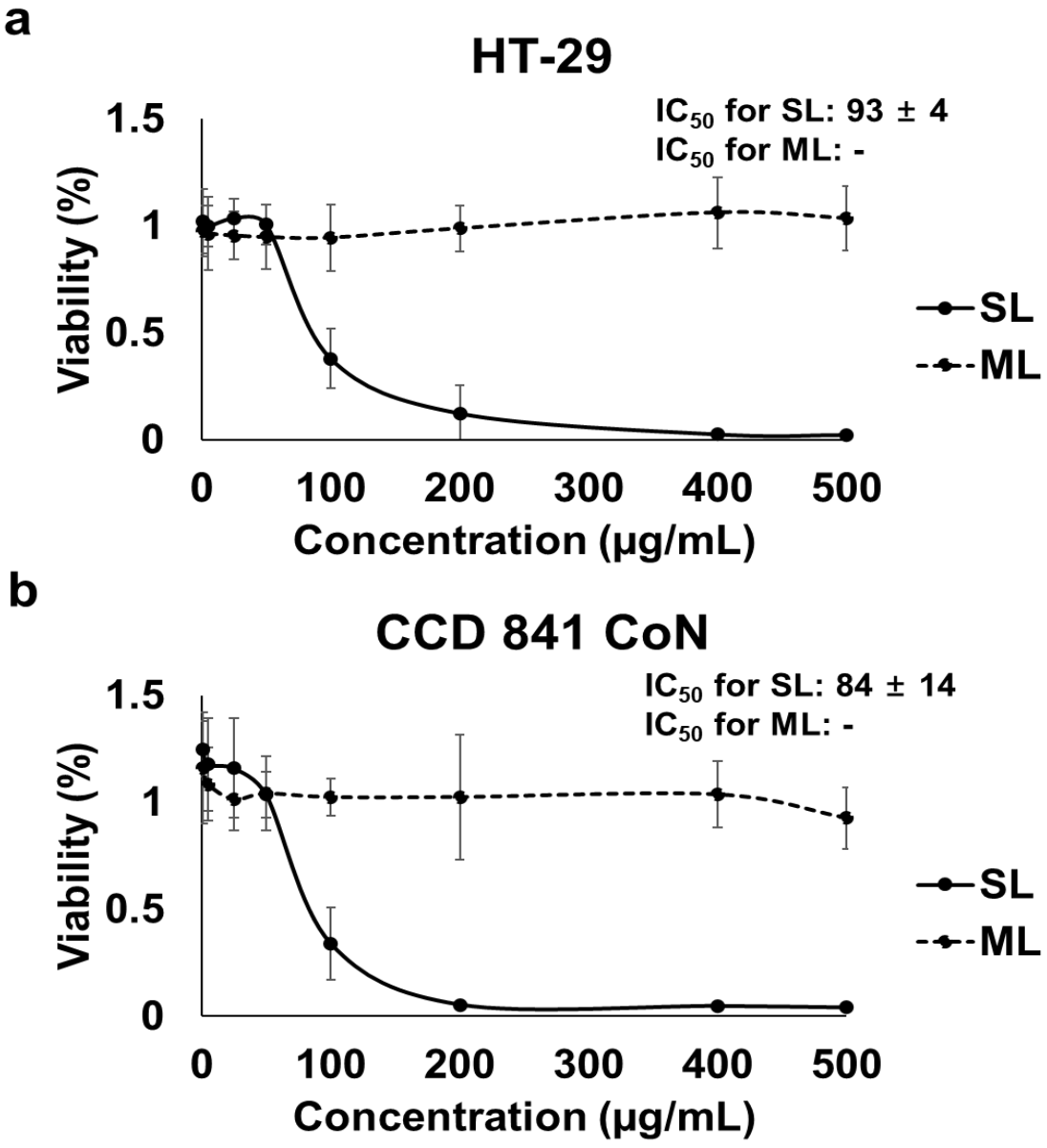

Fig. 5.7. Cytotoxicity of sucrose laurate (SL) and maltodextrin laurate (ML) against two colonic cells; (a) HT-29 (cancerous colonic epithelium) and (b) CCD 841 CoN (normal colonic epithelium).

The SL results indicated similar IC_{50} values previously reported (71.76 – 107.65 μg/mL against epithelial lung and cervix) (AlFindee et al., 2018). To our best knowledge, this research is the first to evaluate ML's cytotoxicity. The lower toxicity of ML could be from the lower DS (fewer laurate molecules esterified) or different synthesis and purification methods (chemical synthesis for SL and enzymatic synthesis for ML), resulting in the less amounts of toxic byproducts in the final samples.

Conclusions

Microbial inhibitory activities of enzymatically synthesized ML were evaluated regarding different food models and storage temperatures compared to commercial SL. Different carbohydrate moieties and the DS mainly differentiated both compounds, indicating distinct inhibitory patterns. ML and SL showed contrary temperature-dependent activities in the growth media study, and different food models showed variable bacterial inhibitory levels by both compounds. That information emphasizes selecting proper antimicrobial agents according to food types, bacterial susceptibility, and storage conditions. Notably, ML was the most suitable for lowering the spoilage of apple juice by *L. plantarum* at all temperatures tested, and so was SL for preventing the contamination of products by both bacteria in cold storage, suggesting their different applications in the food industry. In addition, ML could be a less toxic food additive alternative to commercial SL.

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CHAPTER VI

CONCLUSIONS AND FUTURE RESEARCH

Six different maltodextrin fatty acid esters (MFAs) were synthesized from 2 different maltodextrins and 3 different fatty acid combinations using lipase and food-grade ethanol. These compounds showed a low degree of substitution (moles of fatty acids/moles of glucose units) (0.016 to 0.026) and good aqueous solubility (93 to 101%) at 1% (w/v) level, suggesting their potential alternative to current carbohydrate fatty acid esters made of simple sugars having low aqueous solubility (Nagai et al., 2016). Solubility of compounds is an essential factor for food application because of the various functions in the aqueous system, such as emulsifying property (Guo, Hu, Wang, & Ai, 2017).

