Microemulsions of cinnamon and orange oils as an antimicrobial delivery system for *Salmonella* biofilms on stainless steel

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Abstract

Essential oils (EOs) are natural antimicrobials that can be used to develop intervention strategies to inhibit pathogens, but EOs are lipophilic. Colloidal systems, such as microemulsions, are needed for food industry applications. This dissertation focused on the development and characterization of a microemulsion composed of cinnamon oil (CO, *Cinnamomum zeylanicum*) and orange oil (OO, *Citrus sinensis*) to be used against *Salmonella* Enteritidis H4267 biofilms formed on stainless steel disc surfaces.

First, the antimicrobial activity of CO and OO against *S.* Enteritidis H4267, either used in combination or in a microemulsion system, was determined. CO had a greater antimicrobial effect on *S.* Enteritidis H4267 when used in combination with OO than when individually ($p<0.001$). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) indicated that CO and OO were optimum in a 9:1 volume ratio (MIC and MBC: 750:750 ppm). Physical analyses determined that formulations with, on mass basis, 0% and 2% sunflower lecithin (SL), 20% Tween® 20, 1% and 5% CO-OO (9:1) were transparent, thermodynamically stable microemulsions that had bactericidal activity on *S.* Enteritidis H4267.

The second objective investigated the biofilm forming ability of *S.* Enteritidis H4267 and determined a method to remove biofilms on stainless steel disc surfaces. *S.* Enteritidis H4267 was determined to produce curli and cellulose indicative of biofilm development, and biofilms were formed on stainless steel disc surfaces. Sonication in

0.1% (w/v) peptone water for 30 sec effectively dislodged biofilms from disc surfaces without causing extensive cell death.

Biofilms were treated with microemulsions and emulsion controls for 5 minutes to determine the antimicrobial activity in the third objective. The 2% SL, 5% CO-OO (9:1) microemulsions displayed the greatest antimicrobial activity against biofilms.

This study demonstrated that OO enhances the bactericidal activity of CO when in an oil or microemulsion system, and co-encapsulated oils in microemulsions could be developed for antimicrobial delivery systems. Further research into developing microemulsions with positively charged droplets and with greater microemulsion exposure time should be investigated.

Keywords: microemulsions, essential oils, antimicrobial delivery system, *Salmonella*, biofilms, stainless steel

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List of abbreviations

CDC	U.S. Department of Health and Human Services Centers for Disease Control and Prevention
CFU	Colony forming unit
CRA	Congo Red Agar
CV	crystal violet
d	Day(s)
diH_2O	Deionized water
EOs	Essential oil(s)
FIC	Fractional inhibitory concentration index
g	Gram(s)
h	Hour(s)
l	Liter(s)
LBA_c	Luria Broth agar supplemented with 0.001% (w/v) calcofluor
MBC	Minimum bactericidal concentration
mg	Milligram(s)
MIC	Minimum inhibitory concentration
mm	Millimeter(s)
nm	Nanometer(s)
PBS	Phosphate Buffered Saline
PW	Peptone water

sec	second(s)
spp.	Species (plural)
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
µl	Microliter(s)
USDA	United States Department of Agriculture
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

Chapter 1. Introduction and objectives

1. Introduction

Of the 48 million cases of foodborne illness that occur in the US annually, only 9.4 million cases are related to a foodborne pathogen that has an identified pathogen source.[1-2] Non-typhoidal *Salmonella* is the leading bacterial cause of foodborne illness, and the rates of *Salmonella* cases in the US have remained relatively constant with approximately 1 million illnesses, 19,000 hospitalizations, and 380 deaths annually.[3-6] These numbers are estimated to be lower than actual infection rates since foodborne illness cases are often underreported as symptoms can be commonly be referred to as "stomach flu-like" symptoms.[7-8]

Since 2005, over 35 foodborne illness outbreaks in the US have been traced back to *Salmonella* species (spp.).[9] Poultry, egg sources, and fresh fruit has contributed 50% (1653 cases) of US food-related cases from 2015 to 2019.[9] *S. enterica* subspecies (subsp.) *enterica* serovar Enteritidis (*S.* Enteritidis) is the leading Salmonellae isolate serotyped from laboratory-confirmed cases of foodborne infection.[10] Between 2007 and 2015, the incidence of infection from *S.* Enteritidis increased by 37%.[10-11]

With non-typhoidal *Salmonella* resulting in approximately 380 deaths a year in the U.S., decreasing the rates of *Salmonella* spp. in the food supply is a priority.[12] In the U.S., the economic impact of salmonellosis, the gastrointestinal disease associated with *Salmonella*, can be as high as $10.9 billion per year, and the cost of illness is estimated at over $3.3 million per year.[4, 13-14] While bacterial presence in the food industry is of

concern and can cause foodborne illness, the majority of bacterial life is thought to exist in biofilms.[15]

Biofilms are a composition of a microorganism or microorganisms attached to a surface that are encased within an extracellular polymeric substance (EPS) layer.[16-20] The formation of a biofilm is complex and consists of a variety of intrinsic and extrinsic components. Biofilm formation generally consists of five stages: reversible attachment, irreversible attachment, proliferation, maturation, and detachment.[19] The ability to convert to being immobile instead of planktonic depends on a variety of environmental factors, and once attached, organisms must be able to convert to being irreversibly attached while proliferating within a biofilm.[19, 21] As biofilms mature further and proliferation of microorganisms continues, an increase in biomass occurs within the biofilm. As biofilms become larger, pieces of biofilms can detach from the biofilm structure.[22]

Biofilms can form on practically any surface, and the ubiquitous nature of biofilms is troubling for the food industry.[23-26] If an antimicrobial delivery system were to diffuse through the EPS layer and/or become internalized into the biofilm, the system would be advantageous to a variety of industries. Antimicrobial delivery systems to combat biofilms in the food industry while also avoiding bacterial resistance to disinfectants are of interest.[27] Plant-derived essential oils (EOs) are an attractive option for antimicrobial systems since EOs do not contribute to drug resistance due to the current mechanism of action focusing on the disruption of bacterial membranes.[28-31] The hydrophobicity of EOs also requires colloidal systems, such as emulsions, for utilization.

Colloidal systems with nanoscale oil droplets have unique features uncommon in conventional emulsions.[32-33] Nanoemulsions have a droplet diameter between 20 and 200 nm, and microemulsions have an oil droplet dimension between 4 and 100 nm.[32] Though microemulsions have a small droplet size, they are relatively easy to prepare in comparison to some emulsions.[34] Microemulsions have the added benefit of being transparent and thermodynamically stable oil-water-surfactant mixtures while nanoemulsions are metastable systems that can be clear or turbid in appearance.[32, 35] Microemulsions also have the ability to enable EO compounds to penetrate through bacterial cell walls more efficiently due to their small droplet size and large surface area, which can increase the antimicrobial activity.[36] While there are several benefits of microemulsions, microemulsions are formed only at a particular set of conditions, such as environmental conditions and composition, and may convert to a different colloidal system when the conditions are changed.[32-33] Microemulsions also require a high level of surfactants.[32] Despite these drawbacks, microemulsions can be used to deliver lipophilic compounds, such as EOs, which could be an approach to enhance antimicrobial activity.[37-38]

EOs are an attractive option for antimicrobial systems due to the hydrophobicity of EOs allowing the oil to penetrate through the bacterial cell membrane, which can destabilize the membrane structure.[29-30] The destabilization of the membrane structure causes disruption of the cell's metabolic processes and membrane transport systems, which ultimately leads to the breakdown of membrane integrity.[29-30] As the membrane integrity declines, the increased membrane permeability occurs, which ultimately leads to

cell death.[29] When combined with other organic compounds, the antimicrobial activity of the individual EOs may be enhanced to provide greater antimicrobial ability at reduced levels.[39] Cinnamon oil (CO, *Cinnamomum zeylanicum*) has been extensively researched as an antimicrobial against a variety of foodborne pathogens, including *S.* Enteritidis.[40-43] Orange oils (OO, *Citrus sinensis*) have exhibited inhibitory activity against a variety of *S. enterica* and have displayed antimicrobial activity enhancement of other EOs when used in combination against *Salmonella* spp.[44-45]

CO and OO have been investigated individually for antimicrobial activity against foodborne pathogens, but the possible synergy in antimicrobial activity of CO and OO in combination has not been studied. Microemulsions containing co-encapsulation of EOs with an antimicrobial compound, antibiotics, and coenzyme Q_{10} have been created, so an antimicrobial delivery system containing co-encapsulated CO and OO could also be created.[37-38, 46] Internalization of an emulsion-based system with droplet sizes near the microemulsion range in *Pseudomonas aeruginosa* biofilms have confirmed that nanoscale oil droplets have the ability to penetrate a biofilm's EPS layer.[47] The developed microemulsion containing CO and OO could be utilized as an antimicrobial delivery system for *S.* Enteritidis biofilms.

2. Research objectives

The goal of this dissertation was to develop an antimicrobial delivery system in the form of a microemulsion containing co-encapsulated CO and OO. To develop this

microemulsion system and determine if it has antimicrobial activity against a biofilm, the following three objectives were developed.

2.1. Objective I: Antimicrobial inhibition of **Salmonella** *Enteritidis H4267 via cinnamon and orange oil used in neat form, in combination, or in a microemulsion system.*

The first objective was divided into three phases. The first phase aimed to determine the antimicrobial ability of the selected EOs, CO and OO, prior to the development of a microemulsion containing the oils. The first phase's hypothesis was that CO and OO will have inhibitory activity against planktonic *S.* Enteritidis H4267 when used in combination. This is supported by OO's ability to enhance the antimicrobial activity of other EOs when used in combination against *Salmonella* sp. and CO's bactericidal activity against *S.* Enteritidis alone and in combination with other EOs.[42, 44-45]

The second phase was to develop a microemulsion formulation for encapsulation of CO and OO, and upon microemulsion creation, physical characteristics were investigated. The second phase hypothesis was that through the adjustment of surfactant and oil levels, a microemulsion containing CO and OO can be formed. Microemulsions of oil mixtures have been created and characterized previously.[37-38]

The third phase investigated the antimicrobial activity of the microemulsion systems formed during Objective I, Phase 2 against *S.* Enteritidis H4267. Surfactants can lower the antimicrobial ability of an emulsion system, but EOs can still have an antimicrobial effect in emulsion systems despite the surfactant addition.[48-53] The

hypothesis for the third phase was that microemulsions containing CO and OO would have antimicrobial activity against *S.* Enteritidis H4267.

2.2. Objective II: Development and characterization of **Salmonella** *Enteritidis H4267 biofilms.*

Objective II was split into two phases to investigate the characterization of biofilm development and the removal method for biofilms formed on stainless steel discs. Since there is no current literature on the development and characterization of *S.* Enteritidis H4267 biofilms, phase one aimed to address this lack of knowledge. The occurrence of curli and cellulose production was determined via Congo Red agar and Luria Broth agar supplemented with 0.02% (w/v) calcofluor as well as the development of the biofilm in a microtiter assay. Staining with crystal violet allows for both visual observation as well as a quantitative analysis of biofilm extent. The hypothesis for phase one was that *S.* Enteritidis H4267 can form biofilms. Previous work indicating that *Salmonella* biofilm phenotypes display curli and cellulose production supports the hypothesis.[54-56]

The second phase of Objective II focused on the development of *S.* Enteritidis H4267 biofilms on stainless steel disc surfaces. *S.* Enteritidis H4267 was hypothesized to form biofilms on stainless steel disc surfaces due to previously reported formation of biofilms on stainless steel by other *S.* Enteritidis serovars.[57-58]

2.3. Objective III: Determination and characterization of the antimicrobial activity of microemulsions containing cinnamon oil and orange oil on S. *Enteritidis H4267 biofilms formed on stainless steel disc surfaces.*

Due to the complexity of biofilms, microemulsions that have activity against planktonic cultures may not have activity against a formed biofilm. Additionally, droplet size is of importance for biofilm treatment to determine if a microemulsion would also be able to have an antimicrobial effect on biofilms. The goal of Objective III was to determine if the CO and OO microemulsions developed for Objective I have antimicrobial activity on *S.* Enteritidis H4267 biofilms on the stainless steel surface utilized in Objective II. The hypothesis was that microemulsions containing high volumes of CO and OO will have a greater antimicrobial effect on *S.* Enteritidis H4267 biofilms compared to control emulsions of a larger droplet size. Because of the small droplet size, microemulsions should have greater bioavailability and could have higher antimicrobial ability compared to nanoemulsions that have larger droplet sizes.[32, 41, 43, 59-60]

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Chapter 2. Literature review

1. Foodborne illness

Annually in the United States, an estimated 48 million cases of foodborne illnesses occur, but only 9.4 million foodborne illness-related cases have an identified pathogen source.[1-2] Foodborne illness cases are often underreported as symptoms can be commonly be referred to as "stomach flu" and symptoms include nausea, vomiting, diarrhea, and/or fever.[3-4] Being unsure of the cause, having a belief that reporting would not be beneficial, and the amount of time between consumption of the food and reporting are all causes for the underreporting of foodborne illnesses.[4] The 9.4 million cases that are identified annually result in over 56,000 hospitalizations and 1,400 deaths.[5] Reducing these case numbers is not only of public health concern, but also of economic interest as it was estimated in 2015 that the economic burden of foodborne illnesses was over $15.5 billion.[6]

Of the top five causes of foodborne illnesses, non-typhoidal *Salmonella* is the leading bacterial culprit.[5, 7] Non-typhoidal *Salmonella* species are estimated to be the cause of approximately 1 million foodborne illnesses, 19,000 hospitalizations, and 380 deaths in the US annually.[8] In 2012, it was estimated that *Salmonella*-related foodborne illness resulted in $10.9 billion in losses due to medical need, productivity loss, and mortality costs.[8] Among the human *Salmonella* isolates reported in the US, 99% belong to *S. enterica* with the top two *S. enterica* isolates serotyped from laboratory-confirmed cases being *S.* Enteritidis and *S.* Typhimurium.[9] Between 2007 and 2015, the incidence of

infection from *S.* Enteritidis increased by 37% while *S.* Typhimurium incidence of infection decreased by 66%.[9, 10]

1.1. Intervention methods

The high costs associated with foodborne outbreaks, particularly with pathogenic bacteria, are of great concern for food industries and government agencies.[6, 8, 11] The persistence of bacteria and issues with bacterial removal once in a food processing environment necessitates the need for preventive measures in food facilities.[11] Government regulation requires the implementation of food safety preventive measures, such as Good Manufacturing Practices (GMPs), Hazard Analysis and Critical Control Points (HACCP), and items detailed in the Food Safety Modernization Act (FSMA).[12-15]

A facility's GMPs requires companies to abide by sanitation practices that keep employees trained and proper equipment and facility maintenance to be performed.[13] Testing methods must also be performed regularly to decrease the likelihood of sanitation issues.[12-13] HACCP systems must also be implemented to identify and target control points in the food processing chain to be monitored by sampling.[14] Accurate record keeping is utilized to maintain accountability and ensure sanitation methods are efficient.[14] The passage of the FSMA provided updates to HACCP requirements. In addition to having hazard identification conducted and a preventive control plan being created, facilities were required to provide a supply chain program hazard analysis.[15] If a hazard from a manufacturer requires a preventive control and the control will be applied

in a facility's supply chain, manufacturers are responsible for ensuring that suppliers are approved.[15]

Process flow and equipment design can also play a role in cleaning design and preventing contamination. Ensuring adequate space between processing lines and defined areas between raw and finished product assists with minimizing contamination.[16-17] Easy access points for equipment can also assist with ensuring proper sanitation and cleaning of material surfaces.[17] Clean-in-place (CIP) systems for equipment assists in not only providing a cleaning routine but also increasing employee safety by having an enclosed sanitation system that utilizes chemical agents.[16, 18] A concern with CIP systems is that bacteria can be detached from surfaces early in the cleaning system but could reattach to a surface further in the system line.[18]

Sanitizing and cleaning agents, such as hypochlorite, peroxygens, acids, and quaternary ammonium compounds, are commonly used in the food industry to ensure facility and CIP system cleanliness.[19] The antimicrobial effect of organic acids results from their ability to lower pH and from the toxicity to microorganisms from the undissociated form of the acid.[20-21] All microorganisms have a maximum, minimum, and optimum pH level for growth, and if pH is changed, it can influence the inhibition or growth of the organism.[21] Undissociated acid molecules can easily cross cell membranes of microbial cells and enter the cytoplasm, where the molecules will dissociate due to the cytoplasm pH being more than 6.0.[20] As a result, the cytoplasm pH will be lowered, causing the cell to use energy to force excess hydroniums out of the cytoplasm to regain metabolic pH.[20] Eventually, the cytoplasm pH falls below the level of cell homeostasis,

and the cell dies.[20] While several chemical antimicrobials are available, the presence of organic material can decrease the antimicrobial efficacy of wash solutions.[16-17]

2. *Salmonella*

2.1. Characteristics of Salmonella enterica

Salmonella species (spp.) are rod-shaped bacteria that are facultatively anaerobic.[16, 22] Belonging to the family *Enterobacteriaceae*, *Salmonella* are also Gram-negative, non-sporulating organisms.[23] *Salmonella* grows optimally at 37°C but can also proliferate at temperatures between 5.3°C and 45°C.[16] The optimum pH for growth is near neutral pH at pH 6.5–7.5, but growth for *Salmonella* has been recorded in pH as low as 4.05.[22, 24] Water activity (a_w) levels must also be at or above 0.94 for growth, with higher a_w values being required as the pH decreases, although this is dependent on the environment.[16] While *Salmonella* spp. can catabolize glucose, which results in acid and gas production, the bacterium cannot utilize lactose as a carbohydrate source.[22]

In the genus *Salmonella*, there exists two species: *S. bongori* and *S. enterica. S. enterica* can be further classified into six subspecies (subsp.), including *enterica, arizonae, diarizonae, salamae, houtenae,* and *indica.*[16] Although there are a myriad of *Salmonella* spp. *S. enterica* subsp. *enterica* is usually associated with warm-blooded mammals, and 99% of *Salmonella*-related human infections can be attributed to this particular group of Salmonellae.[25] The *S. enterica* subsp. *enterica* contains over 1,500 serovars and establishes the concept that *Salmonella* can adapt to a variety of ecologically diverse environments to survive.[22, 25] Cold shock proteins are used by *Salmonella* to adapt

to low-temperature environments while certain strains, such as *S.* Seftenberg strain 775W, have high thermotolerance with low water activity.[26] Most *Salmonella* spp. are motile with peritrichous flagella, but *S. enterica* subsp. *enterica* serovar Pullorum and *S. enterica* subsp. *enterica* serovar Gallinarum are non-motile strains due to the lack of functional flagella.[22, 27]

2.2. **Salmonella** *and foodborne illness*

The primary environment for *Salmonella* spp. is in the intestinal tract of animals, such as birds, reptiles, humans, and farm animals, with humans and animals being the primary reservoirs.[16] *Salmonella* can be excreted via fecal matter, and this can result in transmission to humans by insects and other living creatures serving as vectors.[28] Animals can serve as a vector when *Salmonella* is present in the gastrointestinal tract and are excreted in the animal's feces, resulting in an unsanitary environment.[29] *Salmonella* can also be present on the exterior surface of an animal, and contamination can occur when contact is made with fecal matter.[16] As humans and other animals consume contaminated foods and water, and the organisms are shed through fecal matter, the cycle will continue.[28-29] Through these dissemination vehicles, *Salmonella* spp. can eventually be found in water, soils, and farms, resulting in their presence on food commodities through cross-contamination and natural occurrence.[16, 29]

Since 2015, *Salmonella* spp. have been the source of over 35 foodborne illness outbreaks in the US.[30] Over 26% of *Salmonella* cases from 2015 were traced back to fruit sources, such as papayas, cut fruit, and dried coconut.[30] Poultry and egg sources, such as

ground turkey and shell eggs, were the next leading cause of *Salmonella* cases during this time with over 23% of *Salmonella* cases.[30] Together, these food categories had 1653 cases, 557 hospitalizations, and 6 deaths attributed to *Salmonella* infection and made up 50% of *Salmonella* food-related cases from 2015 to 2019.[30]

2.2.1. Salmonellosis

Salmonellosis is a gastrointestinal disease that typically lasts 4–7 days, although chronic salmonellosis can occur.[31-32] *Salmonella* infections can occur in humans when as few as 10 cells per gram are present.[16] Rates of salmonellosis are the highest from May through October.[33] This may be due to the increased occurrence of temperature abused foods and/or cross-contamination of foods at cookouts during the summer months.[29] However, the more likely cause is that higher ambient temperatures provide an environment suited for rapid bacterial replication.[34] Children under the age of 5, older adults, and immunocompromised individuals are at the highest risk for infection, requiring the consumption of fewer cells in order to develop symptoms.[16, 29, 35]

Symptoms for salmonellosis can appear in as few as 4 hours, but the average incubation period until the onset of clinical illness is regarded as 12-14 hours.[16, 32] Salmonellosis can cause lower gastrointestinal tract symptoms within an infected person.[16, 32] These symptoms can include abdominal cramps, diarrhea, vomiting, fever, chills, nausea, and a possible headache.[28, 30] Most individuals recover without needing antibiotic treatment, but in severe cases, *Salmonella* can infect the bloodstream or other

parts of the body.[31] Severe cases result in an individual needing medical attention, and death can occur if the disease is not treated.[35]

Although salmonellosis has been causing illness for over 125 years, it has only been a notifiable disease in the U.S. since 1942.[29, 35] While it is mandatory that reportable disease cases are reported to the state and territorial jurisdictions, it is voluntary that notifiable diseases are reported to the CDC by state and territorial jurisdictions.[36] Since 1942, the rate of reported cases for salmonellosis has increased over time, but this could be attributed to more awareness, surveillance, and sampling.[29, 35] Recently, rates of *Salmonella* cases have remained relatively constant, with approximately 1 million confirmed *Salmonella*-derived foodborne illnesses occurring per year in the U.S.[37] With non-typhoidal *Salmonella* resulting in an annual estimate of 378 deaths a year in the U.S., decreasing the rates of *Salmonella* spp. in the food supply is a priority.[38] This is a priority not only due to the hospitalizations and/or fatalities that may occur, but also due to the economic impact that salmonellosis can have annually.[39] In the U.S., the economic impact of salmonellosis can be as high as $10.9 billion per year, and the cost of illness is estimated at over $3.3 million per year.[8, 31, 40]

3. Biofilms

The first indication possibly describing biofilms was when cell aggregates were seen during dental plaque examinations by Anton von Leeuwenhoek.[41-44] Some claim that Henrici accurately recorded biofilms when observing bacteria that were able to grow on

submerged surfaces in water.[45] Regardless of the initial discovery, biofilms have since been described in a variety of environments outside of an oral cavity and freshwater.[45-48]

The name "biofilm" was established by Bill Costerton in 1978.[41, 46] A basic description is that biofilms are composed of microorganisms attached to either a surface or themselves with an extracellular polymeric substance (EPS) layer, and sessile cells contained within biofilms are different from planktonic cells.[43, 49-52] Since biofilms have been seen in a variety of environments, specific definitions of biofilms vary minutely across foci, but the general concept remains as described.