The esterification reaction of maltodextrin led to the enhancement of emulsion stability because of the resulting amphiphilicity. Maltodextrin esterified with laurate (ML), especially, showed the highest stabilizing ability, which was even better than commercial emulsifiers, indicating their potential as emulsion stabilizers. This result suggests that the increased size of carbohydrate groups may work as a thickened stabilizing layer preventing oil droplet aggregation (Fig. 6.1).

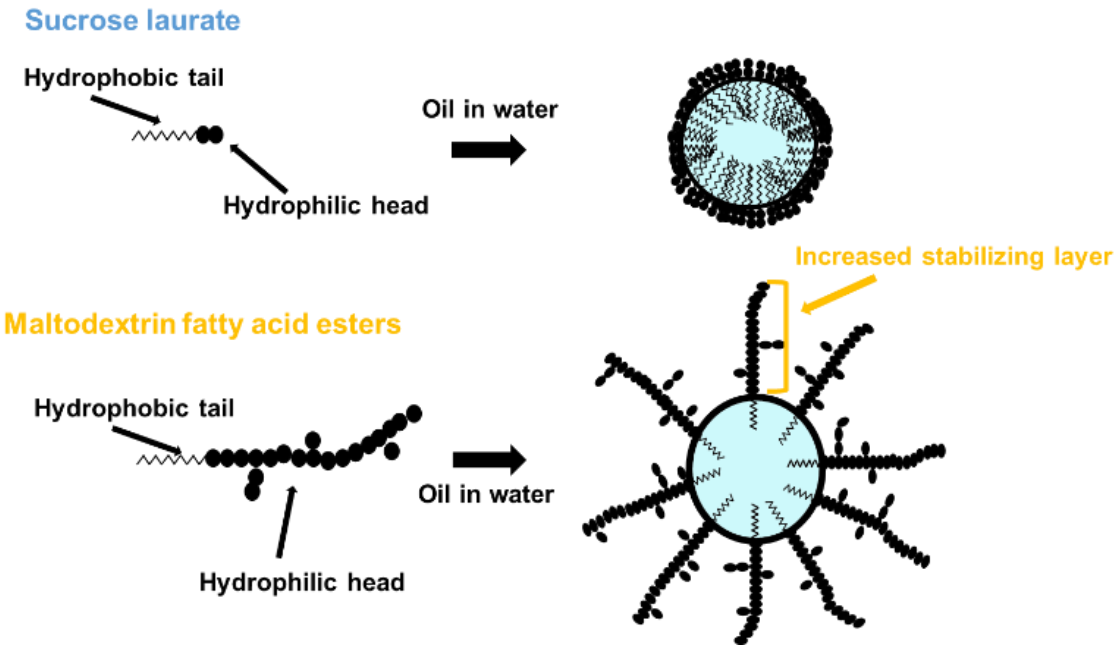

Fig. 6.1. The hypothesized emulsion stabilizing mechanism of maltodextrin fatty acid esters.

In addition, MFAs were tested against several food-related microorganisms in growth media to test their microbial inhibitory properties. These compounds inhibited several bacteria (Gram-positive and Gram-negative) and yeasts, and ML could inhibit all microorganisms tested. This inhibitory spectrum was broader than commercial sucrose laurate (SL), suggesting the potential applicability of MFAs in the food industry. The larger carbohydrate group of ML than SL was assumed as a possible reason for broader inhibitory activities by the increased steric hindrance to destabilize cell membranes, resulting in cell inhibition or death (Fig. 6.2).

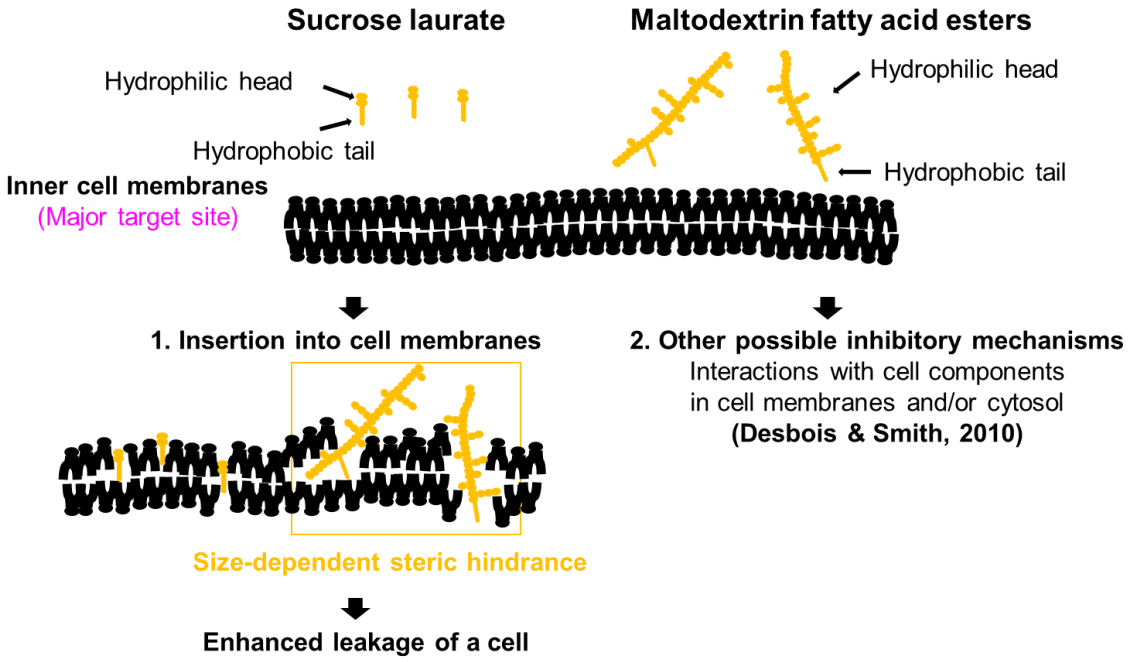

Fig. 6.2. The hypothesized microbial inhibitory mechanism of maltodextrin fatty acid esters.