3.1. Ability of microorganisms to form biofilms

Organisms must synergize with other organisms, sometimes of other species and phyla, with success to survive.[53-54] The production of EPS is an example of how individual cells can coordinate to work in communities. However, methods of intracellular communication for microorganism community building have been debated due to the complexity of the biofilm interior.[53-55]

There are methods of cell communication, such as quorum sensing, chemotaxis or release of extracellular DNA, which do not rely solely on EPS. Quorum sensing was first described when *Vibrio fisheri* was observed secreting autoinducer (AI) molecules.[23, 56] Autoinducer 2 (AI-2) is a quorum sensing signal that has been shown to stimulate biofilm formation.[57-58] Cyclic-di-(3'-5') guanylic acid (c-di-GMP) is a secondary messenger responsible for relaying environmental conditions intracellularly and is highly researched in *S.* Typhimurium.[59-60] Extracellular DNA released from lysed cells within the biofilm

can be important for intracellular communication for certain organisms, such as *Pseudomonas aeruginosa*.[61] The broad spectrum of cell communication systems strengthens the argument that these systems do not exclusively regulate biofilm development, but rather, biofilm development is a component that some cell communication systems contribute towards.[53]

3.2. Biofilm development

The formation of a biofilm is a complex process, consisting of a variety of intrinsic and extrinsic components. Changes in gene expression regulating motility appendages and EPS-producing compounds can assist in bacteria attaching to a surface.[51] The reduction of nutrient availability in the environment can also promote faster formation of biofilms for some organisms.[62-63] Model systems of non-equilibrium, non-living systems have been utilized to attempt to understand the formation and structure of biofilms.[54, 64] While these model systems can be utilized, different systems yield differing results, and the complexities of biofilms cannot give consistent results, especially when a variety of species are taken into consideration.[54]

3.2.1. Attachment

Being able to convert from a planktonic to a sessile state is important for biofilm attachment success.[41, 65-66] The ability to convert to being immobile instead of planktonic depends on a variety of environmental factors and can be dependent on the organism's

ability to mediate attachment.[51] The organisms must then be able to convert to being irreversibly attached and still be able to proliferate within the biofilm.[67]

Attachment for bacteria is thought to be able to occur when an environmental condition, or combination of conditions, triggers planktonic bacterium to seek a sessile state.[51, 68-71] Motility appendages, such as flagella, fimbriae, or pili, and surface protein presence can play a crucial role in biofilm formation, but may not be necessary depending on the environment.[72-75] Bacterial cells can produce signals for a cell to use a glycocalyx structure to attach to a surface.[46, 53] Cells can aggregate at the site of attachment using the organism's motility mechanisms or via an aqueous flow in which the organisms are suspended.[45]

Once attached, cells can either be reversibly or irreversibly attached.[76] When cells are in the reversible attachment stage, bacteria are kept in place through a combination of steric, electrostatic, hydrophobic, and van der Waals interactions.[77] Cells will frequently de- and re-attach during this stage through nearby shear forces, such as an aqueous flow.[76-77] Cells can enter an irreversible attachment stage where cells form a surface monolayer and aggregate together to form a microcolony.[51] The utilization of type IV pili to twitch or "crawl" across surfaces to form these microcolonies leads to successful biofilm formation.[69, 78] The confirmation of irreversible attachment can also indicate sessile bacteria are prepared to produce EPS.[53]

3.2.2. Proliferation, maturation, and detachment

Bacterial settlement can be determined as a "success" when cells adhere and are able to grow and cooperate metabolically in the biofilm community.[67] Single- and multispecies presence can influence the proliferation of organisms contained within the biofilm, and fitness of the organism's level of growth could be related to EPS producing ability.[43, 79] As microorganisms proliferate, EPS are excreted, encasing the biofilm in a scaffolding matrix.[80] During proliferation, cells within biofilms can function as cooperative groups by releasing extracellular signals to upregulate EPS production and other products.[44, 60, 81] These byproducts assist in distinguishing between microcolonies within the biofilm community.[60, 81]

Complexities within the extracellular matrix of the biofilm arise with continued proliferation leading to biofilm maturation. The ability to gain nutrients in a biofilm can be difficult as diffusion must be able to overcome biochemical and structural obstacles.[82] Once nutrients permeate a biofilm, bioavailability of nutrients to cells depends on successful diffusion past the exterior portion of the biofilm structure.[83-84] As more EPS is produced, channels and pores begin to form within the biofilm, creating a highway for transporting water, oxygen, and nutrients throughout the biomass while excreting waste products from the interior portion.[85-86]

As biofilms mature further and proliferation of microorganisms continues, an increase in biomass occurs within the biofilm. Biofilms begin to take on a mushroom-like appearance as biomass accumulates and more channels begin to form.[85] As biofilms become larger, pieces of biofilms can detach from the biofilm structure.[42] Biofilm

detachment can occur via passive dispersal where shear stress from fluid flow in the environment can cause sections of the exterior portion of the biofilm to be removed from the main biofilm structure.[82] It could be that there are also environmental cues for detachment from biofilms, such as chemical or physical gradient changes.[42, 82, 87] Cells can additionally leave through channel or pore openings or revert to planktonic cells and disperse to repeat the biofilm process again.[88]

3.3. Biofilm matrix

The EPS can be described as a mass of polysaccharide fibers that extend outside of the surface of the bacteria and forms a glycocalyx around and between cells.[46] It is considered one of the most crucial characteristics of bacterial biofilms as the EPS matrix acts as a means for direct and indirect cell interaction as well as protection.[89] The EPS also provides protection from extrinsic forces and antimicrobials as well as creating the network of pores and channels for nutrient and oxygen supplies.[86, 90] Microorganisms within biofilms utilize the EPS matrix to communicate with other cells and their environment, both the microenvironment and outer environment.[89] The EPS matrix contains polysaccharides, proteins, lipids, nucleic acids, enzymes secreted from cells, and materials from the surrounding environment.[91] This complex network enables the microbial success of a biofilm community.

A major component of the EPS layer is polysaccharides. Cells utilize polysaccharides to adhere to the EPS matrix present on cell surfaces and onto substrate surfaces.[90] Cellulose, an extracellular polysaccharide, is an indicative factor of bacterial

ability to form biofilms[79, 90, 92] and is a major component of *Salmonella* biofilms.[93] By interacting with curli present in an extracellular matrix, cellulose can provide structure and support cell adhesion.[93] In addition to biofilms being more resistant to antimicrobials, cellulose presence can provide resistance to chlorine treatment against *Salmonella* serovars.[94]

O-antigen polysaccharide is another extracellular polysaccharide that can be produced by *Salmonella* to assist with biofilm development.[94] O-antigen polysaccharide provides *Salmonella* spp. biofilms with the ability to persist through desiccation stress and sublethal injury.[94] The utilization of both cellulose and O-antigen has been shown to enable *Salmonella* to attach and form biofilms on plants and plant food products.[95]

3.4. Biofilms in food processing environments

Food processing environments are ideal environments for microorganisms due to the large volume of water usage, constant food matter available to serve as nutrient sources, and high number of human personnel involved.[16] The large variety of equipment surfaces, material types, and drains and pipes present in a food processing facility are perfect harbor points for bacterial growth.[16, 96] If cleaning, sanitation, and hygienic practices are not implemented correctly, microorganisms can thrive in food processing environments.[16, 97] Additionally, biofilms present in a food processing facility can allow for a persistent pathogen source with detachment allowing for microorganisms to travel throughout a facility.[98-100] Eventually, food products, materials, and contact and non-

contact surfaces can become contaminated, leading to large scale effects, such as food recalls and plant closures.[97-100]

3.4.1. Material surfaces

Equipment and devices used in the food industry can vary from personal protective equipment to water-wash tanks used to clean produce.[16] Stainless steel, aluminum, polypropylene, rubber, silicone and other materials are commonly used in the food industry.[79, 101] Metals, such as stainless steel, aluminum, copper and tin, are commonly used throughout a variety of food industries.[102] While glass is one of the most commonly used materials in the food industry, metal and plastics are more common, with metals being the most important material used in food processing equipment.[102]

The type of bacterial species can affect the surface they attach to. [42, 67] In the mouth, only teeth, epithelial mucosa and the newly formed surface on growing dental plaques act as available surfaces, so organisms must be able to adapt and attach to the surfaces made available in the environment.[67] While the function of devices and equipment used in the food and healthcare industries differ, the materials utilized to create these machines and tools are similar.[47, 101]

Stainless steel is utilized for manufacturing a major component of most processing equipment.[17] The food industry uses stainless steel austenitic grades 304 and 316 the most due to the ease of cleaning, high temperature stability, and resistance to corrosion.[17] Steel surfaces can also be finished differently, such as via cold rolling or

electro-polished.[103] The more polished or finished and smooth a surface, the less likely bacteria will be able to attach and colonize the surface.[103-104]

Surface roughness, a characteristic based on the distance between peaks and valleys present on a material's surface, can also cause increased opportunity for biofilms to form.[16, 104-105] Crevices present in rough surfaces provide bacteria with harborage points.[105-106] These crevices can increase the likelihood of bacterial attachment to occur and colonize surfaces.[66, 107] Deterioration of equipment surfaces can cause biofouling and can also harbor bacteria.[108] With rigorous cleaning protocols and continued mechanical abrasion, food processing equipment surfaces become more rough over time.[19] Prolonged bacterial presence could affect the equipment surface as well, causing corrosion or increased porosity. Biofilms can also contribute to surface corrosion since some bacteria can produce acid that can corrode equipment surfaces.[109]

The surface charge of materials can also contribute to bacterial attachment.[101, 105] Depending on the surface material, the negatively-charged bacterial surface can be repelled. Metals are negatively charged at the surface and hydrophilic.[107, 110] Teflon-coated surfaces are hydrophobic due to having a lower electrostatic-charge.[105, 110] Surface hydrophobicity can be correlated to contact angle, with hydrophobic surface contact angles above 90° being favorable for prevention of fluid pooling.[111] There are some exceptions, such as *Listeria monocytogenes* being able to attach to a variety of material surfaces, whether the materials are hydrophobic or not.[101, 112] Though there is evidence that charges can cause attraction or repulsion towards bacterial attachment, a study by Baker[113] showed that materials, such as polystyrene or hydrophilic glass, show no

statistical difference on attachment rates. Since this study, food processing equipment has advanced, leading food engineers to develop equipment that would be less likely to harbor bacteria.[114]

3.4.2. Treatment

While advancements towards equipment surfaces have been made, surface contact interventions, such as the application of a Teflon film, are not enough to be a standalone prevention of biofilms in the food industry.[115] When food matter is present, antimicrobial coated surfaces can still harbor bacterial growth and attachment.[115] If improper cleaning and sanitization occurs, microorganisms can seize the opportunity to utilize secure attachment to equipment and facility surfaces, and proliferation can occur due to the high nutrient availability that was not removed in routine cleanup.[16, 105, 108]

Treatment must be preventive instead of proactive. A combination of antimicrobial interventions are utilized to create a hurdle effect, with the premise being that with each hurdle applied against the organism, bacterial fitness in the environment decreases.[16] Common intervention practices include (i) mechanical action, such as scraping or sonication, (ii) chemical intervention, seen in the use of quaternary ammonium compounds or membrane-disrupting sanitizers, and/or (iii) thermal intervention which can be the use of chemical interventions in combination with high water temperatures or the removal of heat during freezing processes.[16, 105, 108] An industry lacking in these areas could cause microorganism growth to progress to the point where bacterial contamination of the food can occur.[108]

Increased sanitization and medicinal resistance is also a characteristic of biofilms.[41, 44, 116] Sessile cells contained within a biofilm can become more resistant to sanitizing or antimicrobial methods than their planktonic counterparts.[79, 101, 117-118] Microorganisms within biofilms can proliferate at a slower rate than their planktonic counterparts.[50] Due to the decreased metabolism, increased resistance towards antibiotics or bactericidal interventions can occur.[41] Permeability through the complex biofilm matrix could also be related to the amount of biofilm biomass density; the higher the cell density, the lower the diffusion.[41, 64, 119]

The ability of bacteria to utilize efflux pumps for transport of antibiotics out of the cell and to maintain homeostasis of chemical gradients are critical towards preventing cell death.[41, 47] Successful treatment of bacterial biofilms can also be strain-dependent. When comparing various strains of *S.* Typhimurium and *Escherichia coli* O157:H7, EPS-producing strains in biofilms had higher resistance to quaternary ammonium compounds and chlorine sanitizing solutions compared to planktonic cells.[79] Multispecies biofilms can also contribute to developing an increased antimicrobial or sanitizer resistance in bacteria that have low biofilm-forming ability.[79] Low- to no-curli and cellulose producing *S.* Typhimurium and *E. coli* O157:H7 strains were able to have higher sanitizer resistance in multispecies biofilms compared to single-species biofilms of the same strains.[79] Methods for antimicrobial and sanitizer resistance also vary and are dependent upon the antibiotic or sanitizer type, the species, or the environment.[41] Since biofilms can be diverse and dependent on the environment and presence of other microorganisms, the methods for treatment should be developed case-by-case.

4. Emulsions

Food emulsions are complex systems composed of two immiscible liquids, and fall within the realm of colloidal systems consisting of oil droplets distributed within a liquid medium.[120-122] An oil-in-water (O/W) emulsion will occur when the oil fraction occupies the dispersed phase.[123-124] Oil can also occupy the continuous phase and water-in-oil (W/O) emulsions will form.[123-124] Most emulsions are O/W or W/O, but there are complex multiple emulsions that can be oil-in-water-in-oil (O/W/O), water-in-oil-in-water (W/O/W), and solid-in-oil-in-water (S/O/W) combinations.[122]

4.1. Types of emulsions

Emulsions are commonly classified based on droplet size. Food emulsions are generally in the micrometer (µm) droplet diameter range, while nanoemulsions and microemulsions have droplets with a diameter being in the nanometer (nm) range.[125-128] Emulsions can also be distinguished from one another based on thermodynamic stability and structure.[129]

4.2. Distinguishing between nano- and microemulsions

There are discrepancies about the droplet size of microemulsions, and the definition has been redefined several times.[124] Mason[130] and McClements[120, 129] both state that the main difference between the two are that nanoemulsions are metastable with high

interfacial tension present whereas microemulsions are thermodynamically stable and have extremely low interfacial tension.

4.2.1. Droplet size distribution

Droplet size can determine emulsion properties, such as appearance and stability.[131] Emulsions that have droplets of only one size are described as monodisperse whereas polydisperse describes emulsions with droplets that have a range of sizes.[132] For droplet size distribution, histograms of volume frequency demonstrating size-class of droplets are often utilized.[133] A common method of determining mean droplet size for an emulsion is by determining the area-volume mean diameter (**Equation 1.1**) where n_i is the number of droplets with diameter d_i.[134]

$$d_{3,2} = \frac{\sum n_i \, d_i^3}{\sum n_i d_i^2} \qquad \textbf{1.1}$$

The overlapping size areas for nano- and microemulsions have caused some confusion. McClements[123] describes the droplet radius of emulsions, nanoemulsions and microemulsions to be from 100 nm – 100 µm, 10-100 nm, and 2-50 nm, respectively. Sanchez-Dominguez[135] agrees with McClements' size cutoff, but Narang[136] categorizes microemulsions droplet radii to be below 200 nm. It is important to take droplet size into consideration along with thermodynamic stability to determine whether a formulation is a microemulsion.

4.2.2. Thermodynamic stability

Droplets in nanoemulsions are not thermodynamically stable while those in microemulsions are stable.[130] While large droplets can cause emulsions to be opaque, nanoemulsions can also be translucent.[130] The droplet size in nanoemulsions can be small enough that droplets smaller than optical wavelengths cannot refract light.[130] Over time, nanoemulsions can break down due to coalescence, flocculation, or Ostwald ripening.[137] The rate of nanoemulsion breakdown depends on a variety of factors, such as oil and surfactant type and temperature.[120]

Microemulsions are thermodynamically stable and transparent.[138] Surfactant molecules within O/W microemulsions are highly organized, and non-polar surfactant tails associate closely with each other to form a hydrophobic core capable of dissolving nonpolar components.[120] This requires high concentrations of surfactant to be used for microemulsions to ensure there are enough surfactant molecules to promote these highly organized micelles.[120, 129] The free energy associated with the colloidal dispersion of oil droplets in water must be lower than the free energy of the separate, individual oil and water phase to create a thermodynamically stable microemulsion.[120] However, a microemulsion is only thermodynamically stable under a particular set of conditions, such as temperature and surfactant concentration, and mechanical and/or thermal energy may be required to reduce the energy barrier in a formulation.[120, 129]

4.2.3. Energy method required

Nanoemulsions can sometimes require a high-energy method of preparation whereas microemulsions can utilize low-energy preparation methods.[124] In high energy

methods, nanoemulsions are created when mechanical energy can overcome the surface tension energy barrier.[139] High-pressure homogenization or colloidal mill devices are some of the commercial unit operations utilizing high mechanical energy.[139]

Self-assembly is utilized for microemulsion formation. When microemulsions are created, the interfacial area is increased as the droplet size decreases.[124] This causes the overall entropy to increase which causes the system to be thermodynamically favorable by having negative free energy.[124] Microemulsification can be increased when heat and/or mechanical agitation is applied to the system.[124] Non-ionic surfactants are commonly used to induce phase inversion to form microemulsions.[124] Surfactants cause a lowering of surface tension due to adsorbing strongly at the interface of materials.[131] By lowering the interfacial tension, surfactants also lower the Laplace pressure inside a droplet.[131]

Nanoemulsions and microemulsions can also be created using the Phase Inversion Temperature (PIT) method. PIT is a low-energy method where the composition of an emulsion can be kept constant while the temperature is increased and then rapidly decreased to create the spontaneous formation of droplets that are smaller in size than before.[122, 140] The PIT is the temperature where the affinity of the surfactant for the water and oil phases used is balanced, and rapid cooling once the PIT is reached results in the formation of smaller droplets.[122]

4.3.1. Surfactants

Emulsions are not thermodynamically stable, and interfacial tension between oil and water phases needs to be reduced to have a thermodynamically stable system.[120] Surfactants are molecules that can reduce the interfacial tension between water and oil phases.[141] When surfactants are not used, the volume fraction of each phase heavily influences the type of emulsion that is formed. When a surfactant is present, the surfactant used will determine the type of emulsion formed, regardless of the volume fraction of the phases.[123]

4.3.1.1. Hydrophile-lipophile balance (HLB)

The HLB is a measure of the hydrophobic and hydrophilic parts of a molecule and can be used to classify surfactants.[122-123] The numeric HLB value is based on the weight addition of the surfactant compared to the hydrophile-lipophile property of the surfactant.[142] An HLB value ranges from 1-20 and will indicate whether a surfactant is more soluble in water or oil.[123] Surfactants with an HLB value above 8 are mainly hydrophilic and can form O/W emulsions, while HLB values below 6 are hydrophobic and can form W/O emulsions.[124, 136] An HLB near 7 has relatively equal solubility in both oil and water phases, causing the solubility to not be very high.[122]

4.3.1.2. Surfactant stabilized emulsions

Surfactants are amphiphilic molecules that have a polar, hydrophilic head group with a non-polar, hydrophobic tail.[123-124] Interfacial tension in an emulsion system is lowered by surfactant addition to a solution.[135] When surfactants are added into oil and

water mixtures, the surfactants will self-assemble at the oil-water interface.[130] To ensure a system can have a stable and long shelf life, surfactants are considered necessary.[130]

Surfactant types can also vary and be classified as either non-ionic, anionic, cationic, or Zwitterionic depending on the head group charge.[123-124] No charge on surfactant head groups yield non-ionic surfactants, negative charges yield anionic surfactants, positive charges provide cationic surfactants, and Zwitterionic surfactants have head groups with both charges present.[123] Phospholipids, such as lecithin, are Zwitterionic and are highly utilized in emulsion creation.[124] The tail part of surfactants are made up of single or multiple non-polar hydrocarbon chain.[123] Single chain surfactants are highly hydrophilic so a cosurfactant or electrolyte is required, but double chain surfactants are not mandatory for microemulsion formation.[135]

As mentioned, cosurfactants can also be added into emulsion systems. Cosurfactants are often used to create microemulsions due to their ability to lower interfacial tension.[124, 136] The decrease in interfacial tension is due to the cosurfactant being able to reduce the dielectric constant of the water phase.[136] Medium- to short-chain alcohols can act as cosurfactants by reducing interfacial tension that causes a barrier between the oil and water phases, leading to solubility being higher.[124]

4.3.1.3. Non-ionic surfactants

The food industry utilizes non-ionic surfactants due their biodegradability and the wide range of HLB values available.[123] Common non-ionic surfactants include sugar esters that have head groups derived from items such as, but not limited to, glucose,

sucrose, and raffinose and tail groups originating from palmitic, oleic, or linoleic acid.[123-124, 143]

4.3.1.4. Sorbitan esters and polysorbates

Sorbitan esters of fatty acids are non-ionic surfactants that are also known as Span(s)®.[144] Spans are waxy solids or viscous liquids that are products of a reaction between sorbitol and a fatty acid.[144-145] Sorbitan esters can be modified into polyoxyethylene sorbitan esters or polysorbates. To produce polyoxyethylene sorbitan esters, sorbitan esters are reacted with ethylene oxide and a small amount of a catalyst, such as potassium hydroxide.[146]

Polysorbates, or Tweens, are non-ionic, hydrophilic surfactants that have a high HLB value.[146] Tween® is a steric stabilizer due to the large hydrated polyoxyethylene chains repelling each other.[146] The most common Tween types include Tween 20, or polyoxyethylene (20) sorbitan monolaurate, and Tween 80, or polyoxyethylene (20) sorbitan monooleate.[146] Both Tweens contain 20 oxyethylene units, but the fatty acid associated with each polyoxyethylene sorbitan portion of the molecule differs.[146] Tween 20 is derived using lauric acid while the synthesis of Tween 80 utilizes oleic acid.[146]

4.3.2. Disruptions to emulsion stability

Due to the small droplet size of microemulsions, Brownian motion is the main mechanism describing droplet dynamics.[135] When it comes to gravitational force, emulsions are highly affected.[129] Due to the density difference between continuous and

dispersed phases in thermodynamically unstable emulsion systems, droplets can move due to gravitational influence.[147] While nanoemulsions are more resistant to gravitational separations than emulsions, flocculation, Ostwald ripening, and coalescence can still occur.[129]

There are multiple mechanisms that can cause emulsion stability to be disturbed, such as coalescence, Ostwald ripening, and gravitational separation (**Figure 1.1**). Flocculation occurs when droplets adhere to each other and form flocculates or aggregates of droplets.[137, 148] Coalescence can cause stability issues if the surfactant used does not provide a strong repulsion at the droplet interface.[130] When two droplets come into close contact, the droplets can collide and slightly deform.[123] The increased surface area between the droplets upon contact can disrupt the interfacial tension present on the droplet surface which will disrupt packing of surfactants in the interfacial film.[137, 149] With disrupted interfacial packing, droplet can fuse and a large droplet will form containing the contents of both previously small droplets.[137, 149]

If the dispersed phase has even a slight solubility in the continuous phase, Ostwald ripening can occur.[131, 150] Smaller droplets present in the system have higher Laplace pressure than large droplets.[150] Over time, molecules from the small droplets will diffuse out of the small droplet and diffuse into the large droplet that has a lower Laplace pressure.[150] This will cause an increase in droplet size, causing instability in the emulsion.[150] If surfactant concentration decreases, then a system will undergo Ostwald ripening to decrease the interfacial energy.[151] The difference between Ostwald ripening and coalescence is that Ostwald ripening is a diffusion-driven mass transport whereas

coalescence is a kinetic-driven process that relies on the droplets coming into contact.[131, 137]

4.4. Nano- and microemulsions of essential oils and inhibition of biofilms

Having a smaller droplet size can enable antimicrobial compounds, such as essential oils (EOs), to penetrate through bacterial cell walls more efficiently due to the small droplet size and large surface area increasing antimicrobial activity.[152] Al-Adham *et al.* created a physically stable microemulsion containing 64 ppm sodium pyrithione, an antimicrobial derived from aspergillic acid, that was able to reduce *P. aeruginosa* present in biofilms by up to 3 $\log_{10}$ CFU/ml.[153] When filter plugs used for biofilm growth were exposed to microemulsions prior to bacterial inoculation, *P. aeruginosa* biofilm formation was 1.0 $\log_{10}$ CFU/ml lower than the exposure of the saline control.[153] Once the microemulsion was added to the biofilm-growth system, *P. aeruginosa* growth was hindered within the biofilm and viability of bacteria within the biofilm structure was decreased compared to the saline control.[153] A nanoemulsion containing 25,000 ppm cinnamon oil (CO) was able to decrease *S. aureus* biofilms formed on stainless steel surfaces by 1.0 $\log_{10}$ CFU/cm^2 after a 15 min exposure.[154] The CO nanoemulsion was also able to reduce *S. aureus* from biofilms by more than 1.0 $\log_{10}$ CFU/cm^2 when biofilms were formed in nutrient-rich microbiological media.[154] A curcumin (4,000 ppm) and geraniol oil (20,000 ppm) microemulsion had greater biofilm inhibition of *P. aeruginosa* on plastic surfaces compared to emulsions containing 4,000 ppm curcumin by more than 15%.[155]