As a representative of MFAs, ML with SL was tested against two spoilage bacteria (*Bacillus subtilis* and *Lactobacillus plantarum*) concerning the different food models and storage temperatures (4°C, 22°C, and 35°C). Microbial inhibition by these compounds varied by different food models and storage temperatures, suggesting the effect of food components and temperatures on inhibitory activities targeting cell membranes (Fig. 6.3).

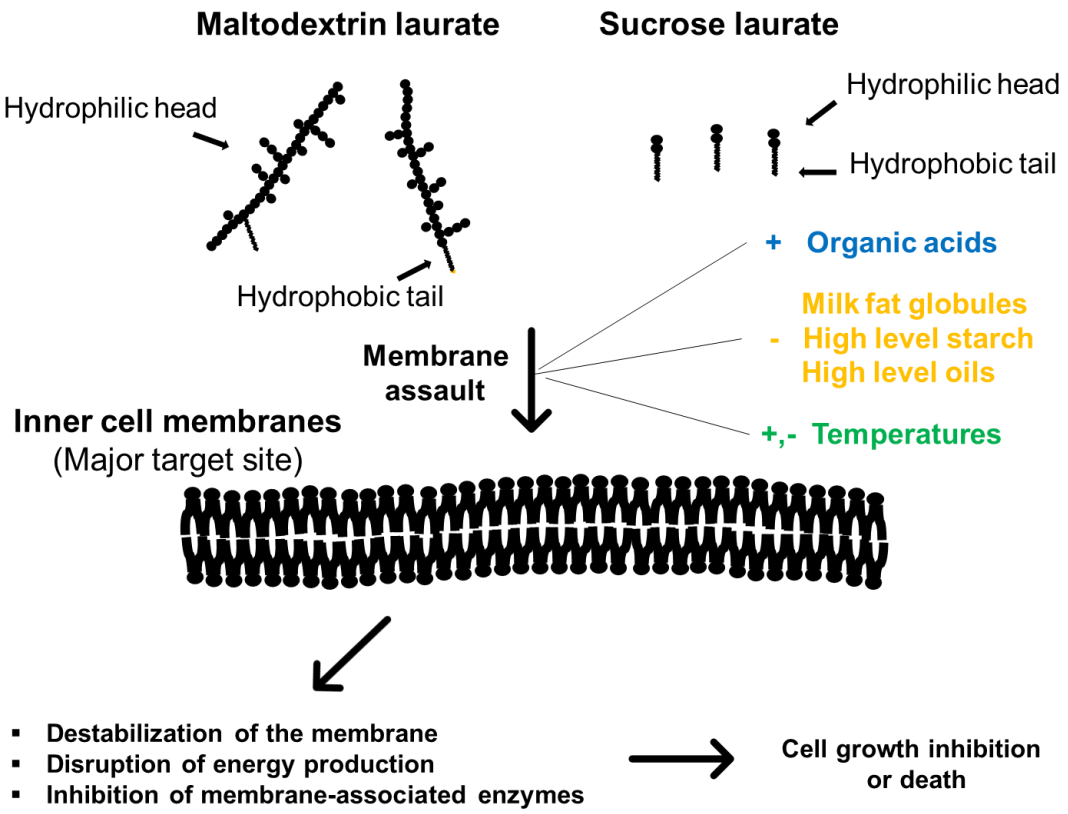

Fig. 6.3. The hypothesized influence of food components and storage temperatures on microbial inhibition by maltodextrin laurate and sucrose laurate

The high inhibition of *L. plantarum* in apple juice was obtained by ML at all temperatures tested, and SL was inhibitive against both bacteria when the food models were stored at 4°C. This different dependence on food types and temperatures can suggest their particular applications in the food industry.

Also, ML was treated against two types of human colonic cells (normal and cancerous) to test its cytotoxicity compared to SL. In particular, ML did not show toxicity against these two cells at the levels tested up to 500 μg/mL, but this was contrary to commercial SL, which indicated high toxicity from around 100 μg/mL. This result may propose ML's reduced potential toxic effect on human intestines.

In general, MFAs were synthesized from two different food-based sources by lipase-catalyzed reaction, and these resulting compounds can be potential emulsion stabilizers or microbial inhibitory agents in the food industry with lower intestinal toxicity.

Future research may be conducted in order to verify the suggested hypotheses. Because maltodextrin contains different sizes of glucose polymers, further studies would be necessary to directly connect the enhanced emulsion stabilizing and microbial inhibitory abilities to only the increased sizes of carbohydrate groups. Thus, the study using purified glucose polymers consisting of uniform chain length may support checking the size-dependent properties.

Specific inhibitory mechanisms of different sizes of purified glucose polymers would be studied to check any size mechanism relationship. That would include techniques, such as K^+ efflux analysis for permeability alteration, oxygen uptake assay for interruption of the electron transport chain, and activity assay of purified enzymes for the inhibitory effect on membrane-associated enzymes (Yoon, Jackman, Valle-González, & Cho, 2018).

Because maltodextrin is made from the simple hydrolysis of starch, it has a competitive price compared with the purified glucose polymers for esterification reaction. So, further studies using ML could include inhibitory analysis against other microorganisms using the various food models. This information could be helpful to find ML's best application in the food industry.

ML showed lower intestinal toxicity than SL, but this result was at the cell-study level, which is simpler than animal and human studies (more comprehensive). Commercial SL was highly digestible from the animal and human studies (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2010). Therefore, analysis of the

digestibility of ML could be beneficial information.

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APPENDICES

APPENDIX A

FOR CHAPTERⅢ

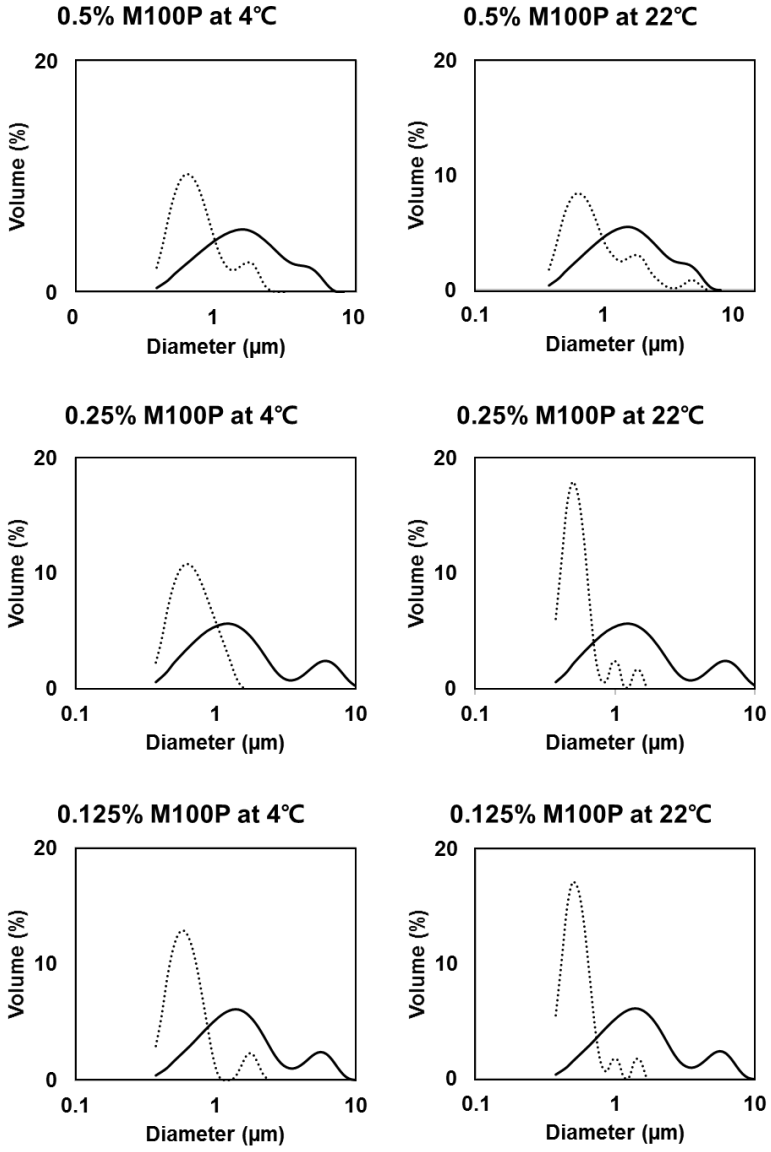

Fig. A.1. Particle size distribution of emulsions stabilized by M100P at 4ºC or 22ºC.

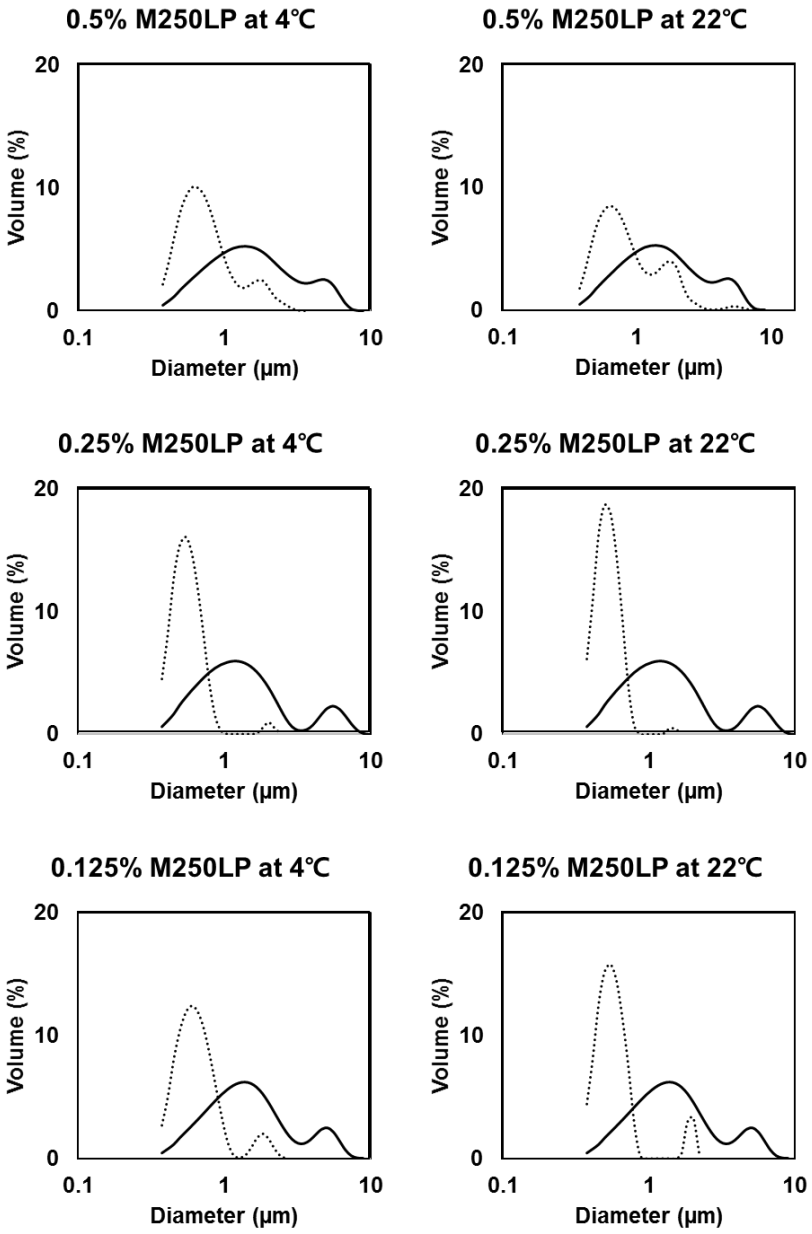

Fig. A.2. Particle size distribution of emulsions stabilized by M250LP at 4ºC or 22ºC.