5. Essential oils

Recently, emulsion antimicrobial delivery systems have been growing in interest.[156] Emulsion systems can be easily modified to deliver a wide variety of antimicrobial agents.[16, 156] Encapsulation has the potential to increase the antimicrobial activity of a compound by increasing the surface area of the particle or droplet, which in turn increases the bioavailability.[120, 157-158] The utilization of EOs as an antimicrobial agent has also increased due to the increasing consumer demand for natural products.[159] EOs and their constituents can have high antimicrobial activity and have been encapsulated in a variety of emulsion systems.[152, 160-165]

EOs are secondary metabolites in plants that assist with plant-plant communication, defense, and pollination and are found in the flower, bud, seeds, bark, fruits, leaves, and roots of a plant.[166-167] EOs have been widely applied in cosmetic products, such as skim creams, perfumes, and soaps, and have been used for spices and seasonings in food.[168] Recently, EOs are gaining interest as antimicrobials due to their antimicrobial, antifungal, antiviral, and insecticidal activity.[166, 169] Additionally, many EOs and their constituents have been approved generally-recognized-as-safe (GRAS) status in the US.[170]

5.1. An overview of EOs

It has been observed that date of extraction, plant origin, state of the vegetable, growing season, and storage can contribute to differing compounds in EOs.[171] EOs can

be obtained by physically extracting the oils from a plant through various pressing, distillation, and solvent extraction methods.[172-174] There is discrepancy on which type of method to use since methods can affect the loss of volatile compounds, be too time intensive, be inefficient, and degrade the EO constituents in the extraction process.[172, 175] This may explain why chemical compositions for EOs vary greatly depending on extraction type and plant origin. Solvent extraction and steam distillation methods are the most common methods used for EO extraction, with cold press methods used for citrus EOs.[172-173, 176]

Primarily, there are two circumstances that dictate whether a plant will be EO-producing: volatiles blended in a unique manner, e.g. scented flowers, and the storage of volatiles from secretions.[172] If the volatiles are stored from secretions, there are special structures, such as idioblasts or glandular trichomes, which contain the volatiles.[177-178] Each EO is comprised of a multitude of compounds in variable concentrations.[166, 169] The primary compounds of EOs typically come from three pathways: the methyl-erythritol-pathway giving way to mono- and diterpenes, the mevalonate pathway giving way to sesquiterpenes, and the shikimic acid pathway giving way to phenylpropenes.[172]

5.1.1. Mono- and sesquiterpenes

Monoterpenes and sesquiterpenes both have a five carbon (C_5) base unit called an isoprene.[168-169] Due to the isoprene unit, monoterpenes have a basic $C_{10}H_{16}$ molecular formula while sesquiterpenes have a $C_{15}H_{24}$ molecular formula.[179] Both can be categorized as acyclic, monocyclic, bicyclic, and tricyclic.[179] Monoterpene structures are

usually derived from the isoprene carbon skeleton after isomerization rearrangement or oxygenation causes conversion to alcohols, aldehydes, ketones, or esters, and sesquiterpenes are generally cyclic structures in nature.[179] Monoterpenes usually constitute ~90% of EOs and include *p*-cymene, limonene, linalool, thymol, and carvacrol.[169] A multitude of EO constituents can be in one plant, such as seen with oranges containing both mono- and sesquiterpenes.[169, 176]

5.1.2. Phenylpropenes

Phenylpropanoids refers to any compound containing a three carbon (C_3) chain attached to a six carbon (C_6) aromatic benzene ring.[179-180] Most phenylpropanoids are derived from cinnamic or *p*-coumaric acids, which are synthesized from L-phenylalanine.[180] Phenylpropanoid metabolism provides a wide array of secondary metabolites that are utilized by several other metabolic pathways.[180-181] Plant resistance towards pests can also be mediated by plant phenylpropanoid production.[182] Cinnamaldehyde from cinnamon bark and leaf oil and eugenol from cloves are common phenylpropanoids.[183-184]

5.2. Mechanism of action against bacteria

Bacterial membranes have three primary functions: to serve as a barrier from external forces, to act as an energy transductor, and to serve as a matrix for transmembrane proteins.[167] Gram-negative bacteria are generally more resistant to EOs compared to Gram-positive bacteria due to the differences in cell wall structure (**Figure**

1.2).[185] Gram-negative cell walls have a thinner peptidoglycan layer than those of Gram-positive bacteria.[16, 186] The Gram-negative peptidoglycan layer is surrounded by an outer membrane which are linked by Braun's lipoprotein.[186] The outer membrane is made up of a phospholipids bilayer that is linked to an inner membrane by lipopolysaccharides (LPS).[186] LPS contains a polysaccharide, known as lipid A, and an O-side chain.[186] Hydrophilic transmembrane channels within the outer membrane limit passage of solutes into the cell and also contribute to Gram-negative bacteria being more resistant to hydrophobic EOs and drug treatments.[186] Though passage of hydrophobic compounds are limited, hydrophobic molecules can still slowly travel through porins present in the cell membrane.[186]

Antimicrobial activity of EOs is driven largely by the breakdown of chemical components in the EO.[169, 187] Generally, antimicrobial activity of the constituents of EO in decreasing order are phenols > aldehydes > alcohols > esters > hydrocarbons.[188] The hydroxyl groups in phenolic compounds plays an important role as the hydroxyl group increases the solubility of the phenolics in lipids when interacting with the fatty acids present on the microbial cell membrane.[189-191] The positioning of the hydroxyl group is also of importance as different placement can affect antimicrobial activity against Gram-positive and Gram-negative bacteria.[189] Stereochemistry is also highly influential in determining effectiveness of the EO; α- and *cis*-isomers tend to be inactive compared to β- and *trans*-isomers, respectively, and the unsaturation of cyclohexane rings enhances antibacterial properties.[189] Regarding terpenoids, antibacterial effectiveness was increased when alkylation had occurred or if a carbonyl group was present.[189]

It is difficult to determine the antimicrobial activity of EOs as the constituents can affect singular or multiple items that can result in cell death. EOs have been shown to degrade microbial cell walls, damage cytoplasmic membranes, disrupt the electron transport chain, and increase cell permeability which ultimately leads to the leakage of cell contents.[167, 191-195] Phenolic compounds can insert into the phospholipid layer in cell walls and bind to proteins, causing a disruption in normal cell function.[166, 191] When *Bacillus cereus* was exposed to 2 micromolar (µM) carvacrol, there was no increase in extracellular ATP while intracellular ATP loss was associated with a decrease in membrane potential.[193] When using a combination of orange oil and bergamot, cell permeability was increased while a decrease in intracellular pH and membrane potential occurred.[195] In both *E. coli* and *Staphylococcus aureus*, cinnamon EO treatment led to an efflux of electrolytes and decreased metabolic activity.[196] A series of simultaneous and/or subsequent events caused by the introduction of EOs and/or their constituents is believed to be the mechanism of action rather than a singular target. For both Gram-negative and Gram-positive bacteria, it is understood that cell death occurs when the structural integrity of the cell membrane is compromised and leads to membrane permeability causing cell death.[166, 191, 197]

Studies trying to identify an antimicrobial mechanism of action primarily investigate EO constituents, but there are emerging that argue that the minor constituents found in whole EO also contribute to the overall antimicrobial activity.[166-167, 196-198] EOs can exhibit synergistic effects when combined with common antibacterial agents as well as other EOs.[199-205] Some combinations of EO constituents have been shown to be more

effective at producing antibacterial results.[199-205] In a study evaluating the effects of 32 EOs on five different bacteria, EOs combined with a cinnamon EO displayed an additive antimicrobial effect against *E. coli, L. monocytogenes, S. aureus, S.* Typhimurium, and *P. aeruginosa*.[203] When applied to lettuce surfaces, combinations of *trans*-cinnamaldehyde, a phenylpropene aldehyde from cinnamon EO, and eugenol, an allylbenzene from clove EO, had enhanced antimicrobial efficacy against *E. coli* O157:H7.[205-206]

5.3. Cinnamon oil

Cinnamomum zeylanicum contains a myriad of constituents.[207] Cinnamaldehyde has been shown to be the primary EO constituent making up anywhere from 58-71% of the formulation of CO.[161, 208-209] Depending on the extraction method, other CO components consist of carboxylic acid, linalool, cinnamic alcohol, and eugenol among other compounds.[207-209]

5.3.1. Mechanism of action

C. zeylanicum EO seems to be able to weaken and alter the membrane of cells and promote intracellular compound leakage.[209] This was observed when *E. coli* cell walls appear to have collapsed after exposure to cinnamon oil (CO).[209] The observed cell shape seem to further the idea that membrane lysis, integrity, and permeability are all acted on by the EO.[196, 210] A separate study also reported that the lipid composition of cell membranes of *E. coli* O157:H7, *S. aureus, S.* Typhimurium, *P. fluorescens,* and *B. thermosphacta* was altered when treated with thymol, carvacrol, limonene, eugenol, or

cinnamaldehyde.[211] A decrease in unsaturated fatty acids among all treated cells were evident after the EO constituent exposure.[211]

5.3.2. Antimicrobial effectiveness against *Salmonella* sp. in food

Cinnamon oil has proven effective at reducing cell counts of various *Salmonella* species in food.[212-215] On organic romaine lettuce and iceberg lettuce, a 0.5% CO wash was compared to treatment with phosphate buffered saline and *S.* Newport was not present until after 3 days at refrigeration (4°C) and temperature abused (8°C) storage conditions.[212] In hummus, 0.5% CO levels were able to hinder the growth of a *Salmonella* cocktail containing *S.* Typhimurium, *S.* Heidelberg, *S.* Kentucky, and *S.* Copenhagen for 7 days at refrigeration (4°C) and temperature abused (10°C) storage.[213] Cinnamon bark and leaf oils were also effective at reducing *S.* Typhimurium levels to be below 2.0 $\log_{10}$ CFU/g on fresh celery after 7 days of storage at refrigeration (4°C) temperature.[214]

5.4. Orange oil (OO)

Citrus EOs contain a high amount of terpene hydrocarbons, which are unstable when exposed to heat or light.[216-217] The primary constituent of OO is limonene and can be found at levels as high as 98%.[176, 218-219] Other compounds found to make up OO are α-pinene, linalool, citral, β-myrcene, and α-terpineol.[176]

5.4.1. Mechanism of action and antimicrobial effectiveness against *Salmonella* spp.

OO has been widely used for antifungal treatments and foodborne pathogen literature is limited.[217, 220] The high oxygenated compound make-up of OO makes it have a lower inhibition level against foodborne pathogens compared to other EOs.[217-218] In *S. aureus*, cold-pressed Valencia OO inhibiting cell wall synthesis when treated when treated with 0.1% OO for 15 minutes with cell lysis occurring within 60 min of OO exposure.[221] OO was shown to not inhibit *S.* Enteritidis when in a lone-EO system.[218] Individual OO constituents had inhibition activity across 11 different *Salmonella* serotypes via disc diffusion assay.[222]

OO has also displayed synergistic antimicrobial effects when combined with thyme oil.[223-224] Higher inhibition was seem with a thyme-OO combination against *S.* Heidelberg, *S.* Montevideo, *S.* Enteritidis than when both EOs were used separately.[223] *S.* Enteritidis on inoculated broiler breast fillets and whole wings had a salt-phosphate marinade solution with 0.5% thyme-OO treatment, and *S.* Enteritidis levels were reduced more than 2.0 $\log_{10}$ CFU/ml on both the breast fillets and wings.[224]

6. Overview of dissertation research

With the increased incidence of infection from *S.* Enteritidis and over 50% of CDC-reported *Salmonella* cases being from contaminated eggs, *S.* Enteritidis H4267, sourced from an egg outbreak, will be used for experimental examination.[225-226] Despite the growing interest in antimicrobial delivery systems for biofilms and the increased incidence of infection from *S.* Enteritidis, an antimicrobial delivery system targeting

biofilms composed of *S.* Enteritidis H4267 has not been investigated.[10, 227] As *S.* Enteritidis is the leading *Salmonella* serovar for human laboratory-confirmed cases, this research can provide knowledge on biofilm-forming characteristics of *S.* Enteritidis H4267 to assist in future antimicrobial delivery system development. The increased bioavailability and thermodynamic stability of microemulsions is an attractive option for the development of an antimicrobial delivery system.[120, 129] The utilization of EOs as an antimicrobial will also assist with reducing the possibility of antimicrobial resistance to a microorganisms via a microemulsion delivery system.[228-229]

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Appendix One

Figures

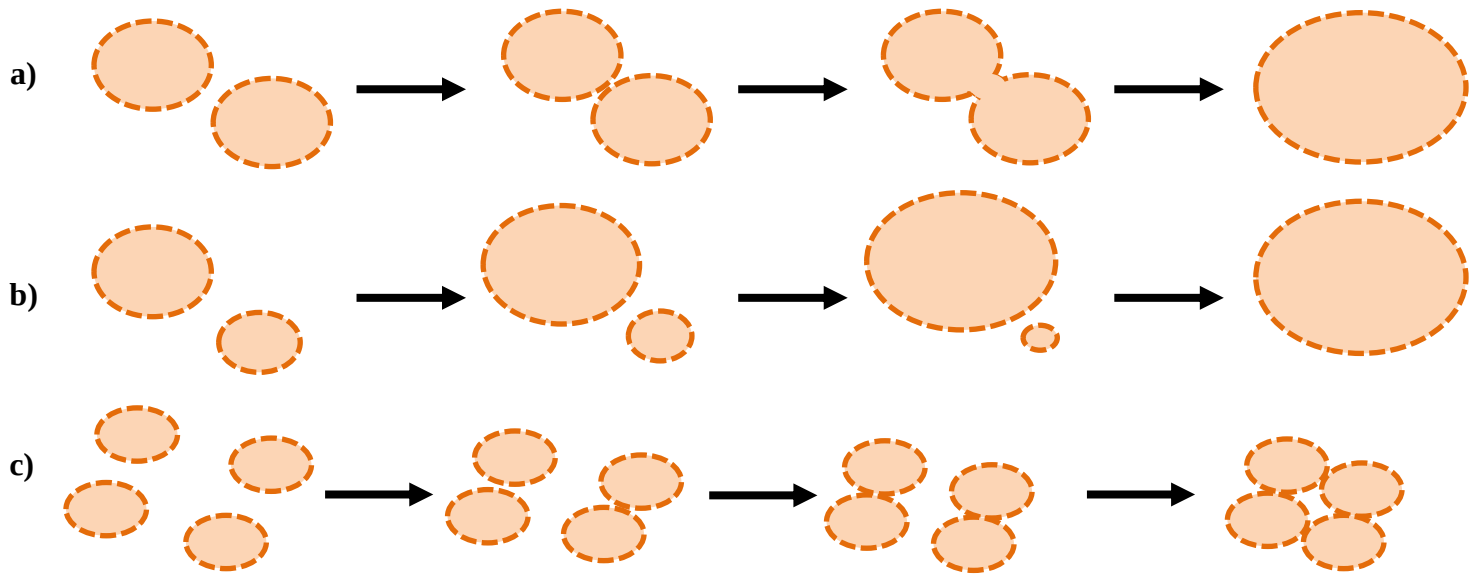

Figure 1.1. Representation of coalescence, Ostwald ripening, and flocculation. Oil droplets demonstrating a) coalescence, b) Ostwald ripening, and c) flocculation. Coalescence occurs when droplets get close together to have a hole formation form in the interfacial film which leads to the formation of one larger droplet. Ostwald ripening occurs when molecules in smaller droplets of the dispersed phase diffuses into droplets of larger sizes. Flocculation occurs when droplets aggregate into flocs.[111]

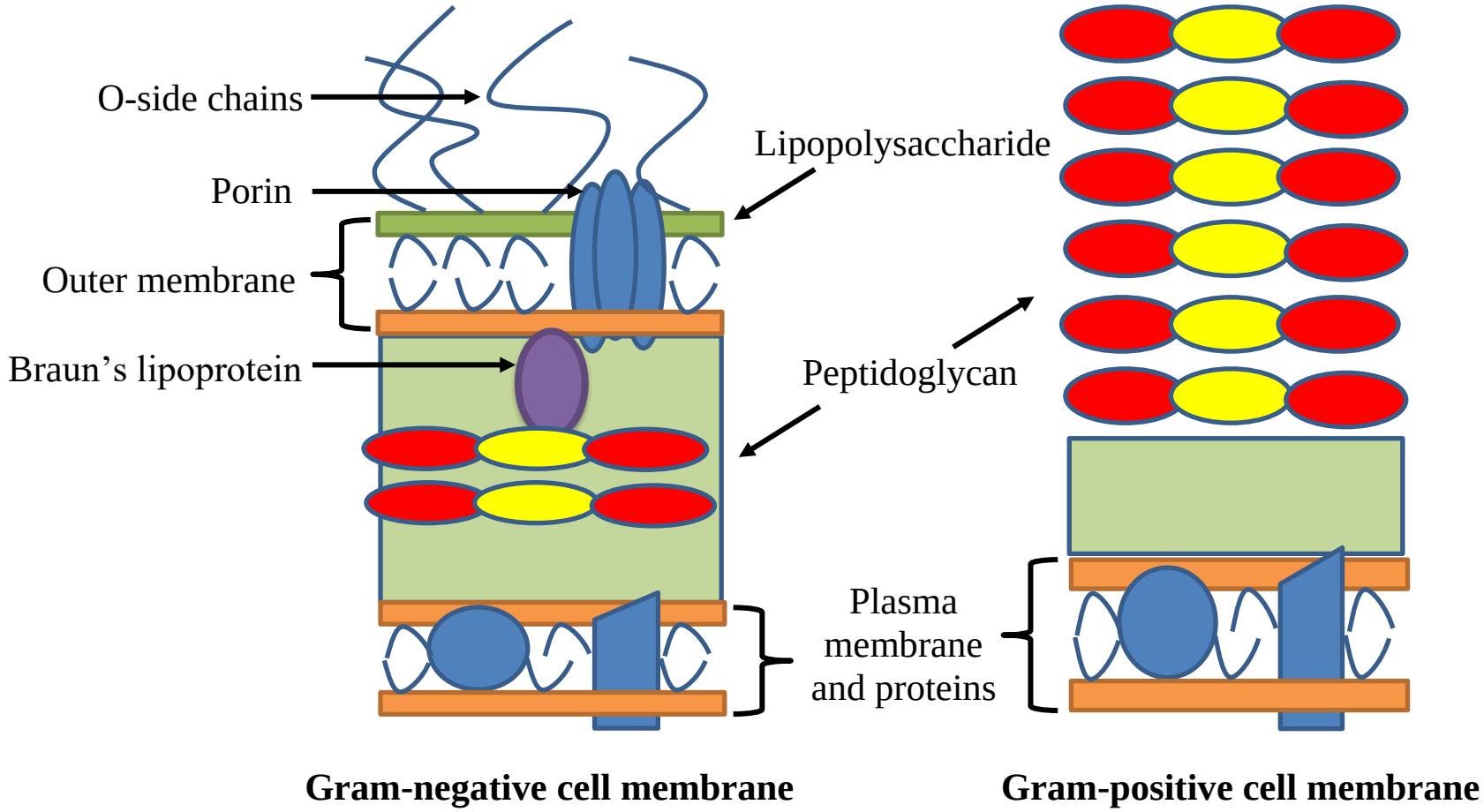

Figure 1.2. Differences between Gram-negative and Gram-positive cell membranes. Adapted from Nikaido *et al*.[186]

Chapter 3. Microemulsions containing cinnamon and orange oil: physical properties and antimicrobial activity against *Salmonella* Enteritidis H4267

Abstract

Essential oils (EOs) are natural antimicrobials that can be used to develop intervention strategies to inhibit pathogens, but EOs are lipophilic. Colloidal systems, such as microemulsions, are needed for food industry applications. This study investigated the antimicrobial activity of cinnamon oil (CO, *Cinnamomum zeylanicum*) and orange oil (OO, *Citrus sinensis*) against *Salmonella* Enteritidis H4267, when used either in combination or in a microemulsion system. Disc diffusion assays indicated that CO had a greater antimicrobial effect on *S.* Enteritidis H4267 when used in combination with OO than when individually ($p<0.001$). Analyses of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) indicated that CO and OO were optimum in a 9:1 volume ratio (MIC and MBC: 750: 750 ppm). Physical analyses determined that, on mass basis, 0% and 2% sunflower lecithin (SL), 20% Tween 20, 1% and 5% CO-OO (9:1) formulations were transparent, thermodynamically stable, and Newtonian fluids. These formulations were therefore determined to be microemulsions. Only formulations of 0% SL, 1% CO-OO (9:1) and 0% and 2% SL, 5% CO-OO (9:1) exhibited bactericidal activity against *S.* Enteritidis H4267. This study shows that OO enhances the bactericidal activity of CO when in an oil or microemulsion system, and co-encapsulated oils in microemulsions could be effective as antimicrobial delivery systems.

1. Introduction

An estimated 48 million cases of foodborne illnesses occur annually in the United States, but only 9.4 million foodborne illness-related cases have an identified pathogen source.[1-2] Reducing these case numbers is not only of public health concern, but also of economic interest as it was estimated in 2015 that the economic burden of foodborne illnesses was over $15.5 billion.[3] Of the top five causes of foodborne illnesses, *Salmonella* is the leading bacterial culprit.[4-5] Among the human *Salmonella* isolates reported in the US, 99% belong to *S. enterica*, with the top two *S. enterica* isolates serotyped from laboratory-confirmed cases being *S.* Enteritidis and *S.* Typhimurium.[6] Between 2007 and 2015, the incidence of infection from *S.* Enteritidis increased by 37% while *S.* Typhimurium incidence of infection decreased by 66%.[6, 7]

Antimicrobial delivery systems are of interest to combat foodborne pathogens in the food industry while also avoiding bacterial resistance to disinfectants.[8] Plant-derived essential oils (EOs) are an attractive option for antimicrobial systems since EOs do not contribute to drug resistance due to the current mechanism of action focusing on the disruption of bacterial membranes.[9-12] EOs are extracted from the leaves, bark, and/or stems of herbs and spices, are highly aromatic, and display a broad range of antioxidant, antiviral, and antimicrobial properties.[13-14] EOs are composed of hundreds of chemical compounds, such as terpenes, terpenoids, and phenylpropenes.[15] The hydrophobicity of EOs allows for penetration through bacterial cell membranes to disrupt cellular metabolic processes and membrane transport systems.[10-11] These disruptions ultimately lead to the breakdown of the cell membrane integrity and cause increased membrane permeability.[10]

The hydrophobicity of EOs also requires colloidal systems, such as emulsions, for utilization.

Colloidal systems with nanoscale oil droplets have unique features uncommon in conventional emulsions.[16-17] Nanoemulsions have a droplet diameter between 20 and 200 nm, and microemulsions have an oil droplet dimension between 4 and 100 nm.[16] While nanoemulsions and microemulsions have overlapping dimensions, nanoemulsions are metastable systems that can be clear or turbid in appearance, and microemulsions have the added benefit of being transparent and thermodynamically stable oil-water-surfactant mixtures.[16, 18] Nanoemulsions of EOs are susceptible to Ostwald ripening as the water-solubility of an EO compound contained within a droplet increases as the size of the oil droplet decreases, which contrasts with stable oil droplets in microemulsions.[16, 19-20] Due to the small droplet size and large surface area, microemulsions have the ability to enable EO compounds to penetrate through bacterial cell walls more efficiently, which can increase the antimicrobial activity.[21] Additionally, microemulsions are relatively easy to prepare in comparison to nanoemulsions that may require high-energy methods for forming nanoscale droplets.[22]

While there are several benefits of microemulsions, microemulsions are formed only at a particular set of conditions, such as environmental conditions and composition, and may convert to a different colloidal system when the conditions are changed.[16-17] Microemulsions also require a high level of surfactants.[16] Despite these drawbacks, the oil body of microemulsions can be used to dissolve lipophilic compounds, which could be an approach to enhance antimicrobial activity. [23-24]

When combined with other organic compounds, the antimicrobial activity of the individual EOs may be enhanced to provide greater antimicrobial ability at reduced levels.[25] Cinnamon (*Cinnamomum zeylanicum*) oil (CO) has been extensively researched as an antimicrobial against a variety of foodborne pathogens, and cinnamon bark oil has displayed enhanced antimicrobial activity against *S.* Enteritidis in the presence of other EOs.[26-29] Citrus oils have exhibited inhibitory activity against a variety of *S. enterica* and have displayed antimicrobial activity enhancement of other EOs when used in combination against *Salmonella* sp.[30-31] Cinnamaldehyde in CO, a phenylpropene aldehyde, and citrus oils containing monoterpenoid alcohols, aldehydes, and monoterpenes, such as linalool, citral, and limonene, have antimicrobial and/or antifungal activity when applied individually.[15, 25, 32-33] The possible synergy in antimicrobial activity of CO and citrus oil has not been studied.

2. Hypothesis

CO and OO are hypothesized to have inhibitory activity against planktonic *S.* Enteritidis H4267 when used in combination due to OOs ability to enhance the antimicrobial activity of other EOs against *Salmonella* sp. and COs bactericidal activity against *S.* Enteritidis alone and in combination with other EOs.[28, 30-31] With the adjustment of surfactants concentrations, a microemulsion containing CO and OO may be formed. Microemulsions with co-encapsulated oils have been created and characterized previously.[23-24] Microemulsions containing CO and OO are hypothesized to have a lower

antimicrobial ability compared to the lone-EOs due to the inclusion of surfactants and/or emulsifiers.[34-39]

This hypothesis was tested in three phases. Phase 1 determined the antimicrobial ability of CO and OO against *S.* Enteritidis H4267 while Phase 2 investigated the development and characterization of microemulsions containing co-encapsulated CO and OO. After microemulsions were developed, Phase 3 determined the antimicrobial activity of the formed microemulsions on *S.* Enteritidis H4267.

3. Methods

3.1. Materials

CO was a product of Sigma-Aldrich Corp. (St. Louis, MO). OO was purchased from Now Essential Oil, Co. (Bloomingdale, IL). Tryptic soy broth (TSB) and tryptic soy agar (TSA) medium powders were products of Remel (San Diego, CA) and were dissolved in 1 l of deionized water (diH_2O) prior to autoclave sterilization. Tween® 20 was a product of Acros Organics (Morris Plains, NJ), and sunflower lecithin (SL) was purchased from Perimondo LLC (New York, NY). Ethanol (200 proof) utilized was a product of Decon Laboratories, Inc. (King of Prussia, PA).

3.2. Bacterial culture

S. Enteritidis H4267 was obtained from the Department of Food Science culture collection at the University of Tennessee (Knoxville, TN) and was maintained at -20ºC in

25% glycerol. The strain was transferred two times in TSB with 22±2 h incubation at 37ºC prior to use.

3.3. Disc diffusion assay

Disc diffusion assay experiments were adapted from O'Bryan *et al*.[30] *S.* Enteritidis H4267 was serially diluted to ~10^6 CFU/ml, and 100 µl of the diluted culture was spread onto TSA. Four sterile paper discs with a diameter of 6 mm (Thermo Fisher Scientific, Waltham, MA) were aseptically placed onto the inoculated agar surface. CO and OO were pipetted at various volume ratios up ranging from 1-10 µl volume per paper disc, followed by incubation at room temperature (21±2°C) for 30 min, inversion, and incubation at 37ºC for 22±2 h. The positive control was *Salmonella*-spread plates with paper discs without EOs; the negative control was uninoculated plates with paper discs without EOs. After incubation at 37ºC for 22±2 h, diameters of inhibition zones around each disc were measured using a ruler with a precision of 1 mm. Plates were completed in triplicate for each level of EOs (n = 4, N = 12).

3.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EOs

A microbroth dilution method for MIC determination was utilized.[40] The bacterial culture was diluted to ~10^6 CFU/ml in TSB, and 100 µl of the diluted culture was added to wells in a 96-well microtiter plate (Corning Inc., Corning, NY). CO and OO stock solutions were solubilized at 30% v/v and 20% v/v in 70% and 80% ethanol, respectively,

to completely dissolve EOs. The EO stock solutions were diluted in TSB to 5000 ppm, followed by half-dilution with TSB to an EO concentration as low as 25 ppm as working solutions. The ethanol levels corresponding to EO working solutions were confirmed to have no inhibition on *S.* Enteritidis H4267 at the studied conditions. A 100 µl aliquot of an EO working solution was added to a well. Plates were covered, and optical density (OD) was measured (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT) at 630 nm before and after incubation at 37°C for 24 h. The positive controls were the diluted bacterial culture, and the CO and OO stock solutions; the negative controls were the EO working solutions alone and sterile TSB. The MIC was determined to be the lowest EO level that had an OD change ($\Delta OD_{630\ nm}$) of <0.05.[41] The MBC was determined by spread plating 100 µl of the mixture from negative wells and the bracketing wells onto TSA plates. Plates were incubated at 37°C for 22±2 h. The MBC was determined as the antimicrobial solution level that resulted in at least a 3-log reduction of viable cells.[41]

3.5. Antimicrobial activity of CO-OO combinations

Interactions of CO and OO used in combination were assessed using a checkerboard method.[41] Wells were loaded with varying volumes of the EO working solutions, prepared as above with 25-5000 ppm CO or OO, totaling 100 µl in volume, and 100 µl of diluted *S.* Enteritidis H4267 culture (~10^6 CFU/ml). The MIC and MBC were determined as previously described. If an antimicrobial combination had an MIC,

the fractional inhibitory concentration (FIC) index was calculated (**Equation 2.1**) where antimicrobial A is CO and antimicrobial B is OO.[42]

$$\text{FIC} = \frac{MIC\ of\ A\ in\ combination}{MIC\ of\ A\ alone} + \frac{MIC\ of\ B\ in\ combination}{MIC\ of\ B\ alone} \qquad \textbf{(2.1)}$$

Synergistic interaction would be an FIC index value of <1, while additive and antagonistic interaction of the antimicrobial combination would be 1 and >1, respectively.[42]

3.6. Microemulsion preparation

Microemulsion preparation followed a previous work, with modification for compositions.[24] CO and/or OO were added to scintillation vials on mass basis at 0% to 25% levels along with 20% Tween 20 in CO-OO volume ratios of 1:1 or 9:1. A 12% stock solution of SL was prepared with diH_2O by stirring for 8 h at room temperature (21±2°C) and added to solution vials, on mass basis, at 0% to 6%. Deionized water was added to vials to bring the total emulsion mass to 15.0 g, and the mixture was hand-agitated until visually homogenous, followed by heating in an 80ºC water bath for 5 min. After heating, vials were hand-agitated in an ice water bath (5±0.5ºC). Formed emulsions were visually assessed for transparency, used as a preliminary indication of microemulsion formation prior to further characterization of physical properties.[16, 43]

3.7. Physical properties of emulsions

Droplet size. Droplet size distribution was determined using dynamic light scattering (Zeta Sizer S, Malvern Panalytical Ltd., Worcestershire, UK) at a 173º scattering angle. Emulsions were diluted 100-fold in diH_2O to fit instrument sensitivity. Measurements were performed thrice for each sample, and emulsions were completed in triplicate (n = 3, N = 9).

Thermal and storage stability of droplets. Emulsions were stored at refrigeration (5±2ºC) or freezing (-20±5ºC) temperatures for 24 h and were returned to room temperature (21±2ºC) for 24 h to determine droplet size distribution and the thermodynamic stability. Emulsions were also measured for droplet size distributions after storage at room temperature for 0, 30, and 60 d. The ability to restore droplet size distribution after temperature fluctuations and long-time storage, as determined by thermodynamic stability, is one way to differentiate between a nanoemulsion and a microemulsion.[16]

Rheological property. Emulsion viscosity was determined using a model AR2000 rheometer (TA Instruments, New Castle, DE) with a concentric cylinder geometry (cup inner diameter 30 mm, bob out diameter 28 mm). Shear rate ramps were performed from 0.1 to 100 s^{-1} at 25ºC with a 13 ml emulsion loading volume.

Zeta(ζ)-potential. ζ-potential of samples was measured using the same dynamic light scattering instrument as described above. Samples were diluted 100-fold in diH_2O and were completed in triplicate (n = 3, N = 9).

3.8. Antimicrobial activity of microemulsions

After microemulsions were identified, disc diffusion assays using a 10 µl volume of microemulsion per disc was performed as described previously in Section 3.2. The positive control was *Salmonella*-spread plates with sterile paper discs only; the negative controls included uninoculated plates with paper discs only or paper discs treated with 10 µl of Tween 20, diH_2O, or SL. After incubation at 37ºC for 22±2 h, diameters of inhibition zones were measured (n = 4, N = 12).

The assays for MIC and MBC determination as presented previously in Section 3.3 were adapted for microemulsions. Microemulsions were diluted 0-, 50-, 100-, 200-, and 300- fold in diH_2O, and 100 µl of the diluted microemulsion was added to a treatment well with 100 µl of culture with ~10^6 CFU/ml bacteria. The positive control was the diluted bacterial culture. The rest of experiments followed the same protocol previously described.

3.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to identify significant differences among treatments (α = 0.05). Pairwise significant differences were

determined using Tukey's test after conducting ANOVA. Analyses were conducted using SAS v9.4 (SAS Inc., Cary, NC, USA).

4. Results

4.1. Disc diffusion assay for oils

Discs that displayed no inhibition were recorded as the size of the disc diameter (6 mm) (**Figure 2.1**) (All tables and figures are located in the appendix of this chapter). Discs treated with neat OO did not show inhibition while those treated with neat CO and CO-OO combinations displayed zones of inhibition (**Table 2.1**). CO-OO combinations at all volume ratios showed statistically larger ($p<0.05$) inhibition zones than neat CO treatment. However, there was no significant difference between treatments with various CO-OO combinations. The CO-OO ratios of 1:1 and 9:1 were chosen for further study due to the 1:1 ratio being lower in cost and the 9:1 ratio having the highest CO concentration, which is the EO that displayed bactericidal activity.

4.2. MIC, MBC, and interactions of CO and OO

Stock solutions of CO and OO did not differ ($\alpha=0.05$) in ΔOD_{630nm} values compared to the *S.* Enteritidis H4267 positive control (data not shown). Wells treated with up to 2500 ppm neat OO had ΔOD_{630nm} values >0.05 (**Table 2.2**). The CO-OO combination at 9:1 ratio displayed MICs and MBCs at 750: 500 ppm and 1,000: 250 ppm, respectively, while the CO-OO combination at 1:1 ratio resulted in an MIC and MBC with CO (1,250: 750 ppm) at a level the same as that of the neat CO.

4.3. Physical properties of microemulsions

Thermal and storage stability of droplet size. Only the 0 and 2% SL formulations displayed transparent emulsions after storage for 1 d at room temperature and were selected for thermal and storage stability experiments (**Figure 2.2**). While all emulsions had a slight increase in droplet size after temperature abuse, the emulsions displayed a transparent appearance and stayed within microemulsion droplet size range after temperature abuse in freezer (-20±5ºC) or refrigeration (5±2ºC) (**Figure 2.3**). The 0% SL, 5% CO-OO (1:1) and 2% SL, 1% CO-OO (1:1) indicated changes in intensity peaks after temperature abuse.

Concurrent to the temperature abuse studies, transparent emulsions were measured for droplet size at over 60 days (**Figure 2.4**). There was no change in droplet dimension after 60 d and single intensity peaks remained. The formulations of 0 and 2% SL with 1% CO-OO (9:1, 1:1), 0% SL with 5% CO-OO (9:1), and 2% SL, 5% CO-OO (9:1) were classified as microemulsions due to having droplet dimensions ranging from ~8-14 nm over 60-day storage, remaining transparent in appearance, and returning to microemulsion droplet size with single intensity peaks after temperature abuse.

Microemulsions displayed a linear correlation (R^2 values >0.99) between shear rate and shear stress and had an intercept of zero (**Figure 2.5**), indicating the formulations are Newtonian fluids. The ζ-potential for all microemulsions was slightly below 0 mV (**Figure 2.6**).

4.4. Antimicrobial activity of microemulsions determined with disc diffusion assay

All microemulsion formulations displayed inhibition of *S.* Enteritidis H4267 via disc diffusion (**Table 2.3**). There was no difference (α=0.05) between the microemulsion formulations in relation to mean inhibition zone diameter.

4.5. MIC and MBC of microemulsions

All microemulsions displayed inhibition against *S.* Enteritidis H4267 at 0-fold dilution only (data not shown). Since water did not demonstrate inhibition of growth via disc diffusion (**Table 2.3**), dilution with water was anticipated to promote bacterial growth. Once plated, only 0% SL, 1% CO-OO (9:1), and 0% and 2% SL, 5% CO-OO (9:1) microemulsion systems had a bactericidal effect (**Table 2.4**). The ability for the 2% SL, 5% CO-OO (9:1) microemulsion to have bactericidal activity, while the 1% (9:1) formulation did not, suggests that a higher level of CO is needed to overcome the addition of SL. The 0% SL, 1% CO-OO (1:1) microemulsion displayed the most bactericidal activity for the tested 1:1 microemulsion formulations, but the resulting colony count did not fit the MBC criteria of having a 3-log reduction.[41]

5. Discussion

The average inhibition zone of the neat CO treatment (11.0±1.7 mm) against *S.* Enteritidis H4267 is within the range of other studies on a variety of other *S.* Enteritidis strains (11.0±3.9 mm).[28] The lack of inhibition of *S.* Enteritidis by OO also aligns with previous studies indicating that 10 µl of OO did not display inhibition against *S.*

Enteritidis via disc diffusion.[28, 30] Inhibition of *S.* Enteritidis by OO was previously indicated to be above 1,000 ppm, a lower level than the 2,500 ppm in the present study testing on *S.* Enteritidis H4267.[28, 30-31] The MIC and MBC of CO in this study were both 1250 ppm. Ebani *et al.*[28] found that CO had an MIC of 1,260 ppm on five different *S.* Enteritidis strains, and Raybaudi-Massilia *et al*.[27] indicated CO levels above 1,000 ppm were needed for *S.* Enteritidis inhibition. Although antimicrobial combinations had MICs, synergistic interaction utilizing the FIC index was not able to be determined since an MIC for OO was not able to be determined. This suggests that OO is enhancing the antimicrobial activity of CO, but the synergistic interaction between CO and OO cannot be determined based on estimating the FIC index.[42]

A characteristic of microemulsions is the thermodynamic stability.[16] Droplets should stay within 4-100 nm in diameter, and microemulsions should remain transparent over time.[16, 43] When removed from the optimal temperature environment, microemulsions can become unstable, but when returned to optimal temperature conditions, the emulsion should return to a microemulsion.[16, 43] The 0% SL, 5% CO-OO (1:1) and 2% SL, 1% CO-OO (1:1) are more indicative of nanoemulsions, metastable formulations that break down over time and are not thermodynamically stable.[17] Nanoemulsions may have multiple or single peaks in droplet size distribution, while microemulsions have a single narrow peak.[43] The high surfactant level present in the formulations can encapsulate oils but will also contribute free surfactant micelles that can transfer oil molecules between droplets through Ostwald ripening, which has been seen with other OO nanoemulsions.[44-46] Ostwald ripening can occur in systems where the

dispersed phase has solubility in the aqueous phase.[47-49] Citrus oils contain large amounts of hydrophilic components.[15] Small water-soluble components can diffuse and become larger droplets due to Ostwald ripening.[48] Since components in CO and OO are expected to have some water solubility, both Ostwald ripening and coalescence can occur in those systems after temperature abuse.[15] The microemulsions displaying Newtonian fluid characteristics agree with literature of other microemulsion systems demonstrating Newtonian characteristics.[24, 50-51] Since the majority of bacterial surfaces have a negative charge, the ability of microemulsions to be attracted to bacterial surfaces can be hindered if the negative charge is strong enough.[52]

The addition of surfactants can hinder the antimicrobial ability of oils when encapsulated.[34, 53] Although Tween 80 has displayed inhibition against a variety of microorganisms,[35] the Tween 20 control did not inhibit *S.* Enteritidis H4267 at the volumes applied to discs. The level of Tween 20 utilized is above a critical concentration (4% Tween 20 total in an emulsion) that could cause *S.* Enteritidis H4267 cell death and is acting as a nutrient source and growth promoter.[35, 54-57] Lecithin presence can also increase the growth of microorganisms by supplying fatty acids to microbial cells, which can trigger events that ultimately increase biomass.[34] It has been reported that lecithin below 0.005-1.0% (w/v) improved antimicrobial properties of EOs, but lecithin above these concentrations showed no antimicrobial activity.[58-60] The lack of antimicrobial activity via disc diffusion from Tween 20 and lecithin further strengthen the conclusion that the antimicrobial activity is being driven by the co-encapsulated CO and OO.

CO nanoemulsions have shown increased antimicrobial ability against both Gram positive and Gram negative bacteria when CO content in the lipid phase increased, but MICs of the emulsion system were not lower than that of neat CO.[53] Heating during microemulsion preparation could have contributed to the conversion of cinnamaldehyde, a primary component in CO that has antimicrobial properties, into benzaldehyde, an oxidative product that has been reported to have no inhibitory effect against a variety of Gram positive and Gram negative organisms.[53, 61-62] Room temperature storage of OO has shown that chemical components oxidize over time into secondary constituents, and heat can expedite the process.[63] Tween surfactants, known to undergo hydrophobic interactions with EO constituents can reduce EO antimicrobial interaction with bacteria.[36-39] Further research into determining the extent of EO degradation should be investigated prior to altering preparation methods utilizing low-temperature methods and altering surfactant concentration.

6. Conclusion

OO enhanced the antimicrobial ability of CO against *S.* Enteritidis H4267, both in simple oil combination and in microemulsion systems. Multiple microemulsions were formulated with CO-OO present in 9:1 and 1:1 volume ratios at up to 5% oil concentration. Microemulsions were thermodynamically stable for 60 d and after temperature abuse, were Newtonian fluids, and had a slight negative charge. Only microemulsions containing 9:1 CO-OO ratios had bactericidal activity against *S.* Enteritidis H4267, with 2% SL, 1% CO-OO (9:1) only having inhibitory activity.

Microemulsions containing co-encapsulated CO and OO could be used as an antimicrobial delivery system against *S.* Enteritidis H4267.

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Appendix Two

Tables

Table 2.1. Mean inhibition zone diameter of treatments of cinnamon oil (CO), orange oil (OO), or their different volume ratio combinations in total volume of 10 µl against *S*. Enteritidis H4267.

System tested	Mean inhibition zone diameter (mm)[b]
CO (10 µl)	11.0±1.7 (B)
CO (9 µl)	10.9±1.7 (B)
CO (8 µl)	10.5±1.2 (B)
CO (7.5 µl)	10.6±1.3 (B)
CO (7 µl)	11.9±1.8 (B)
CO (6 µl)	11.7±1.8 (B)
CO (5 µl)	11.5±1.3 (B)
CO (2.5 µl)	11.5±1.9 (B)
OO (1-10 µl)	6.0±0.0 (C)
CO-OO (9:1)	18.6±2.0 (A)
CO-OO (4:1)	18.5±1.5 (A)
CO-OO (3:1)	18.6±1.7 (A)
CO-OO (7:3)	18.6±1.6 (A)
CO-OO (3:2)	18.3±0.7 (A)
CO-OO (1:1)	18.8±0.6 (A)
CO-OO (0.5:0.5) [a]	18.8±0.8 (A)

Table 2.1 Continued

Negative control (uninoculated)	6.0±0.0 (C)
Positive control (without oil)	6.0±0.0 (C)

[a] The treatment was an exception with 2.5 µl of each oil.

[b] Treatments with a zone diameter equivalent to the bore diameter of 6 mm indicate no inhibition. Different letters indicate significantly different (α=0.05) means of inhibition zones.

Table 2.2. Minimum inhibitory concentration (MIC, ppm) and minimum bactericidal concentrations (MBC, ppm) of cinnamon oil (CO), orange oil (OO), and their combinations at 1:1 or 9:1 mass ratio against *S*. Enteritidis H4267.[a]

Oil	MIC	MBC [a]
CO	1,250	1,250 (A)
OO	>2,500	None [b]
CO-OO (1:1)	1,250: 750	1,250: 750 (A)
CO-OO (9:1)	750: 750	750: 750 (A)

[a] Different letters indicate significantly different (α=0.05) means of MBC plate counts on tryptic soy agar.

[b] None = no MBC was obtained.

Table 2.3. Mean inhibition zone diameter of samples containing, on mass basis, 20% Tween 20, varying amounts of deionized water, 0 or 1% sunflower lecithin (SL), 1% or 5% oil with cinnamon oil (CO) and orange oil (OO) at a mass ratio of either 9:1 or 1:1 against *S.* Enteritidis H4267, in comparison to individual components.

System tested	Mean inhibition zone diameter (mm)[a]
0% SL, 1% CO-OO (1:1)	10.1±1.2 (A)
0% SL, 1% CO-OO (9:1)	9.8±1.6 (A)
2% SL, 1% CO-OO (9:1)	9.4±1.3 (A)
0% SL, 5% CO-OO (9:1)	11.0±2.0 (A)
2% SL, 5% CO-OO (9:1)	10.5±1.5 (A)
Deionized water	6.0±0.0 (B)
Tween 20	6.0±0.0 (B)
12% SL	6.0±0.0 (B)
Positive control (no treatment)	6.0±0.0 (B)

[a] Treatments with a zone diameter equivalent to the bore diameter of 6 mm indicate no inhibition. Different letters indicate significantly different (α=0.05) means of inhibition zones on tryptic soy agar plates.

Table 2.4. Minimum inhibitory concentrations (MIC, ppm) and minimum bactericidal concentrations (MBC, ppm) of samples containing, on mass basis, 20% Tween 20, varying amounts of deionized water, 0 or 1% sunflower lecithin (SL), 1% or 5% oil with cinnamon oil (CO) and orange oil (OO) at a mass ratio of either 9:1 or 1:1 against *S.* Enteritidis H4267.[a]

System tested	MIC	MBC [a]
0% SL, 1% CO-OO (1:1)	2,500: 2,500	None [b] (B)
0% SL, 1% CO-OO (9:1)	4,500: 500	4,500: 500 (A)
2% SL, 1% CO-OO (9:1)	4,500: 500	None (B)
0% SL, 5% CO-OO (9:1)	22,500: 2,500	22,500: 2,500 (A)
2% SL, 5% CO-OO (9:1)	22,500: 2,500	22,500: 2,500 (A)

[a] Different letters indicate significantly different (α=0.05) means of MBC plate counts on tryptic soy agar.

[b] None = no MBC was obtained.