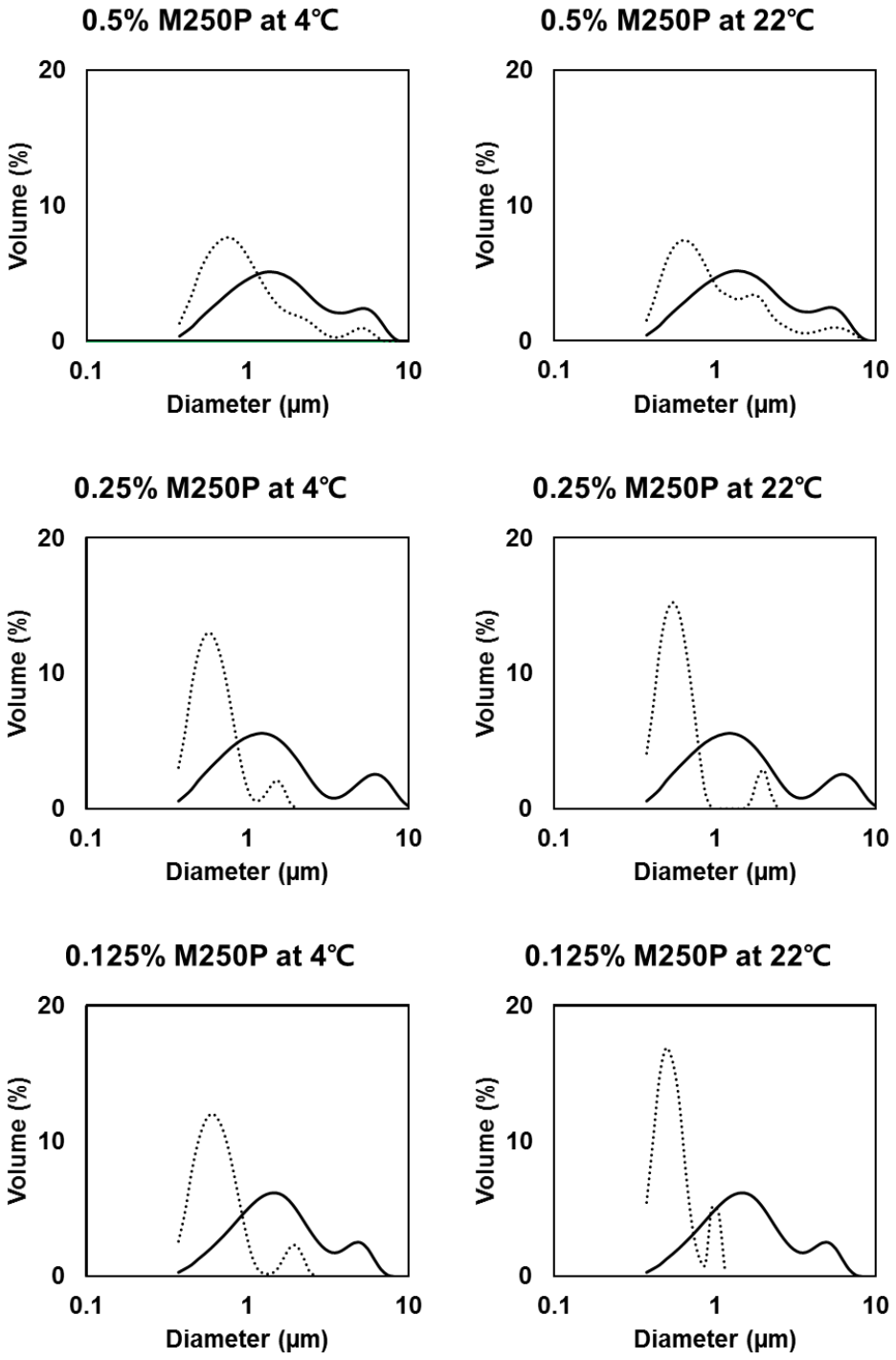

Fig. A.3. Particle size distribution of emulsions stabilized by M250LP at 4ºC or 22ºC.

APPENDIX B

FOR CHAPTERIV

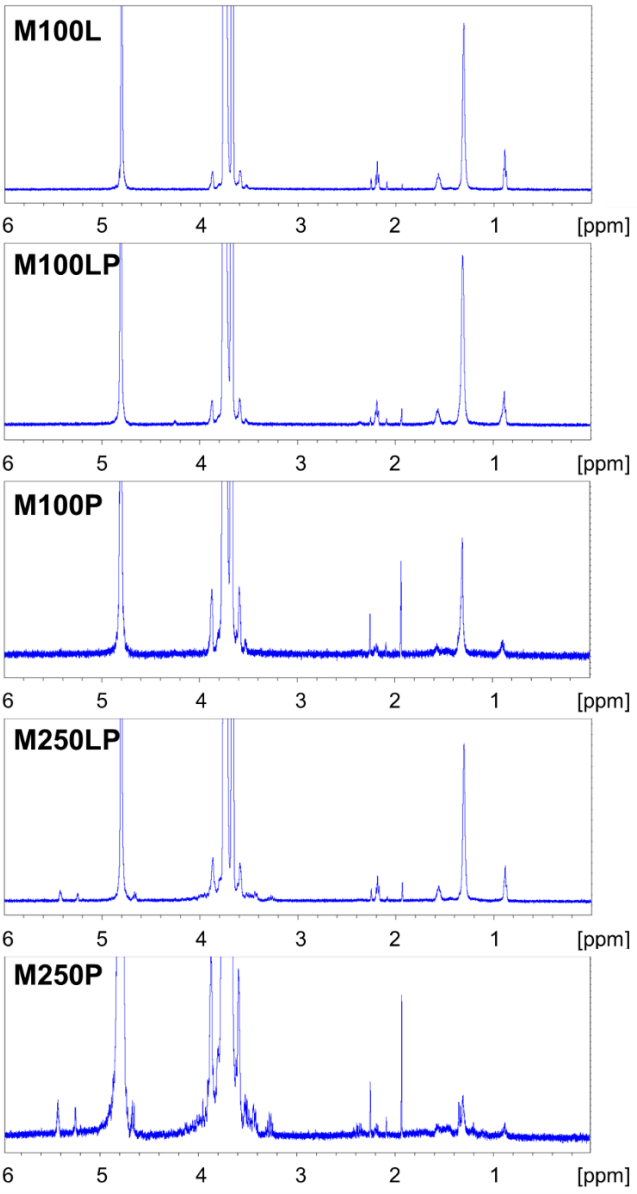

Fig. B.1. ^{1}H NMR spectra of maltodextrin fatty acid ester.

APPENDIX C

FOR CHAPTER V

Table C.1. The inhibition of *Bacillus subtilis* in low-fat milk (1%) by maltodextrin laurate (ML) or sucrose laurate (SL) during storage at different temperatures.

Temperature	Treatment	Day				
		0	1	3	5	7
4°C	Control	6.40 ± 0.16^{bcdef}	6.01 ± 0.20^{defgh}	4.94 ± 0.48^{ij}	3.63 ± 0.40^{kl}	1.90 ± 0.77^{m}
	ML 5 mg/ml	6.40 ± 0.16^{bcdef}	6.22 ± 0.06^{cdefg}	4.52 ± 0.24^{jk}	2.95 ± 0.36^{l}	0.25 ± 0.29^{n}
	SL 5 mg/ml	6.40 ± 0.16^{bcdef}	0.63 ± 0.44^{n}	0.00 ± 0.00^{n}	0.00 ± 0.00^{n}	0.00 ± 0.00^{n}
22°C	Control	6.40 ± 0.16^{bcdef}	6.72 ± 0.24^{abcd}	6.78 ± 0.37^{abcd}	6.53 ± 0.17^{abcdef}	6.50 ± 0.08^{abcdef}
	ML 5 mg/ml	6.40 ± 0.16^{bcdef}	6.41 ± 0.14^{bcdef}	5.98 ± 0.15^{defgh}	5.67 ± 0.14^{fghi}	5.43 ± 0.28^{ghij}
	SL 5 mg/ml	6.40 ± 0.16^{bcdef}	5.64 ± 0.20^{fghi}	6.35 ± 0.08^{bcdefg}	5.86 ± 0.21^{efgh}	5.39 ± 0.27^{ghij}
35°C	Control	6.40 ± 0.16^{bcdef}	6.78 ± 0.18^{abcd}	7.17 ± 0.27^{ab}	6.67 ± 0.28^{abcde}	6.16 ± 0.39^{cdefgh}
	ML 5 mg/ml	6.40 ± 0.16^{bcdef}	6.65 ± 0.32^{abcde}	7.30 ± 0.17^{a}	7.02 ± 0.21^{abc}	6.36 ± 0.15^{bcdefg}
	SL 5 mg/ml	6.40 ± 0.16^{bcdef}	6.26 ± 0.14^{cdefg}	6.03 ± 0.10^{defgh}	5.64 ± 0.69^{fghi}	5.15 ± 0.34^{hij}

*Different small letters indicate the significant difference within each row and column ($p<0.05$).

Table C.2. The inhibition of *Bacillus subtilis* in whole milk by maltodextrin laurate (ML) or sucrose laurate (SL) during storage at different temperatures.

Temperature	Treatment	Day				
		0	1	3	5	7
4°C	Control	6.46±0.03^{fgh}	5.62±0.11^{mnop}	4.29±0.12^{q}	3.38±0.14^{rs}	2.26±0.12^{uv}
	ML 5 mg/ml	6.46±0.03^{fgh}	5.43±0.28^{op}	3.66±0.27^{r}	2.84±0.09^{t}	1.68±0.20^{w}
	SL 5 mg/ml	6.46±0.03^{fgh}	3.15±0.17st	2.67±0.16^{tu}	1.98±0.11^{vw}	1.08±0.15^{x}
22°C	Control	6.46±0.03^{fgh}	6.56±0.24^{efgh}	6.79±0.11^{bcdef}	6.55±0.23^{fgh}	6.60±0.13^{efgh}
	ML 5 mg/ml	6.46±0.03^{fgh}	5.91±0.24^{klmno}	5.93±0.19^{klmn}	5.99±0.12^{jklmn}	6.04±0.15^{ijklmn}
	SL 5 mg/ml	6.46±0.03^{fgh}	6.13±0.04^{hijklm}	5.88±0.08^{lmno}	5.61±0.15^{nop}	5.40±0.19^{p}
35°C	Control	6.46±0.03^{fgh}	7.06±0.11^{bcde}	7.07±0.09^{bcd}	6.77±0.15^{cdef}	6.40±0.08^{fghijk}
	ML 5 mg/ml	6.46±0.03^{fgh}	7.71±0.06^{a}	7.23±0.10^{ab}	7.08±0.09^{bc}	6.65±0.13^{defg}
	SL 5 mg/ml	6.46±0.03^{fgh}	5.58±0.13^{nop}	6.43±0.23^{fghij}	6.44±0.07^{fghi}	6.29±0.17^{ghijkl}

*Different small letters indicate the significant difference within each row and column ($p<0.05$).

Table C.3. The inhibition of *Lactobacillus plantarum* in apple juice by maltodextrin laurate (ML) or sucrose laurate (SL) during storage at different temperatures.

Temperature	Treatment	Day				
		0	1	3	5	7
4°C	Control	6.53±0.18^{cd}	6.61±0.07^{bcd}	6.64±0.06^{bcd}	6.61±0.06^{bcd}	6.63±0.09^{bcd}
	ML 5 mg/ml	6.53±0.18^{cd}	2.72±0.25^{mno}	2.61±0.48^{nop}	2.66±0.48^{mno}	2.63±0.73^{mnop}
	SL 5 mg/ml	6.53±0.18^{cd}	4.31±0.15^{gh}	4.23±0.12^{ghi}	4.06±0.19^{ghij}	3.74±0.12^{ghijk}
22°C	Control	6.53±0.18^{cd}	7.13±0.10^{abc}	7.17±0.06^{abc}	7.15±0.07^{abc}	7.04±0.14^{abc}
	ML 5 mg/ml	6.53±0.18^{cd}	3.08±0.12^{klmn}	2.81±0.08^{lmno}	2.23±0.27^{op}	1.71±0.42^{p}
	SL 5 mg/ml	6.53±0.18^{cd}	5.21±0.12^{ef}	4.57±0.30^{fg}	3.49±0.34^{ijklm}	3.67±0.20^{hijkl}
35°C	Control	6.53±0.18^{cd}	7.57±0.18^{a}	7.39±0.11^{ab}	6.91±0.30^{abc}	5.99±0.23^{de}
	ML 5 mg/ml	6.53±0.18^{cd}	3.49±0.30^{jklm}	6.69±0.47^{bcd}	7.39±0.30^{ab}	7.45±0.13^{ab}
	SL 5 mg/ml	6.53±0.18^{cd}	7.21±0.12^{ab}	7.61±0.07^{a}	7.22±0.07^{ab}	5.95±0.30^{de}