Figures

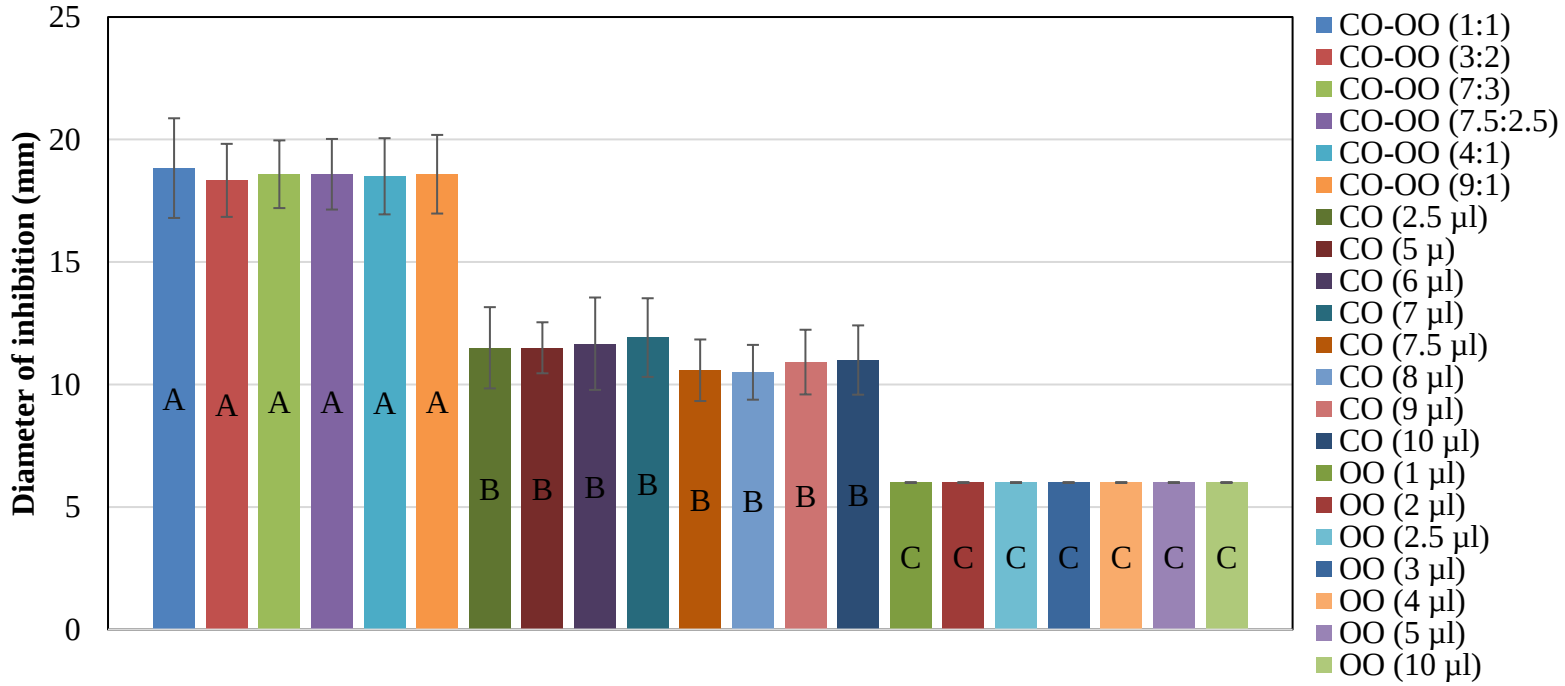


Figure 2.1. Disc diffusion assay diameter of inhibition zones of CO and/or OO against *S.* Enteritidis H4267. CO and/or OO in neat form or combination in volume ratios against S. Enteritidis H4267 on filter discs on tryptic soy agar plates. Discs without inhibition were recorded as the size of the disc (6 mm). Different letters indicate significantly different (α=0.05) means via Tukey's Studentized Range (*n*=4, N=12).

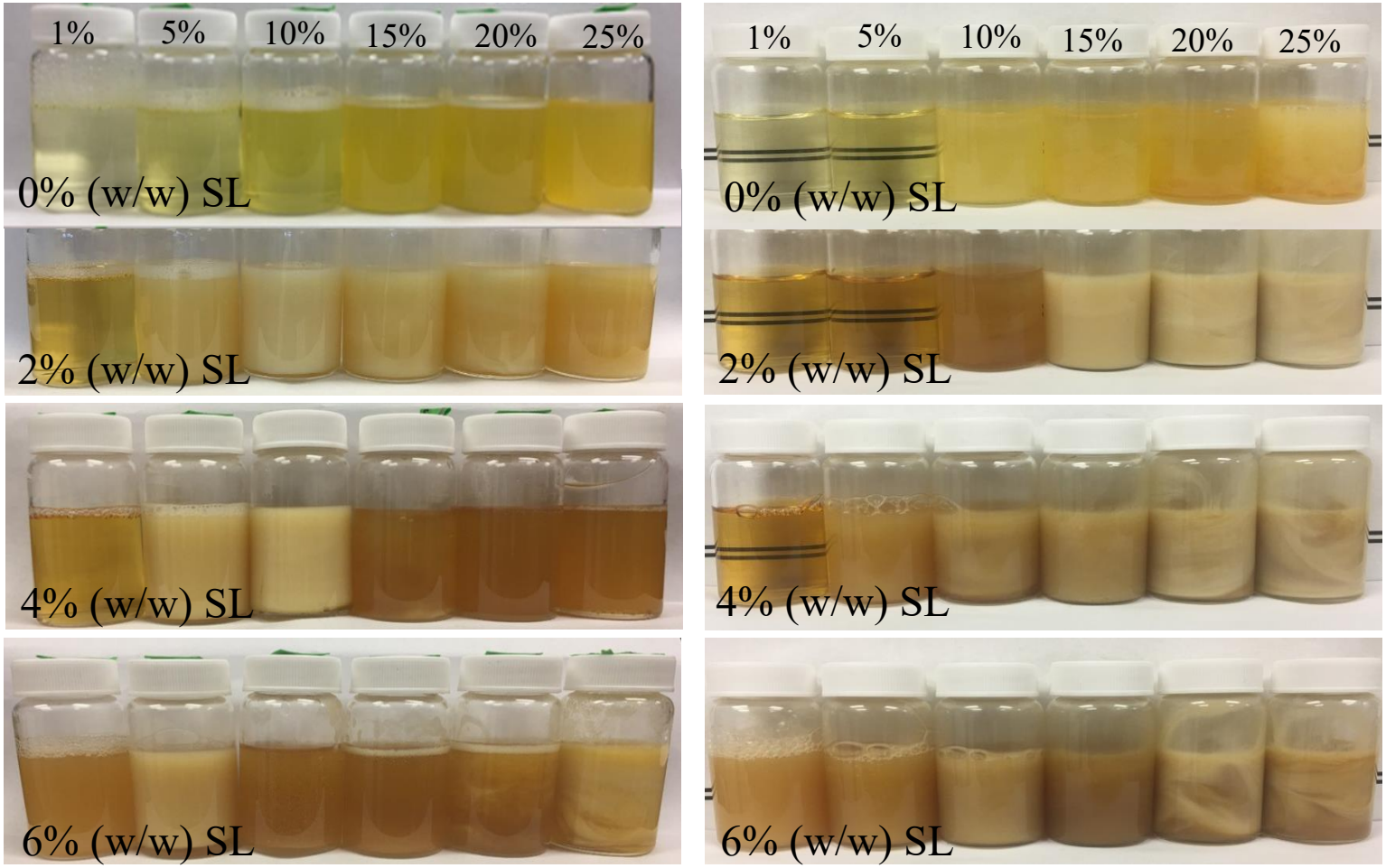


Figure 2.2. Emulsion transparency after creation. Visual clarity of emulsions containing 0 to 6% (w/w) sunflower lecithin (SL), 20% (w/w) Tween 20, deionized water, and 1-25% (w/w) CO and OO in either a 1:1 (left) or 9:1 (right) volume ratio.

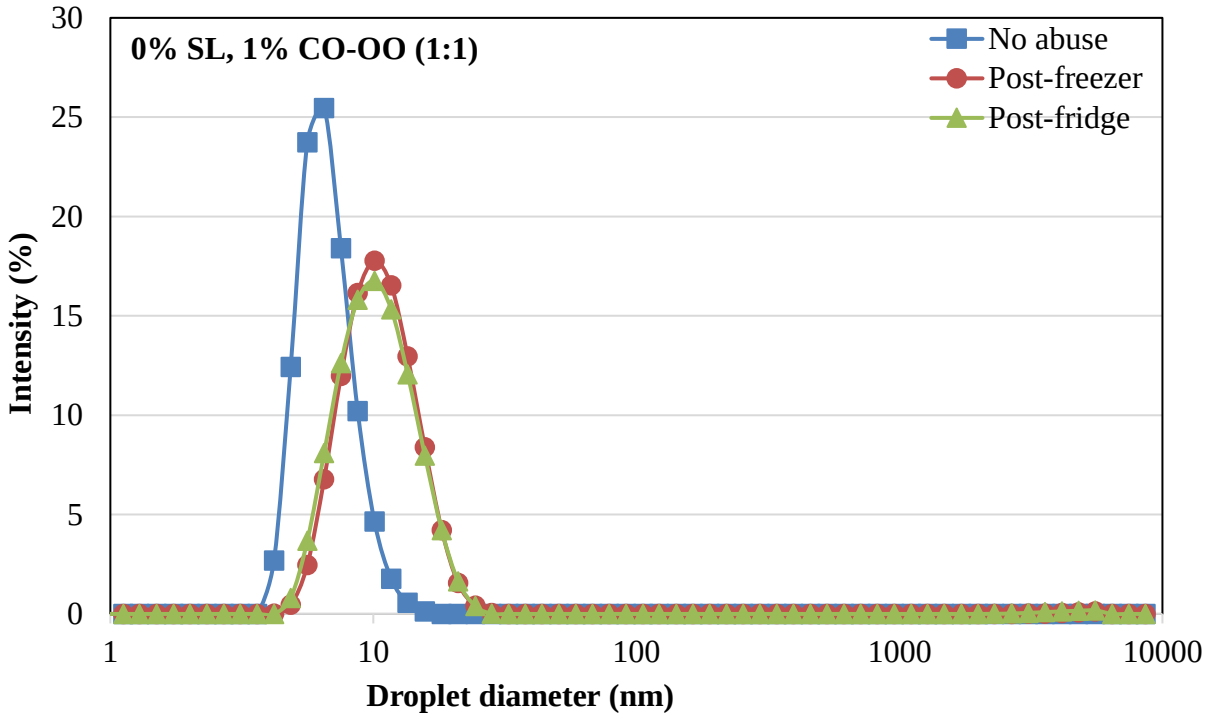
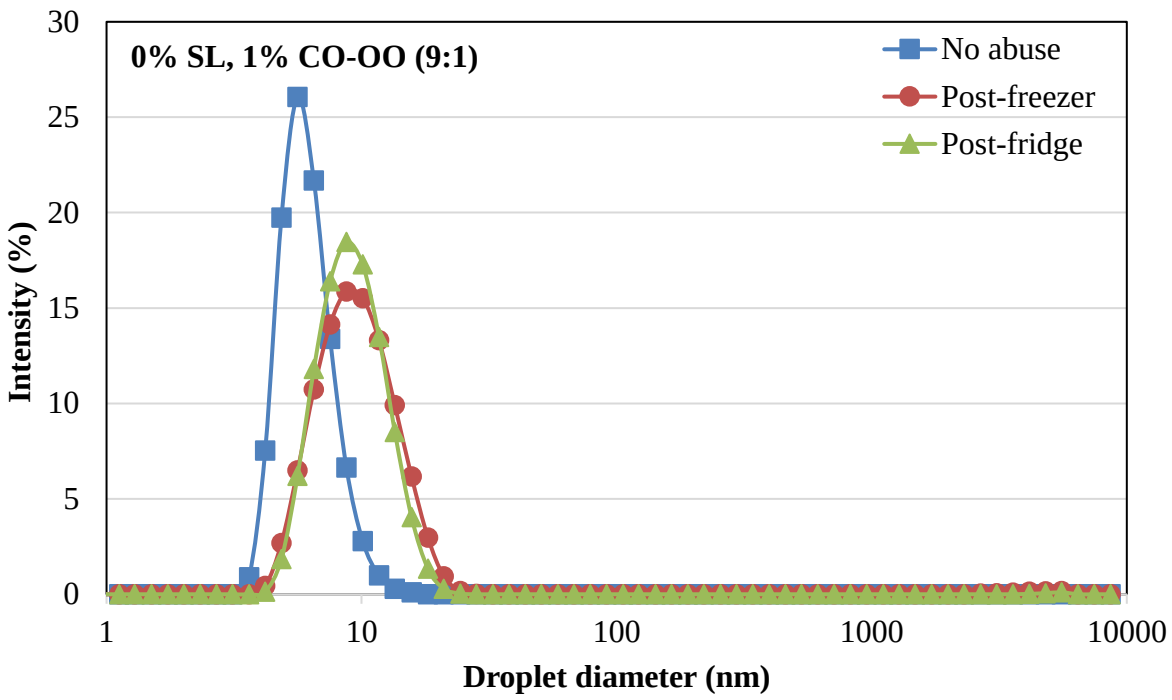


Figure 2.3. Droplet size distribution of emulsion formulations pre- and post-temperature abuse. Droplet size distributions of emulsion formulations held at room temperature (21±2°C) and after being held in a freezer (-20±5°C) or refrigerator (5±2ºC) for 24 h and warming to room temperature for 24 h. Samples contained, on mass basis, 0% or 2% sunflower lecithin (SL), 20% (w/w) Tween 20, 1% or 5% (w/w) cinnamon oil (CO) and orange oil (OO) in either 1:1 or 9:1 volume ratios, and deionized water.



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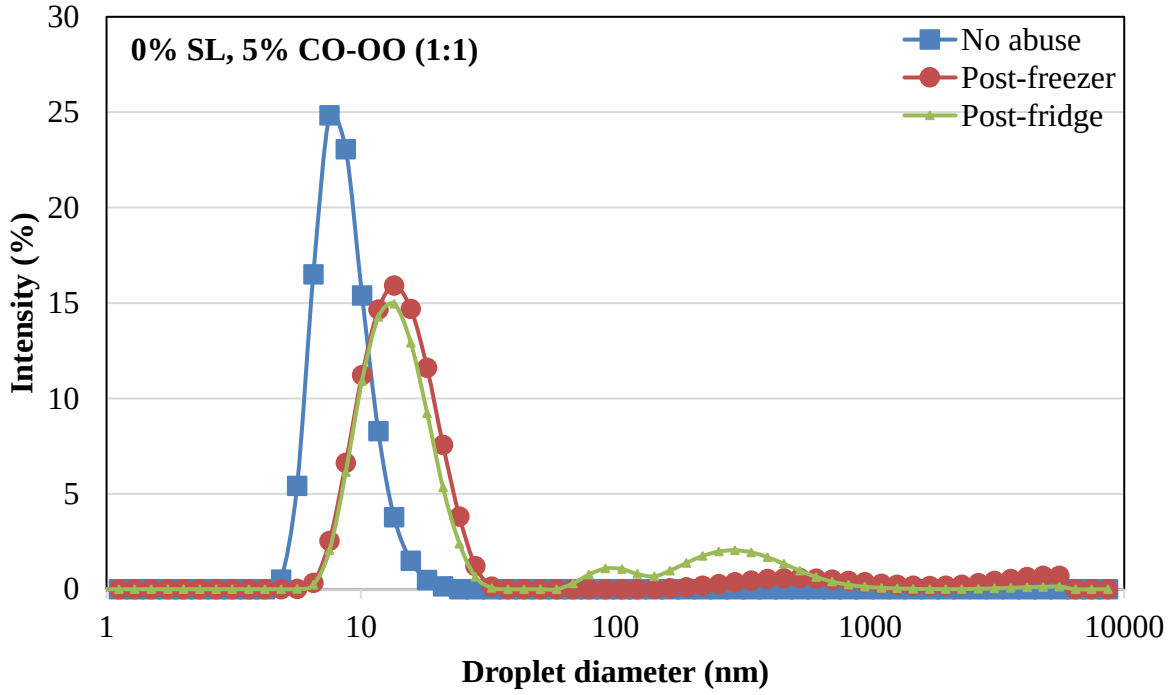

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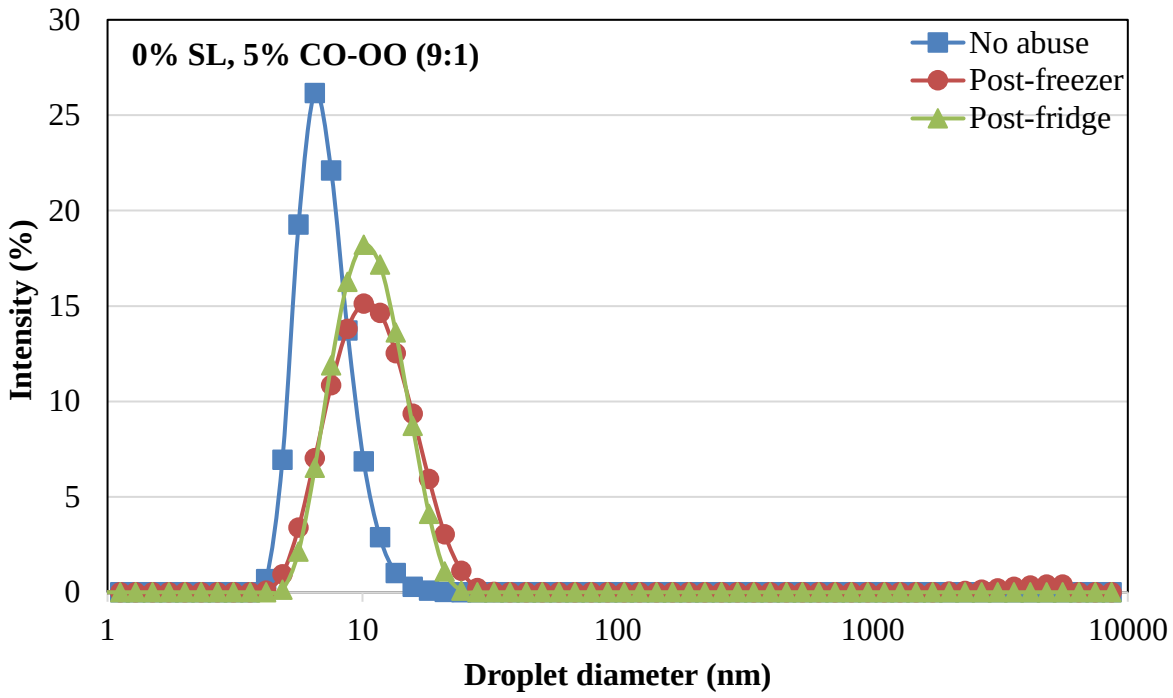

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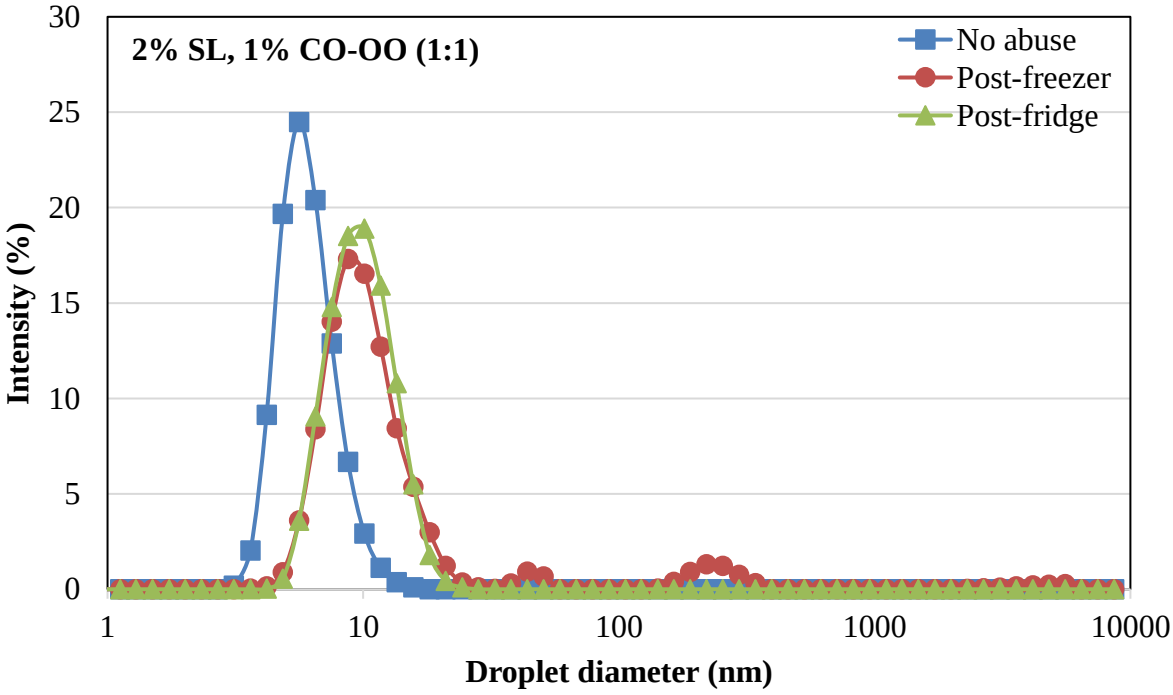

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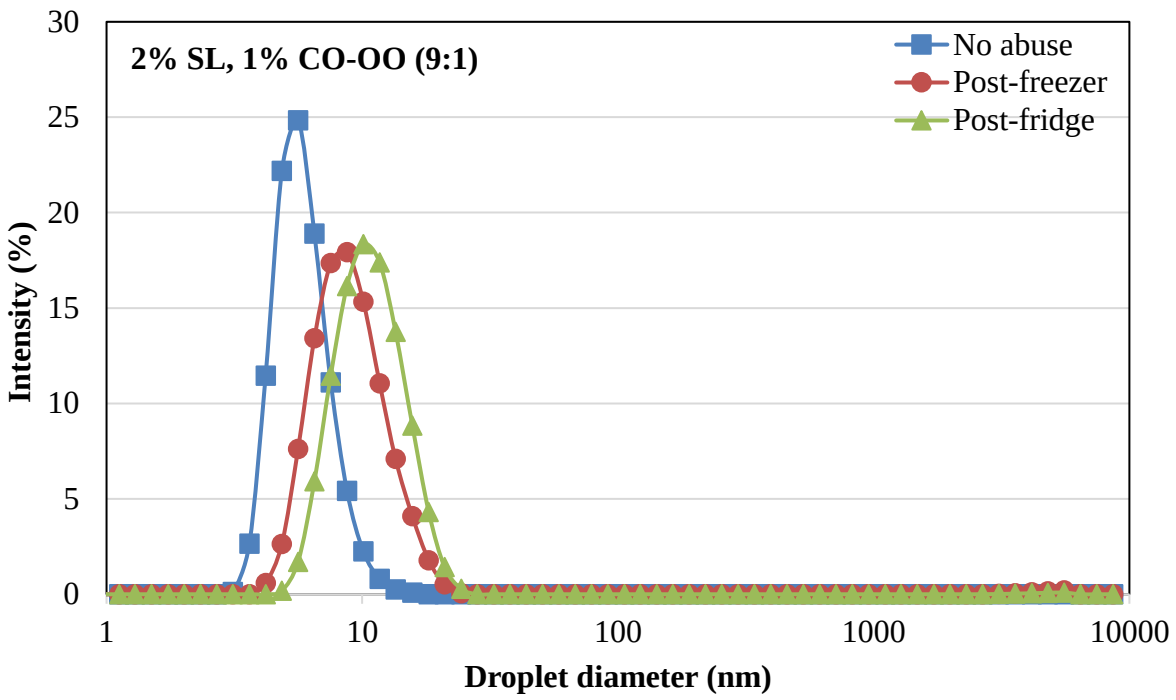