*Different small letters indicate the significant difference within each row and column ($p<0.05$).

Table C.4. The inhibition of *Lactobacillus plantarum* in tomato ketchup by maltodextrin laurate (ML) or sucrose laurate (SL) during storage at different temperatures.

Temperature	Treatment	Day				
		0	1	3	5	7
4°C	Control	6.61±0.12^{bcdef}	6.53±0.06^{bcdefg}	6.51±0.25^{bcdefg}	6.52±0.12^{bcdefg}	6.43±0.12^{bcdefg}
	ML 5 mg/ml	6.61±0.12^{bcdef}	6.08±0.65^{efg}	6.55±0.11^{bcdef}	6.50±0.10^{bcdefg}	6.36±0.12^{cdefg}
	SL 5 mg/ml	6.61±0.12^{bcdef}	4.58±0.14^{h}	4.44±0.26^{h}	4.34±0.25^{h}	4.10±0.38^{h}
22°C	Control	6.61±0.12^{bcdef}	7.17±0.20^{ab}	7.57±0.10^{a}	7.58±0.08^{a}	7.60±0.09^{a}
	ML 5 mg/ml	6.61±0.12^{bcdef}	6.87±0.09^{abcde}	7.55±0.14^{a}	7.39±0.17^{a}	7.38±0.05^{a}
	SL 5 mg/ml	6.61±0.12^{bcdef}	6.18±0.14^{defg}	6.50±0.06^{bcdefg}	6.94±0.17^{abc}	6.97±0.14^{abc}
35°C	Control	6.61±0.12^{bcdef}	7.20±0.27^{ab}	6.99±0.33^{ab}	6.22±0.38^{defg}	0.33±0.38^{i}
	ML 5 mg/ml	6.61±0.12^{bcdef}	6.84±0.16^{abcdef}	6.92±0.13^{abcd}	5.81±0.25^{g}	4.33±0.04^{h}
	SL 5 mg/ml	6.61±0.12^{bcdef}	5.94±0.48^{fg}	6.17±0.40^{efg}	6.43±0.05^{bcdefg}	6.36±0.14^{cdefg}

*Different small letters indicate the significant difference within each row and column ($p<0.05$).

Table C.5. The inhibition of *Lactobacillus plantarum* in French dressing by maltodextrin laurate (ML) or sucrose laurate (SL) during storage at different temperatures.

Temperature	Treatment	Day				
		0	1	3	5	7
4°C	Control	6.42±0.11^{a}	6.07±0.05^{a}	5.62±0.29^{a}	5.58±0.20^{ab}	5.42±0.25^{ab}
	ML 5 mg/ml	6.42±0.11^{a}	5.97±0.16^{a}	5.66±0.25^{a}	5.69±0.16^{a}	5.64±0.25^{a}
	SL 5 mg/ml	6.42±0.11^{a}	3.83±0.23^{b}	3.63±0.07^{b}	3.60±0.19^{bc}	3.61±0.05^{b}
22°C	Control	6.42±0.11^{a}	6.31±0.11^{a}	6.45±0.18^{a}	6.38±0.13^{a}	6.31±0.09^{a}
	ML 5 mg/ml	6.42±0.11^{a}	6.47±0.12^{a}	6.47±0.17^{a}	6.51±0.14^{a}	6.51±0.02^{a}
	SL 5 mg/ml	6.42±0.11^{a}	5.41±0.31^{ab}	5.45±0.37^{ab}	5.78±0.51^{a}	5.80±0.41^{a}
35°C	Control	6.42±0.11^{a}	5.52±0.06^{ab}	3.95±0.19^{b}	0.92±1.07^{de}	0.00±0.00^{e}
	ML 5 mg/ml	6.42±0.11^{a}	5.77±0.22^{a}	4.00±0.30^{b}	1.39±1.60^{de}	0.00±0.00^{e}
	SL 5 mg/ml	6.42±0.11^{a}	3.67±0.18^{b}	2.20±0.40^{cd}	0.69±0.80^{de}	0.00±0.00^{e}

*Different small letters indicate the significant difference within each row and column ($p<0.05$).

CURRICULUM VITAE

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Educations

08-2017 – 12-2021	**Ph.D. in Nutrition and Food Sciences (in progress)** Department of Nutrition, Dietetics, & Food Sciences Utah State University (U.S) GPA: 3.94/4.0
03-2015 – 02-2017	**M.Sc. in Biofood industry** Department of International Agricultural Technology Seoul National University (Korea) GPA: 4.07/4.3
03-2009 – 02-2015	**B.Sc. in Microbiology** Department of Life Sciences Gyeongsang National University (Korea) GPA: 3.96/4.5

Presentations

National meetings	
07-2021	**Poster presentation at** FIRST **(**IFT 21 Online meeting)
06-2021	**Poster presentation at** World Microbe Forum (Online)
07-2020	**Oral and Poster presentation** at IFT 20 (Online)
06-2020	**Poster presentation** at ASM Microbe 2020 (Online)
04-2020	**Poster presentation** at AOCS 2020 (Online) (Abstract accepted)
06-2019	**Poster presentation** at IFT 19 Annual Meeting
06-2016	**Oral and Poster presentations** at KMB 43rd Annual Meeting & International Symposium
Regional/Local meetings	
04-2019	**Poster presentation** at IFT Bonneville Section Supplier Night & Candy Expo

Publications

In progress	**Park N,** Walsh MK. Influence of different food models

and storage temperatures on the bacterial inhibition by maltodextrin laurate and sucrose laurate and investigation of their cytotoxicity. Food Control (IF: 4.258).