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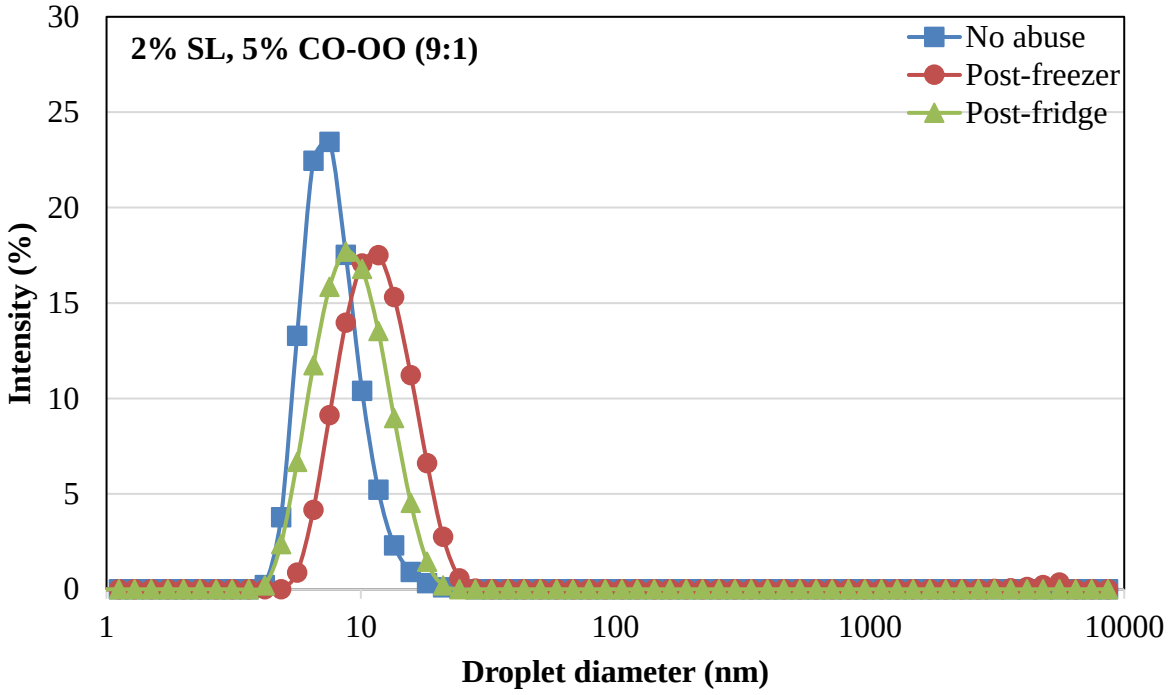

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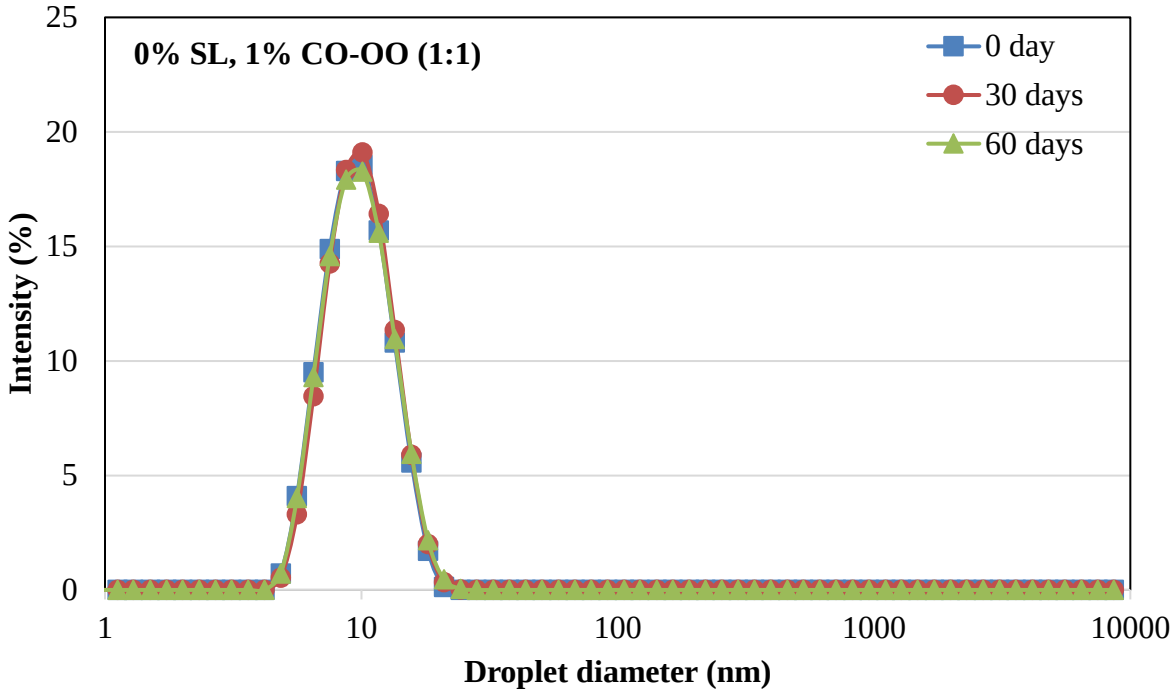
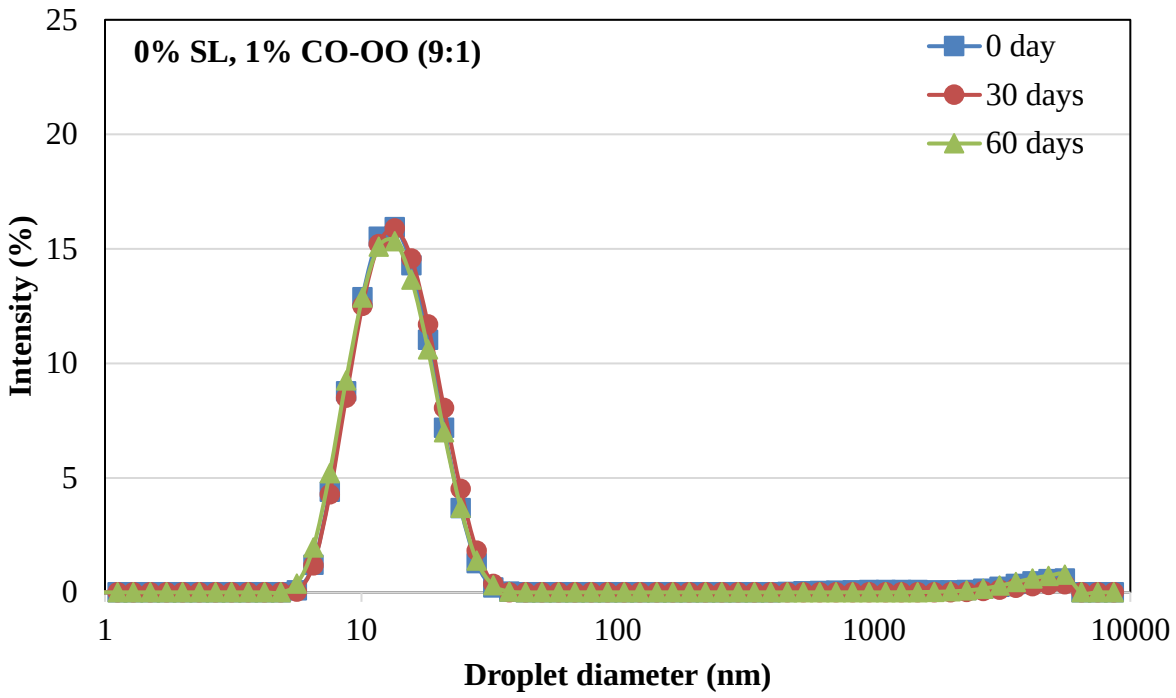


Figure 2.4. Droplet size distribution of emulsion formulations held over 60 d. Droplet size distribution of emulsions held for 60 days at room temperature (21±2°C). Samples contained, on mass basis, 0% or 2% sunflower lecithin (SL), 20% (w/w) Tween 20, 1% or 5% (w/w) cinnamon oil (CO) and orange oil (OO) in either 1:1 or 9:1 volume ratios, and deionized water.



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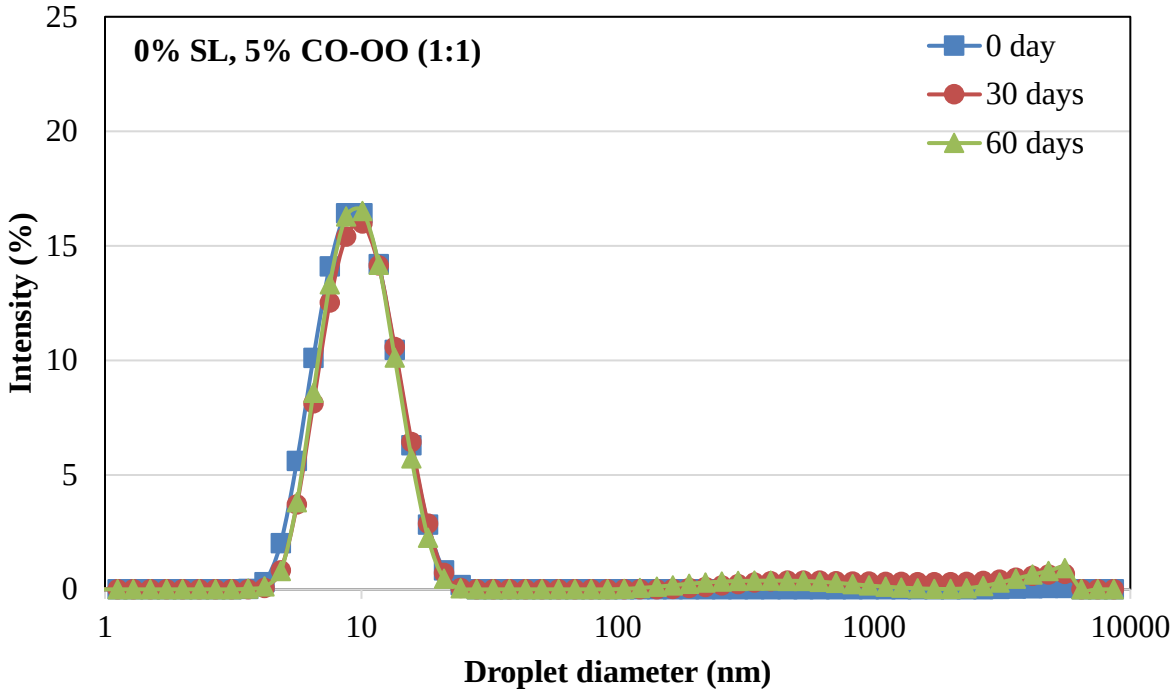

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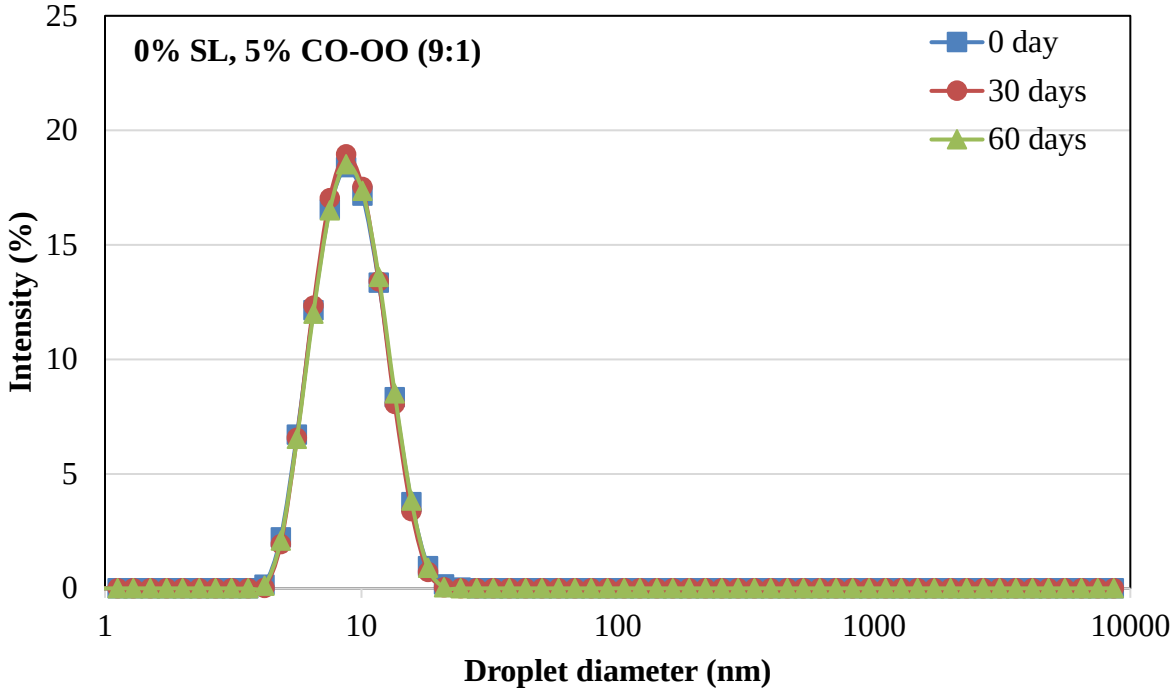

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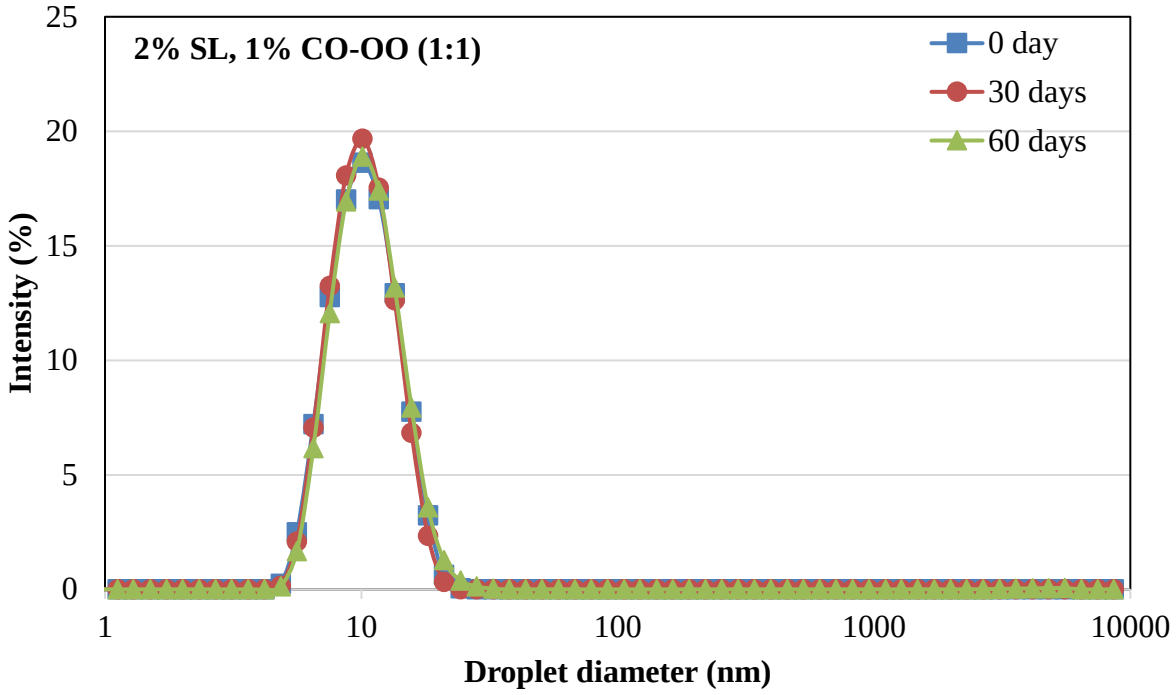

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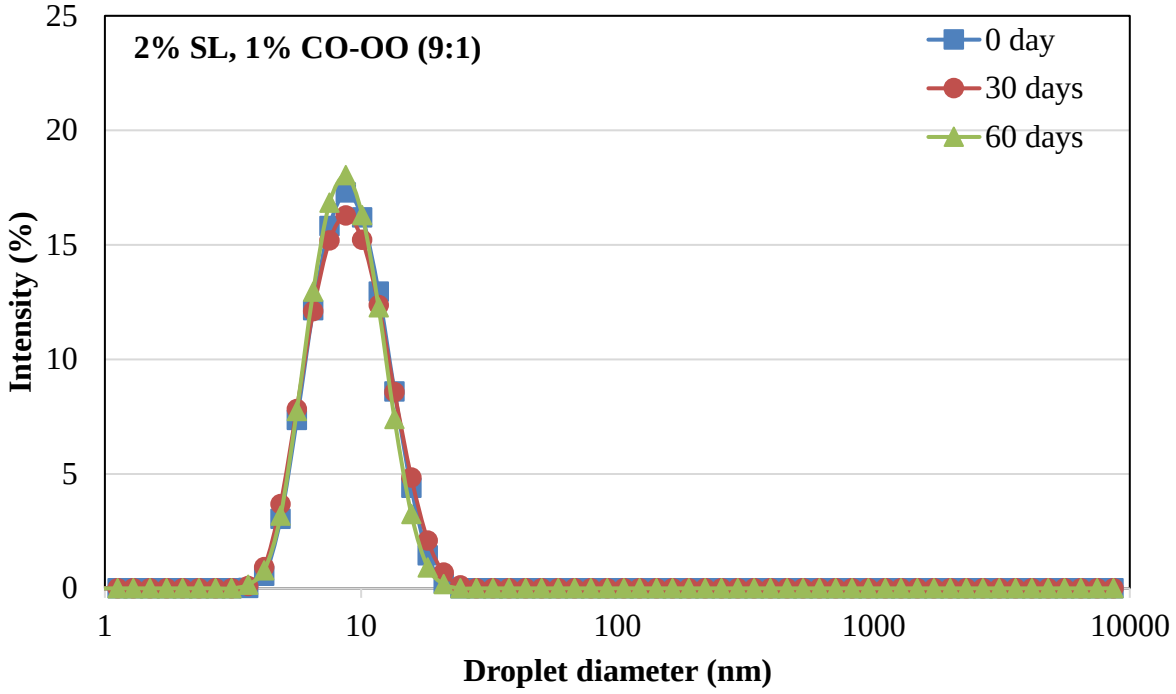

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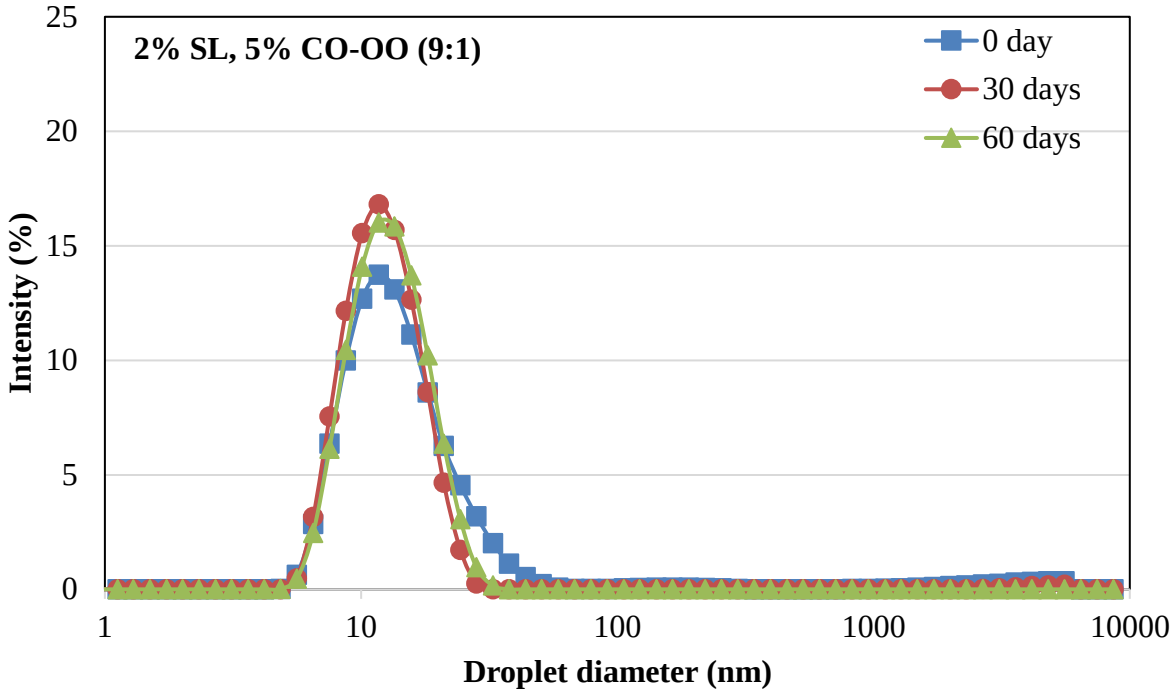

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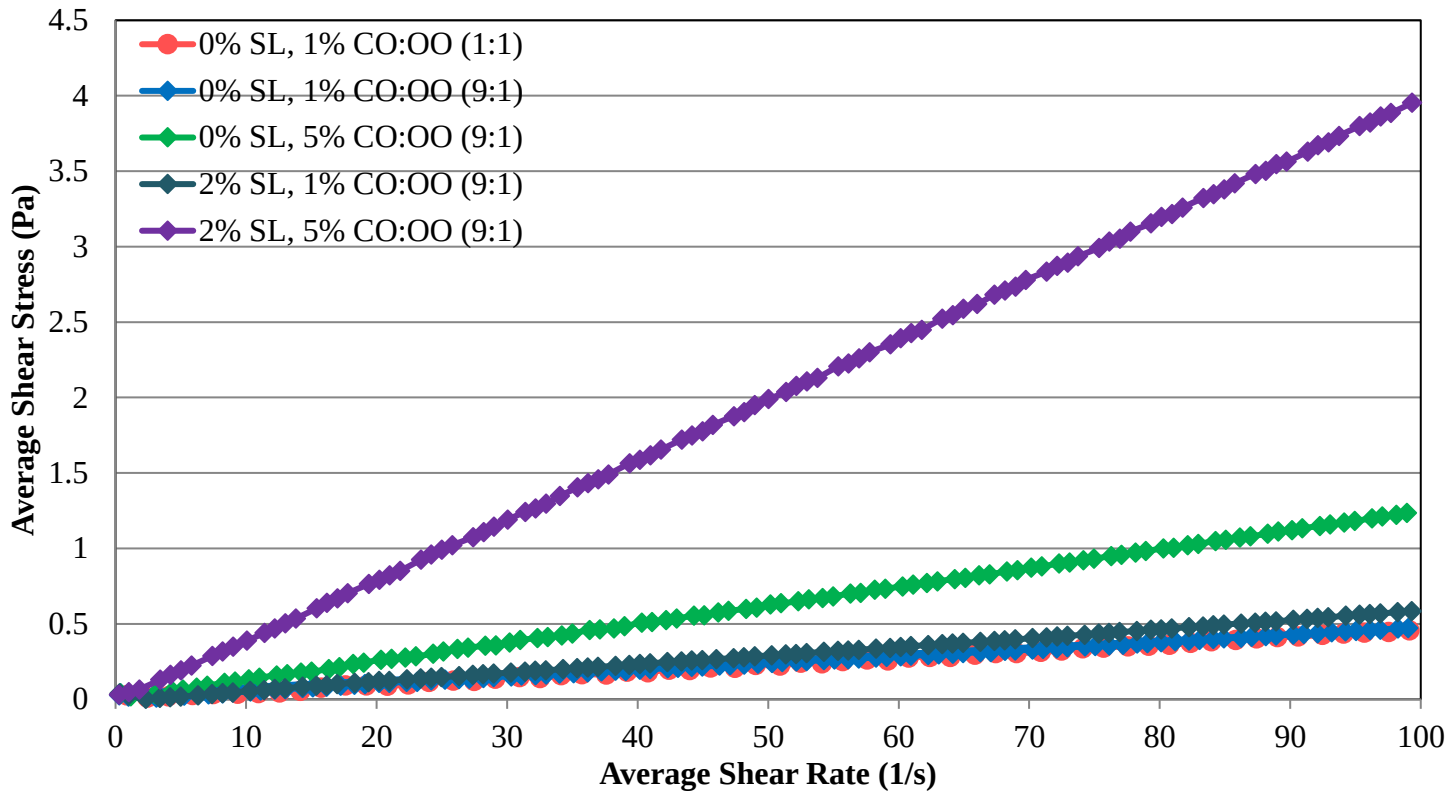

Figure 2.5. Shear rate ramps of microemulsions. Shear rate ramps at 25°C of microemulsions containing, on mass basis, 20% Tween 20, varying amounts of deionized water, 0 or 2% sunflower lecithin (SL), 1% or 5% oil with cinnamon oil (CO) and orange oil (OO) at a mass ratio of either 9:1 or 1:1.