07-2021 **Park N**, Walsh MK. Microbial inhibitory properties of maltodextrin fatty acid esters against food-related microorganisms. LWT- Food Science and Technology (IF: 4.006).

12-2019 **Park N**, Walsh MK. Physical and emulsion stabilizing properties of maltodextrin fatty acid polymers produced by the lipase-catalyzed reaction in ethanol. Carbohydrate Polymers (IF:6.044).

11-2018 **Park N**, Nguyen TT, Lee GH, Jin SN, Kwak SH, Lee TK, Choi YH, Kim SB, Kimura A, Kim D. Composition and biochemical properties of L-carnitine fortified Makgeolli brewed by using fermented buckwheat. Food Science & Nutrition (IF: 1.747).

12-2018 Hur J, Nguyen TT, **Park N**, Kim J, Kim D. Characterization of quinoa (Chenopodium quinoa) fermented by *Rhizopus oligosporus* and its bioactive properties. AMB Express (IF: 2.226).

12-2018 Lee TK, Nguyen TT, **Park N**, Kwak SH, Kim J, Jin SN, Son GM, Hur J, Choi JI, Kim D. The use of fermented buckwheat to produce L-carnitine enriched oyster mushroom. AMB Express (IF:2.226).

07-2017 **Park N**, Lee TK, Nguyen TT, An EB, Kim NM, You YH, Park TS, Kim D. The effect of fermented buckwheat on producing L-carnitine-and γ-aminobutyric acid (GABA)-enriched designer eggs. Journal of the Science of Food and Agriculture (IF: 2.422).

11-2016 Kim J, Nguyen TT, Kim NM, Moon YH, Ha JM, **Park N**, Lee DG, Hwang KH, Park JS, Kim D. Functional properties of novel epigallocatechin gallate glucosides synthesized by using dextransucrase from *Leuconostoc mesenteroides* B-1299CB4. Journal of Agricultural and Food Chemistry (IF: 3.571).

Patents

09-2019 Kim D, **Park N**, Lee TK, Jin SN, Choi S. Method for producing functional Makgeolli rich in L-carnitine using

	fermented buckwheat. (Registration No. 1020280670000).
08-2018	Kim D, Lee, TK, **Park N**, Choi S, Jang T, Choi, J. Method for cultivation of mushrooms containing L-carnitine using L-carnitine containing fermentation products and the cultivated functional mushrooms containing L-carnitine (Registration No. 1018944230000).
07-2018	Kim D, Choi S, Seo Y, Nguyen TT, **Park N**, Cho J, Park TS, An EB. Method for producing L-carnitine rich fermentation products and the fermentation products produced by the same. (Registration No. 1018735140000).

Research skills

Overall knowledges	Fermentation, microbiology, biochemistry, and food science.
Chemical analysis	Antioxidants, polyphenols, reducing sugars, proteins, and others
Chromatography	HPLC/UV, RI, or ELSD, UPLC/MS or MS/MS, HPAEC/PAD, HPLC/SEC/MALS
Large-scale purification	20 L Vacuum concentrator, 20 L Freeze dryer, MPLC/RI/UV, Prep-HPLC
Fermentation technique	1.2 L Fermenter (Batch and continuous cultures)
Emulsion analysis	Droplet size distribution and emulsion stability analysis
Antimicrobial assay	Analysis of minimum inhibitory or bactericidal or fungicidal concentrations
Cell morphology assay	Microbial sample fixation and scanning electron microscopy
Cytotoxicity assay	MTT analysis
Physical & thermal assay	NMR spectroscopy and differential scanning calorimetry
Statistical analysis	SAS and SPSS

Research experiences

08-2017 – 08-2021	**Research assistant** Department of Nutrition, Dietetics, & Food Sciences Utah State University
03-2017 – 06-2017	**Researcher** Institute of Food Industrialization, Institutes of Green Bio Science & Technology Seoul National University

03-2015 – 02-2017	**Graduate student researcher** Institute of Food Industrialization, Institutes of Green Bio Science & Technology Seoul National University

Awards

07-2021	**Registration Scholarship** at First (IFT 21 online meeting)
06-2021	**ASM Student and Postdoctoral Travel Award** at World Microbe Forum
07-2020	**2nd place** in the Food Microbiology Division Z John Ordal Graduate Student Oral Competition at IFT20
02-2020	**Doctoral Student Researcher of the Year Award** from the Department of Nutrition, Dietetics, and Food Sciences
06-2016	**Encouragement Award** for the oral presentation competition at 43rd Annual Meeting & International Symposium, KMB

Scholarships/Assistantships

08-2020 – 08-2021	Research assistantship
08-2020 – 05-2021	AGRI Don & Ming Wang Graduate Fellowship Scholarship
08-2019 – 05-2020	AGRI Don & Ming Wang Graduate Fellowship Scholarship
08-2019 – 05-2020	AGRI Dr. Niranjan R. Gandhi & Mrs. Josephine N. Gandhi Graduate Assistantship
08-2018 – 05-2019	AGRI Dr. Niranjan R. Gandhi & Mrs. Josephine N. Gandhi Graduate Assistantship
08-2017 – 05-2018	Research assistantship
03-2009 – 02-2017	GPA-based Scholarships (3 times / M.Sc, 4 times / B.Sc)

Special activities

08-2019 – 12-2019	Teaching assistant (Food product development)
08-2018 – 03-2019	Cache valley Toastmasters
05-2016 – 06-2016	Teaching assistant (Laboratory class)
02-2016 – 02-2016	Internship at Hokkaido University, Japan
10-2013 – 08-2014	Exchange student at Nagoya University, Japan
03-2013 – 06-2013	International student assistant (GNU buddy)
02-2011 – 02-2013	Military service, Korea

References

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