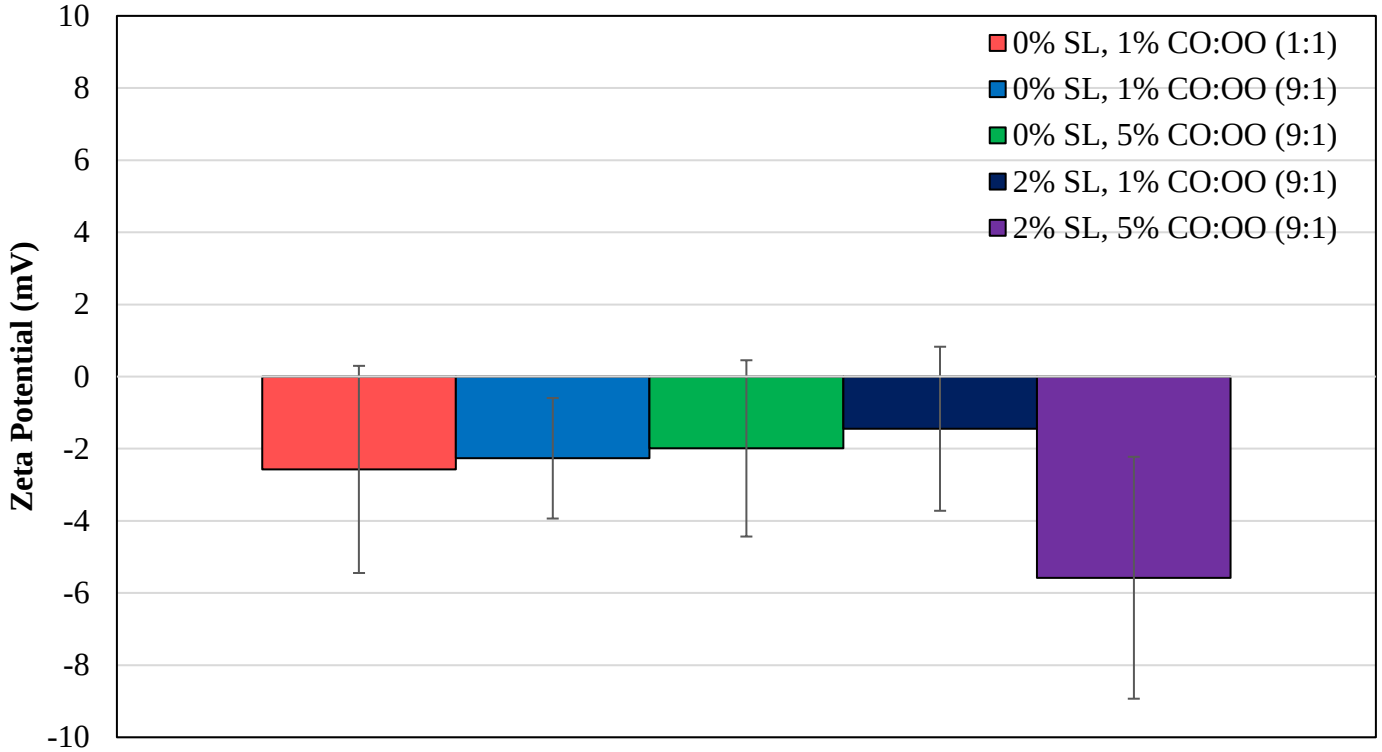

Figure 2.6. Zeta-potential of microemulsions. Zeta-potential of microemulsions containing, on mass basis, 20% Tween 20, varying amounts of deionized water, 0 or 2% sunflower lecithin (SL), 1% or 5% oil with cinnamon oil (CO) and orange oil (OO) at a mass ratio of either 9:1 or 1:1.

Chapter 4. Development and characterization of *Salmonella* Enteritidis H4267 biofilms

Abstract

Salmonella is the leading bacterial culprit of foodborne illness in the US and *S.* Enteritidis, the leading *Salmonella* serovar associated with foodborne illness, has been shown to produce biofilms on stainless steel surfaces.[1-4] Determining biofilm-forming capabilities is important as *Salmonella* spp. can have differing capabilities.[5] Biofilm-forming abilities were determined with red, dry, and rough morphotypes present Congo Red Agar (CRA), fluorescence of colonies on Luria Broth agar with 0.02% (w/v) calcofluor (LBA_c) plates and via microtiter assay. *S.* Enteritidis H4267 displayed curli- and cellulose producing capabilities on CRA and LBA_c while also displaying formed biofilms on microtiter well walls after incubation at 37°C for 24 h. Biofilms were then formed on stainless steel discs, and sonication was evaluated for removal efficacy. Stainless steel discs that were rinsed and sonicated in 0.1% (w/v) peptone water (PW) for 30 sec (25% power, 5 kHz) had the greatest *S.* Enteritidis H4267 recovery while not causing high cell death. This research demonstrates that *S.* Enteritidis H4267 can form biofilms on microtiter well walls and stainless steel surfaces, which has not been shown previously. Biofilms can also be effectively removed from stainless steel discs with rinse in 0.1% PW followed by 30 sec sonication at 25% power for enumeration purposes.

1. Introduction

Equipment and devices used in the food industry can vary in use and material type.[6] Glass and plastic are commonly used in the food industry, but metals, used more often, are the most important material for food processing equipment.[5, 7-8] The large

variety of equipment surfaces, material types, high water usage, and human personnel present in a food processing facility all contribute to bacterial growth being present in a food facility.[6, 9] If cleaning and hygienic practices are not implemented consistently, microorganisms can thrive in a food facility and biofilms can form.[6, 10]

A biofilm is a community of microorganisms that are attached to a surface and encased in an exterior matrix.[9, 11] Biofilms can form on a variety of surfaces and become difficult to remove since biofilm communities have greater antimicrobial resistance.[12-14] Biofilms present in food processing environments can serve as a persistent pathogen source as biofilm pieces can detach, allowing for microorganisms to travel throughout a facility.[15-17] Products, materials, and contact and non-contact surfaces within a facility can become contaminated, which can lead to large scale effects, such as food recalls and plant closures.[10, 15-17]

Salmonella is the leading bacterial culprit of foodborne illness in the US with an estimated one million cases of foodborne illness attributed to *Salmonella* infection.[1-2, 18] *S.* Enteritidis is the leading *Salmonella* serovar associated with foodborne illness and has been shown to produce biofilms on stainless steel, glass, and plastic surfaces.[3-4, 19-22] The majority of *Salmonella* biofilm extracellular matrices are composed of curli and cellulose.[23] Both curli and cellulose are able to provide structure and promote cell adhesion to surfaces to form biofilms.[23] Curli are thin, aggregative, amyloid fimbriae that are important for both surface adhesion and host infection.[24-25] Cellulose is a polysaccharide that has been shown to be a major contributor towards *Salmonella* biofilm formation and antimicrobial resistance.[23, 26-27] Each *Salmonella* serovar can have differing

biofilm-forming capabilities, and determining the curli- and cellulose-forming abilities is needed when developing a biofilm.[5, 28]

Congo Red agar (CRA) and Luria Broth agar with 0.02% (w/v) calcofluor (LBA_c) have been used to determine biofilm forming abilities of bacteria.[5, 23, 28] Biofilms can also be formed in a microtiter well and be indirectly quantified by solubilizing crystal violet (CV)-stained cells in 30% (v/v) acetic acid.[29-30] CV will bind to proteins present in microbial cell walls and can indicate bacterial presence while solubilization of the CV in 30% acetic acid can quantify the extent of microbial abundance.[29-31]

Various methods exist for biofilm removal from a surface.[32-33] Swabbing, sonication, or scraping of material surfaces as well as rinsing prior to enumeration are all methods that have been utilized.[32-33] Sonication has been described as being superior to both swabbing and scraping for biofilm removal, but the length of sonication must be limited as prolonged exposure can cause cell death, which can lead an experimental design to have false negative results.[33-34]

The formation of *S.* Enteritidis H4267 biofilms has not been investigated and can provide insights to the food safety industry. Biofilm-producing abilities must be investigated to better understand this serotype. Biofilm formation on stainless steel is of interest, but experimental methods must be investigated to determine an optimal procedure.

2. Hypothesis

S. Enteritidis H4267 is hypothesized to be able to form biofilms. Previous work indicates that *Salmonella* biofilm phenotypes display curli and cellulose production.[5, 21, 25] Like other *S.* Enteritidis serovars, *S.* Enteritidis H4267 is hypothesized to be able to form biofilms on stainless steel surfaces.[4, 20] This hypothesis was tested using two phases. Phase 1 determined the biofilm-forming ability of *S.* Enteritidis H4267 utilizing CRA, LBA_c, and a microtiter assay. Phase 2 investigated the formation of *S.* Enteritidis H4267 biofilms on stainless steel discs and removal using sonication.

3. Methods

3.1. Materials

Congo Red powder was a product of Alfa Aesar (Haverhill, MA). Brain Heart Infusion broth and Luria Broth Agar (LBA) pre-made powders were from Becton Dickinson & Company (Franklin Lakes, NJ). Bacteriological peptone used for 0.1% (w/v) peptone water (PW) was also from Becton Dickinson. Powdered agar and calcofluor (fluorescent brightener #28) were from Sigma-Aldrich Corp. (St. Louis, MO). Sucrose, acetic acid, and glass microscope slides were from Thermo Fisher Scientific (Waltham, MA). Phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific as a 10X-strength powder and was diluted to be 1X strength in deionized water (diH_2O). The pH of PBS after dilution was determined using an Accumet AE150 pH meter (Thermo Fisher Scientific). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased as pre-made powders from Remel Inc. (San Diego, CA) and rehydrated

in 1 l of diH_2O prior to autoclave sterilization. Crystal violet (CV) solution was also from Remel. Ethanol (200 proof) was from Decan Laboratories Inc. (King of Prussia, PA). Petri plates were from VWR International (Radnor, PA). Conical tubes and 96-well microtiter plates were from Corning Inc. (Corning, NY). Stainless steel 316 discs were from BioSurface Technologies Corp. (Bozeman, MT) and were 0.5" diameter, 0.14" height/thickness, and 1.0 g weight.

3.2. Bacterial culture

S. Enteritidis H4267 was obtained from the Department of Food Science culture collection at the University of Tennessee (Knoxville, TN) and was maintained at -20ºC in 25% glycerol. The strain was transferred two times in TSB with 22±2 h incubation at 37ºC prior to use.

3.3. Determination of curli production of S. *Enteritidis H4267 via CRA*

The procedure for determination of curli production using CRA was adapted from Freeman *et al.*[35] and Hassan *et al.*[36] Congo Red stain was created by dissolving 5 g/l of Congo Red powder in 1 l of diH_2O and autoclaving the mixture prior to use.[35] The CRA base was created by dissolving 37 g/l of Brain Heart Infusion powder, 50 g/l of sucrose, and 10 g/l of agar powder in 1 l of diH_2O.[35] After the CRA base mixture was cooled to ≤55°C, 0.8 g/l of Congo Red was aseptically added using the Congo Red stain to create the CRA.[35-36] The CRA was stirred for 1 min on a stir plate to create a homogenous solution prior to pouring into Petri plates. One hundred µl of *S*. Enteritidis H4267 (~10^6

CFU/ml) was spread plated onto CRA plates, and plates were inverted and incubated at 37°C for 22±2 h. Plates were then visually assessed for the appearance of dry, dark brown colonies that are indicative of curli-production on CRA.[37-38] Plates were completed in duplicate and triplicated (*n*=2, N=6).

3.4. Determination of cellulose-binding production of S. *Enteritidis H4267 via* LBA_c

The Uhlich *et al*.[39] method for determining cellulose binding to calcofluor was utilized. LBA_c was created by dissolving 30.5 g/l of LBA powder in 1 l of diH_2O and dissolving 200 mg/l of calcofluor prior to autoclaving.[39] One hundred µl of *S*. Enteritidis H4267 (~10^6 CFU/ml) was spread plated onto LBA_c plates. Plates were inverted and incubated at 37°C for 22±2 h and were visually assessed for the appearance of white to off-white colonies. Plates were then exposed to long-wave UV light (365 nm) using a handheld UV lamp (Model ENF-280C, Spectronics Corp., Westbury, NY) to observe fluorescence which is indicative of cellulose binding to calcofluor.[39] Plates were completed in duplicate and triplicated (*n*=2, N=6).

3.5. Determination of biofilm formulation of S. *Enteritidis H4267 via crystal violet assay*

The procedure for determining the presence of biofilms from *S.* Enteritidis H4267 was adapted from O'Toole[29] and Merritt *et al.*[30] A 0.1% (w/v) CV solution was created using CV and sterile diH_2O. One hundred µl of *S*. Enteritidis H4267 (~10^7 CFU/ml) was transferred to a well in a 96-well microtiter plate. The negative control utilized was sterile

TSB. Plates were covered and incubated at 37°C for 24 h. Planktonic bacteria were then removed from the wells by dispensing the liquid into a waste receptacle in a biosafety cabinet. Plates were rinsed in sterile diH_2O, and the excess diH_2O was discarded. Plates were tapped to remove residual diH_2O remaining in wells, and 125 µl of 0.1% (w/v) CV solution was added to each treated well. Plates were incubated at room temperature (21±2ºC) for 10 min, and the CV solution was discarded. Plates were tapped to remove residual CV solution in the wells prior to undergoing two successive rinses in sterile diH_2O to remove any residual CV solution. Plates were air dried inside a biosafety cabinet for 24 h at room temperature (21±2ºC). After air-drying, plates were visually assessed for CV-staining on microtiter walls. Each replicate had six treatment wells and were completed in triplicate (*n*=6, N=18).

3.6. Quantification of the extent of S. *Enteritidis H4267 biofilm formation on microtiter walls by CV assay*

The development of *S.* Enteritidis H4267 biofilms on microtiter well surfaces was adapted from O'Toole[31] and Merritt *et al.*[30] A 30% (v/v) acetic acid solution for solubilizing the CV stain was created using acetic acid and sterile diH_2O. One hundred µl of *S*. Enteritidis H4267 (~10^7 CFU/ml) was transferred to a well in a 96-well microtiter plate. The negative control utilized was sterile TSB. Plates were held at 37°C for 0, 6, 12, 18, 24, or 48 h prior to undergoing CV staining described in the biofilm formation via CV assay section. After air-drying for 24 h at room temperature (21±2ºC), wells had 200 µl of 30% (v/v) acetic acid added to solubilize the CV present on the well walls. The acetic

acid was drawn up into a pipet tip and dispensed back into the well five times to create a homogeneous mixture of solubilized CV in acetic acid.[30] Plates then underwent an optical density (OD) reading at 630 nm (OD_{630nm}) using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The negative control for OD_{630nm} readings was 30% (w/v) acetic acid. Each replicate had five treatment wells and were completed in triplicate (*n*=5, N=15). The attachment of *S.* Enteritidis H4267 to microtiter walls was indirectly quantified using the OD_{630nm} absorbance values.

3.7. Preparation of S. *Enteritidis H4267 inoculum*

Ten milliliters of an overnight culture of *S.* Enteritidis H4267 was aliquoted into a 15 ml conical tube and centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) at 1372 x *g* for 15 min. The supernatant was discarded, and 10 ml of PBS was added to the conical tube. The tube was vortexed to resuspend and disperse the pellet. The resuspended pellet underwent centrifugation again, and the process was repeated two more times with PBS to have a total of three washes. After the third wash, the supernatant was discarded, and the pellet was re-suspended in 10 ml of sterile 0.1% (w/v) PW.

3.8. Creation of S. *Enteritidis H4267 biofilms on stainless steel disc surfaces*

Ten microliters of *S.* Enteritidis H4267 culture (5.7±0.1 $\log_{10}$ CFU/ml) from the prepared inoculum in Section 3.6 was inoculated into 10 ml TSB tubes and vortexed. One sterile stainless steel disc was added to tubes. Tubes were statically incubated at 37°C and were collected at 0, 6, 12, 18, 24, and 48 h time points. This process was completed in

triplicate (*n*=1, N=3). The *S*. Enteritidis H4267 inoculum was also spread plated on TSA at 0 h to verify ingoing concentration to TSB tubes. The negative control was sterile TSB tubes containing discs.

3.9. Sonication as the removal method for S. *Enteritidis H4267 biofilms formed on stainless steel disc surfaces*

At each time point, the TSB broth was transferred to a sterile, labeled tube. The disc was aseptically transferred using sterile forceps to a sterile Petri dish containing 20 ml of 0.1% (w/v) PW to remove any loosely attached cells from the disc surface. The disc underwent two successive rinses in sterile 0.1% PW to have a total of three washes. After the third wash, discs were placed in a conical tube containing 9.0 ml of 0.1% PW. Conical tubes containing discs were placed in a tube rack in an ice water bath for sonication. The sonicator probe (Vibra Cell VC750 with CV33 probe, Sonics & Materials, Inc., Newtown, CT) was submerged 2 mm below the medium surface in the conical tube, and the tubes underwent sonication (25% power, 5 kHz) for either 0, 0.5, 1, 3, or 5 min to dislodge the attached organisms on the disc surface. The sonicator probe was cleaned with 70% ethanol and rinsed with sterile diH_2O prior to use in another sample. The negative control were discs that did not undergo sonication (0 min).

3.10. Enumeration of S. *Enteritidis H4267 post-sonication*

The collected TSB and the PW containing the sonicated disc were serially diluted in 0.1% PW and spread plated onto TSA for enumeration. Discs that did not undergo

sonication (0 min) were rinsed, placed in 9.0 ml of 0.1% PW, and spread plated onto TSA plates for enumeration. Plates were inverted and incubated at 37°C for 22±2 h. Microbial count data were logarithmically (base 10) transformed. An optimal sonication time length was defined to be the sonication time that was shortest and provided a colony count that did not display indications of causing cell death due to prolonged sonication exposure.

3.11. Visual observation of cell suspensions of S. *Enteritidis H4267 in TSB and 0.1% PW containing the sonicated disc*

After spread plating for enumeration, the collected TSB and 0.1% PW containing the sonicated disc was utilized for a wet mount. One 10 µl loopful of either TSB or 0.1% PW were transferred to a heat-fixed, glass microscope slide and viewed under a phase contrast upright microscope (Fisherbrand, Thermo Fisher Scientific). Clustered and individual colonies present were recorded, and the microscope stage was moved to another section of the sample to record observations. This process was repeated to have a total of five viewed images within a sample and was repeated for all triplicates (n=5, N=15).

3.12. Visual observation of removal efficacy of biofilms formed on stainless steel disc surfaces using a CV staining method

After an optimal sonication time length was determined, experiments were conducted to visually determine the extent of biofilm removal from stainless steel discs and to ensure that rinsing in 0.1% PW was sufficient at removing planktonic cells on disc

surfaces. *S.* Enteritidis H4267 biofilms on stainless steel surfaces were grown in TSB with a stainless steel disc as mentioned in the biofilm creation on stainless steel discs section. Discs were removed from the TSB after 0, 6, 12, 18, 24, and 48 h, and underwent separate treatments. Discs were either not rinsed and not sonicated (NR-NS), rinsed three times with 0.1% PW but not sonicated (R-NS), or rinsed three times with 0.1% PW and sonicated prior to being placed in 0.1% CV solution for 10 min (R-S). After 10 min, discs were rinsed two times with sterile 0.1% PW to remove residual CV on the surface. Discs were then placed in a sterile Petri dish and allowed to dry at room temperature (21±2ºC) for 24 h prior to observations.

3.13. Solubilization using 30% acetic acid of CV-stained stainless steel discs for biofilm removal methodology efficacy

After drying for 24 h at room temperature (21±2ºC), experiments were conducted to indirectly determine the extent of biofilm removal from stainless steel discs. Stained discs were placed into a sterile conical tube, and 1 ml of 30% (v/v) acetic acid was added to the tube. The tubes were vortexed for 5 sec every 2 min for a total of 10 min to ensure disc surfaces were exposed to acetic acid. After 10 min, 200 µl of the liquid was transferred to a well in a 96-well plate. This was repeated to have two samples per disc (n=2, N=6). Plates were measured for OD_{630nm} using a Synergy HT Microplate Reader. The negative control was 30% acetic acid.

3.14. Statistical analyses

Logarithmically transformed enumeration data and absorbance readings were analyzed using one-way ANOVA to identify statically significant differences ($\alpha = 0.05$). Significant differences were determined using Tukey's test ($p<0.05$). Analyses were conducted using SAS v9.4 (SAS Inc., Cary, NC, USA).

4. Results

4.1. S. *Enteritidis H4267 biofilm-forming abilities via CRA, LBA$_c$, and microtiter walls*

S. Enteritidis H4267 displayed red, dry, and rough ("rdar") colonies on CRA after incubation (**Figure 3.1**) (All tables and figures are located in the appendix of this chapter). After incubation at 37°C for 24 h, white colonies were present on LBA$_c$ plates. When LBA$_c$ plates were exposed to long-wave UV light, colonies fluoresced to be bright blue (**Figure 3.1**). After CV-staining, purple lines were present on the walls of *S.* Enteritidis H4267-treated wells (**Figure 3.2**).

4.2. Quantification of the extent of S. *Enteritidis H4267 biofilm formation on microtiter walls over time*

Solubilization of the CV dye via 30% acetic acid indicated that the extent of biofilm formation increases gradually over time ($p=0.002$) (**Figure 3.3**). The 30% acetic acid measurement at 48 h was significantly higher than other 30% acetic acid control time points ($p<0.0001$). This is believed to be due to the presence of air bubbles in the microtiter wells after being homogenized prior to OD_{630nm} readings. At 48 h, three wells

displayed well-bottom CV staining, causing the 48 h time point to be significantly different compared to the other *S.* Enteritidis H4267-treated wells ($p<0.0001$). A q-test, where each time point of a replicate was averaged, was conducted to determine if the three wells at 48 h were outliers, but there were no outliers in time points at 90%, 95%, and 99% confidence (data not shown). The three wells at 48 h were identified as not being outliers, and the values recorded were not adjusted.

4.3. Enumeration of S. *Enteritidis H4267 post-sonication*

The 0.5 and 1 min sonication times resulted in the highest recovery of *S.* Enteritidis H4267 after 48 h (**Figure 3.4**). All sonicated discs were equivalent in recovery at 0 and 12 h, but greater recovery counts were seen at 6 h with 0.5 min sonication (**Table 3.1**). While 3 and 5 min sonication times were still able to recover *S.* Enteritidis H4267 from stainless steel discs, the bacterial counts using 3 and 5 min sonication times were significantly lower ($p<0.0001$) than the 0.5 and 1 min sonication times after 18 h. At 48 h, 0, 3, and 5 min sonicated discs were different ($p<0.0001$) from 0.5 and 1 min sonication treatments. The 0.5 and 1 min sonication times were the closest in bacterial count to the TSB levels at each time point after 6 h. The 0.5 min sonication time length for biofilm removal from stainless steel discs was chosen for further experimentation due to being the shortest sonication time while not causing high cell death.

4.4. Visual observation of cell suspensions of S. *Enteritidis H4267 in TSB and 0.1% PW containing the sonicated disc*

When viewing wet mount images of the 0.1% PW that contained the sonicated disc, the number of bacterial clusters increased over time (**Figure 3.4**). When compared to the TSB that contained the stainless steel disc, more individual cells are observed as being present in TSB than in 0.1% PW (**Figure 3.5**). After 0 h, bacterial clusters were visible in both 0.1% PW and TSB. After 18 h, the percentage of clustered and individual cells in PW and TSB were not statistically different ($p<0.0001$).

4.5. Removal efficacy of biofilms formed on stainless steel disc surfaces

After discs that were stained with CV dried at room temperature (21±2ºC) for 24 h, stained discs were evaluated for visible CV-staining. The NR-NS discs displayed the most visible CV-staining after 12 h with a slight purple-blue color being present on disc surfaces (**Figure 3.6**). Discs that were R-NS began to show a faint purple-blue color at 24 h. The R-S discs displayed no visible presence of CV staining on disc surfaces after 48 h. From a visual observation, rinsing with PW removes residual matter from the disc surface with rinsing and sonication causing further removal of residual surface matter.

The CV-stained discs underwent solubilization in 30% acetic acid after visual assessment. All treatments were similar to the 30% acetic acid control at 0 h (**Figure 3.7**). The NR-NS disc had the highest absorbance value, indicating that this series of discs had the greatest bacterial presence on the disc surface. After 18 h, the R-NS discs were not

statistically different from NR-NS discs ($p<0.0001$). The R-S discs were the closest treatment to the control.

5. Discussion

Cellulose production by *S.* Enteritidis H4267 was exhibited by the rdar phenotype on CRA plates and white colonies on LBA_c fluorescing under long-wave UV light. The rdar phenotype displayed on CRA plates is indicative of curli and cellulose production, particularly for *Salmonella* spp..[5, 22-23, 35-36] If an organism can produce cellulose, colonies present on LBA_c plates will fluoresce under a long-wave UV light source.[22-23] Calcofluor dye fluoresces under long-wave UV light when the dye binds to polysaccharides containing (1,3)- or (1-4)-β-D-glucopyranosyl units, such as cellulose.[23]

The location of CV staining on the microtiter walls is indicative of aerobic growth, which can be expected since *S.* Enteritidis is a facultative anaerobic microorganism.[29-30] Using CV staining, Keelara *et al*. found that 13 different *Salmonella* serotypes were able to form biofilms on microtiter walls while Agarwal *et al.* showed that 150 *Salmonella* serotypes, including *S.* Enteritidis, have increased biofilm formation after 24 h on microtiter walls.[40-41] Anaerobic bacteria, such as *P. aeruginosa*, have displayed biofilm presence on the bottom of microtiter wells after 24, 48, and 72 h of incubation with CV staining.[42] At 48 h, CV staining was present on the bottom of three microtiter wells, indicating that *S.* Enteritidis H4267 was precipitating to the bottom of the well and/or was utilizing facultative anaerobic growth.[29-30] When grown in glass culture tubes, *S.* Pullorum formed biofilms at both the air-liquid interface and settled on the bottom of

the tube when incubated at 37°C for 48 h.[43] The last stage of biofilm development is detachment, so clusters of *S.* Enteritidis H4267 can detach from microtiter walls over time.[44-45] *S.* Enteritidis biofilms have been shown to detach in clusters of aggregated cells after extended time, which can explain the increased sedimentation in the bottom of wells at 48 h.[20]

As sonication times increased, there was a lower bacterial recovery at 18, 24 and 48 h compared to the shorter 0.5 and 1 min sonication times. Extended sonication times can cause a reduction in recovered biofilms, so shortening sonication time was anticipated to have reduced cell death.[34, 46-47] When *S.* Anatum was exposed to sonication (100% power, 40 kHz) for 3 and 6 min, bacterial counts indicated that recovered cells were over 1.0 $\log_{10}$ CFU/cm^2 lower after 3 min sonication and more than 2.0 $\log_{10}$ CFU/cm^2 lower for 6 min treatments than the non-sonicated control.[48] After 5 min of sonication (20 kHz), *S.* Typhimurium levels were reduced by over 50% while *Streptococcus faecalis* and *Enterococcus faecalis*, both Gram-positive bacteria, were only lowered by approximately 30%, but there was no cell count investigated to determine the $\log_{10}$ CFU/ml reduction.[49] Gram-negative bacteria are also more susceptible to sonication than Gram-positive ones.[34, 50] Gram-positive bacteria have extensive peptidoglycan and teichoic acid cross-links that cause the cell wall to be more robust and resistant to ultrasound.[50] Reducing the sonication exposure and power level of ultrasound frequency are recommended for dislodging bacteria from surfaces while simultaneously not causing cell death.[51]

The increasing level of biofilm development over time has caused the gradual increase in clustered cells present in 0.1% PW and TSB. As biofilm stage progresses, accumulation of bacterial population in the biofilm occurs.[52] Sonication disrupts the biofilm structure present on disc surfaces, and pieces and individual bacterial cells are removed from biofilms.[33-34] The *S.* Enteritidis H4267 clusters found in the TSB after 0 h most likely originate from a biofilm, but the biofilm could be on the stainless steel or from the culture tube walls near the air-liquid interface. *S.* Enteritidis biofilms can form at the liquid-air interface in culture tubes, and cell aggregates can be present in the culture fluid.[21, 53] Based on the biofilm enumeration data, biofilms are also being formed on stainless steel disc surfaces as early as 6 h after incubation. The microscopy data support the notion that biofilms on either glass or stainless steel discs could be reaching the "detachment" phase of biofilm development, and clustered communities of *S.* Enteritidis H4267 would be detaching from the main biofilm.[20, 44-45] This also supports the conclusion that the bottom-stained wells at 48 h were from an accumulation of detaching *S.* Enteritidis H4267 aggregates. Austin *et al*.[20] found that *S.* Enteritidis biofilms had cell aggregates detach from stainless steel surfaces over time. As microbial counts are reported in "colony" forming units, clustered cells could result in an underestimation of bacterial load since standard counting procedures do not take clusters of cells into account.[54]

Rinsing surfaces prior to biofilm removal is a common experimental method utilized to ensure that the recovered cells are coming from the biofilm and not planktonic cells.[20, 28, 55-57] Since CV can stain viable and non-viable cells, there is not enough

information to determine if the CV stain present on the disc surfaces was biofilms containing viable or non-viable *S.* Enteritidis H4267 communities.[30-31] The R-S discs being the closest rinse and/or sonication treatment to the 30% acetic acid control further supports the experimental method of utilizing stainless steel disc rinsing with 0.1% PW to remove residual bacterial cells and sonication to dislodge any bacterial presence on disc surfaces. By rinsing discs prior to sonication, the bacterial cells in the 0.1% PW suspension will more accurately reflect the level of *S.* Enteritidis H4267 present in biofilms.

6. Conclusion

S. Enteritidis H4267 displayed the rdar phenotype indicative of *Salmonella* spp. on CRA and had colonies fluoresce on LBA_c plates when exposed to long-wave UV light. Both methods indicated the curli- and cellulose-forming abilities of *S.* Enteritidis H4267. Microtiter assays demonstrated biofilm formation on microtiter cell walls as early as 6 h of incubation at 37°C with CV solubilization demonstrating increased biofilm formation over 48 h. Biofilms formed on stainless steel discs and were removed from the discs with minimal cell death when rinsed and sonicated in 0.1% (w/v) peptone water (PW) for 30 sec (25% power, 5 kHz). This research demonstrates that *S.* Enteritidis H4267 can form biofilms on microtiter well walls and stainless steel surfaces, which has not been shown previously. Biofilms can also be effectively removed from stainless steel disc surfaces with rinses in 0.1% PW followed by 30 sec of sonication at 25% power in 0.1% PW prior to enumeration on TSA.

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Appendix Three

Tables

Table 3.1. Enumeration of stainless steel discs that were sonicated at various times after being immersed in *S.* Enteritidis H4267 in tryptic soy broth for various incubation times. [a]

Incubation time (h)	Sonication time (min) [b, c]	Log_{10} CFU/ml or g [d]
	0	<1.0 est. [e] (A_0)
	0.5	0.3±0.1 est. (A_0)
0	1	0.3±0.5 est. (A_0)
	3	<1.0 est. (A_0)
	5	<1.0 est. (A_0)
	0	0.3±0.5 est. (B_6)
	0.5	1.5±0.3 est. (A_6)
6	1	0.8±0.6 est. (AB_6)
	3	0.3±0.2 est. (AB_6)
	5	0.0±0.0 est. (AB_6)
	0	2.5±0.1 (B_{12})
	0.5	4.8±0.5 (A_{12})
12	1	4.8±0.1 (A_{12})
	3	4.6±0.3 (A_{12})
	5	4.6±0.1 (A_{12})
18	0	4.1±0.1 (C_{18})

Table 3.1 Continued

18 (Cont.)	0.5	5.3±0.5 (A_{18})
	1	5.2±0.0 (A_{18})
	3	4.7±0.2 (B_{18})
	5	4.7±0.1 (B_{18})
	0	4.5±0.0 (C_{24})
	0.5	5.9±0.0 (A_{24})
24	1	5.9±0.1 (A_{24})
	3	5.0±0.0 (B_{24})
	5	4.9±0.1 (B_{24})
	0	4.7±0.0 (B_{48})
	0.5	6.0±0.2 (A_{48})
48	1	5.7±0.3 (A_{48})
	3	4.9±0.0 (B_{48})
	5	4.8±0.1 (B_{48})

[a] Stainless steel discs were immersed in tryptic soy broth during incubation and were sonicated in 0.1% (w/v) peptone water prior to enumeration on tryptic soy agar plates.

[b] Samples were sonicated at 25% power (5 kHz) in 0.1% (w/v) peptone water for 30 sec.

[c] Negative control was 0 min, positive control was the tryptic soy broth medium the discs were incubated in for the duration of the incubation time (data not shown).

Table 3.1 Continued

[d] Different letters indicate significantly different (α=0.05) means when comparing sonication time treatment at each time point. Subscript indicates the incubation hour for ease of comparing different letters.

[e] Samples that had <25 CFU/g or ml on tryptic soy agar after 22±2 h incubation at 37°C were labeled as "est." for estimated count value.[54]

Figures

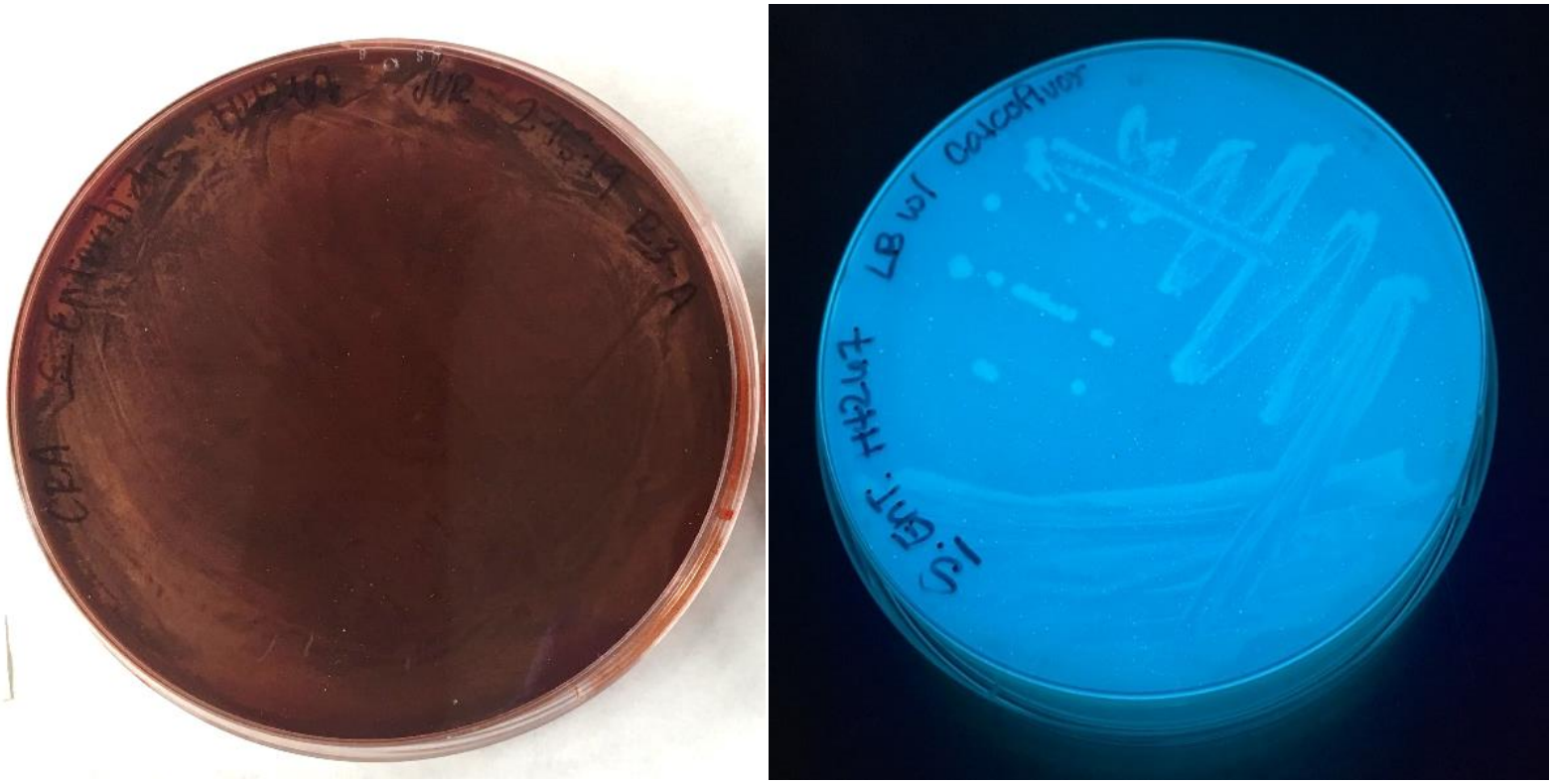

Figure 3.1. *S.* Enteritidis H4267 growth on Congo Red Agar (CRA) and Luria Broth agar with 0.02% (w/v) calcofluor (LBA_c) plates. *S.* Enteritidis H4267 growth on CRA (left) indicating red, dry, and rough colonies and on LBA_c (right) indicating colony fluorescence under long-wave UV light at 365 nm.

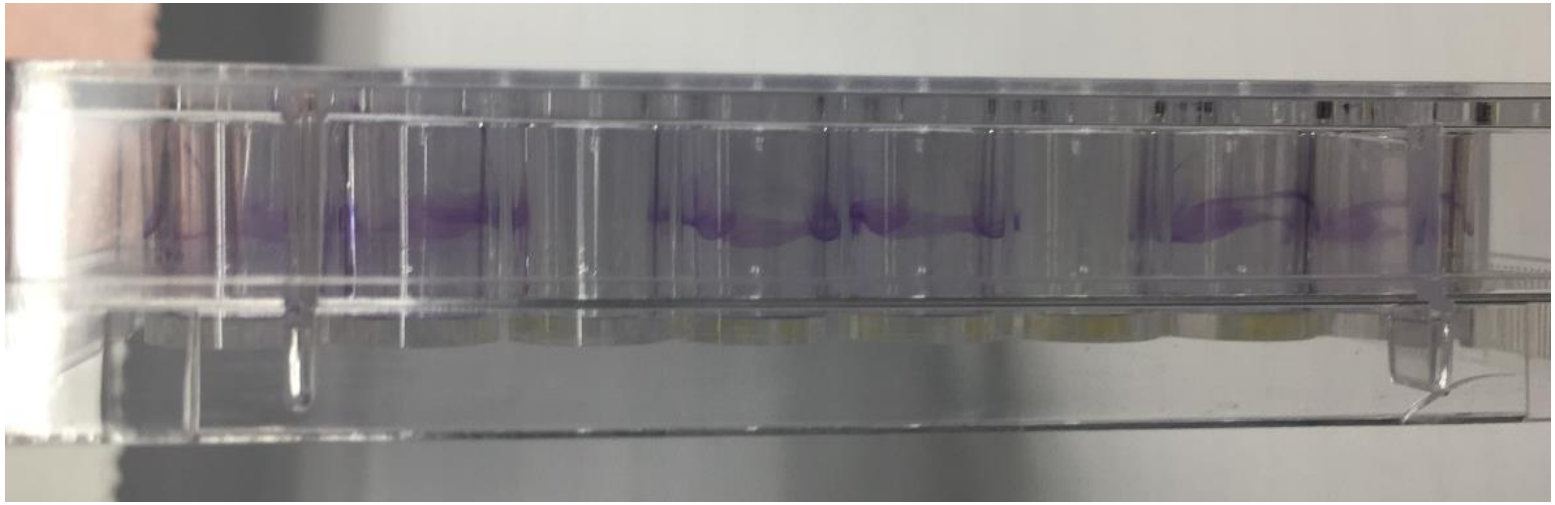

Figure 3.2. Biofilm formation of *S.* Enteritidis H4267 on microtiter well walls. *S.* Enteritidis H4267 visible on microtiter well walls after staining with a 0.1% (w/v) crystal violet (CV) solution. Stained wells are from *S.* Enteritidis H4267 treated wells that were aerobically incubated for 24 h at 37°C. Treated wells had *S.* Enteritidis H4267 removed and washed from the well for CV staining before being dried at room temperature (21±2°C) for 24 h (*n*=6, N=18).

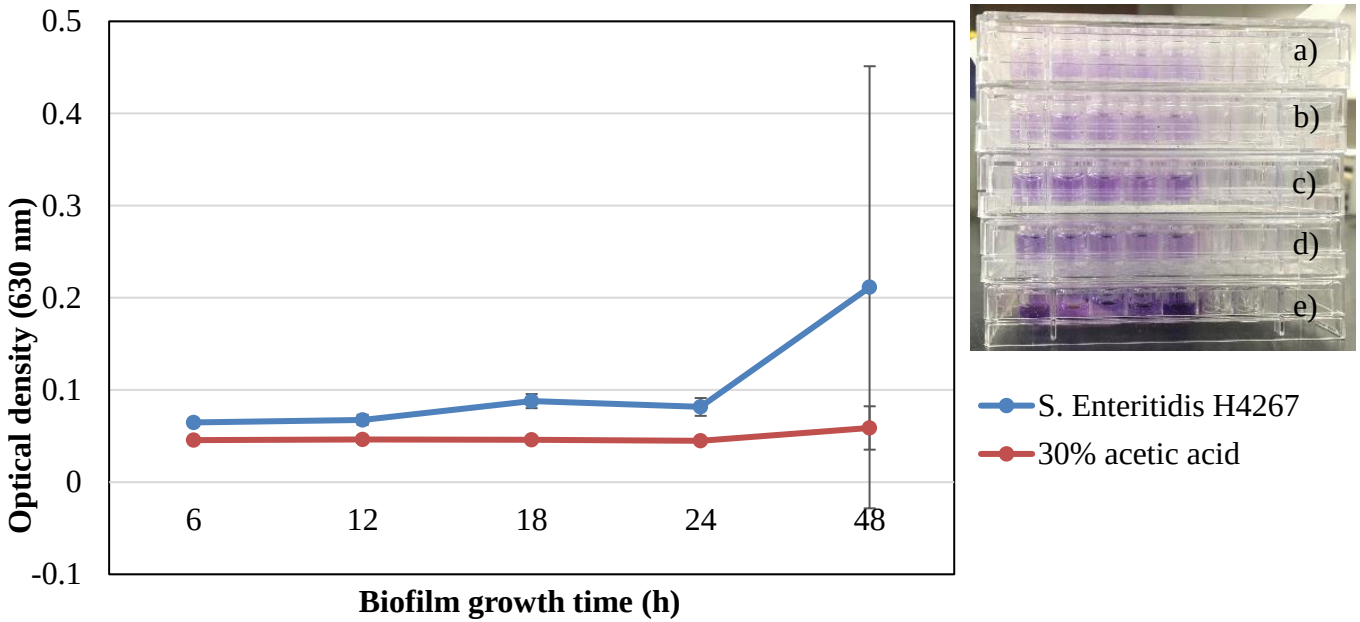

Figure 3.3. Solubilization of crystal violet (CV)-stained *S.* Enteritidis H4267 biofilms in microtiter wells. Extent of S. Enteritidis H4267 over time on microtiter well walls after staining with 0.01% (w/v) CV dye and solubilization of dye in 30% (v/v) acetic acid. Stained wells are from S. Enteritidis H4267 treated wells that were aerobically incubated at 37°C for 6 to 48 h. Treated wells had S. Enteritidis H4267 removed and washed from the well for CV staining before being dried at room temperature (21±2°C) for 24 h, and CV stain was solubilized in 200 microliters of 30% acetic acid for 10 min (*n*=5, N=15). Insert image is of microtiter plates after 6 (top), 12 (second from top), 18 (middle), 24 (second from bottom), and 48 (bottom) h of incubation at 37°C and solubilization of the CV stain.

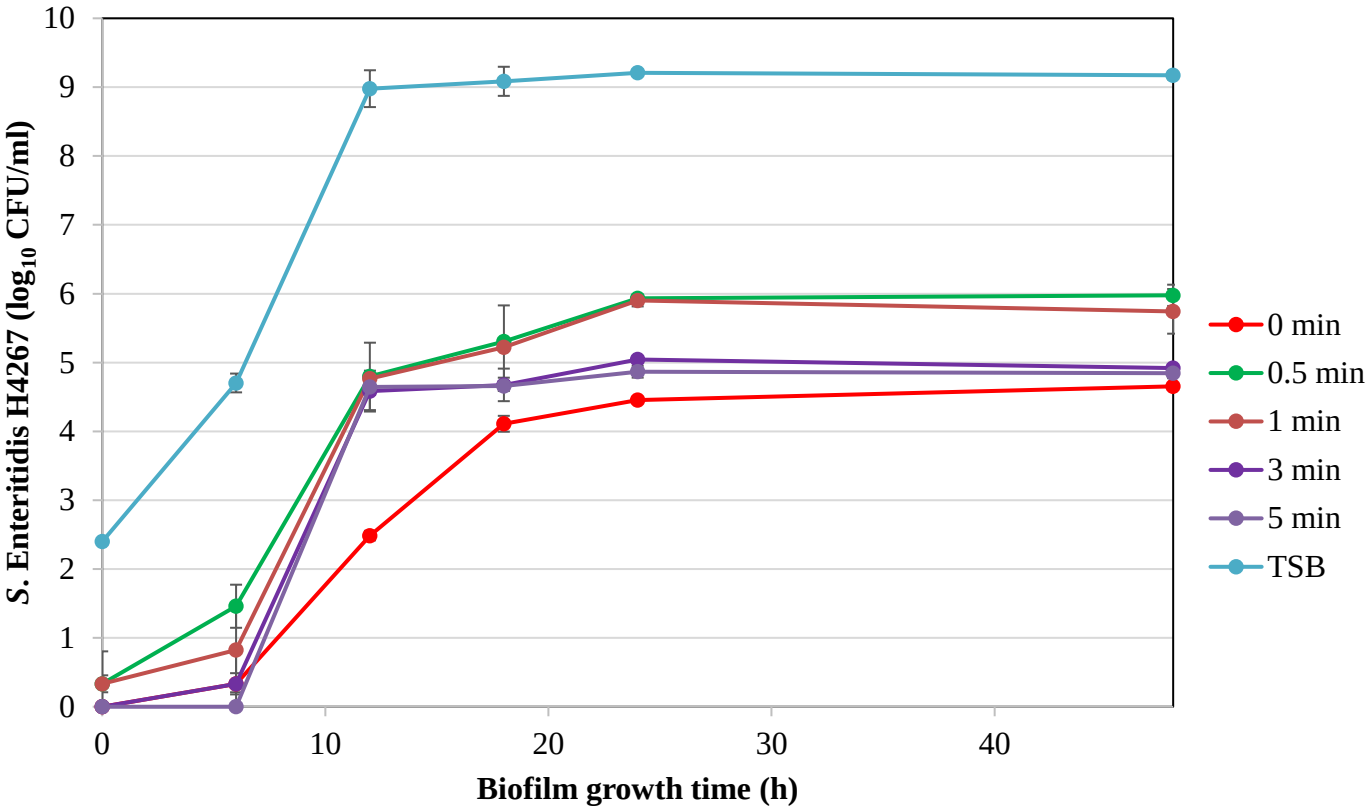

Figure 3.4. Recovered *S.* Enteritidis H4267 from tryptic soy broth (TSB) and biofilms formed on stainless steel discs. *S.* Enteritidis H4267 recovered from TSB or stainless steel discs sonicated in 0.1% (w/v) peptone water for various lengths of time. Stainless steel discs were statically incubated at 37°C in TSB inoculated with *S.* Enteritidis H4267 until time point collection.

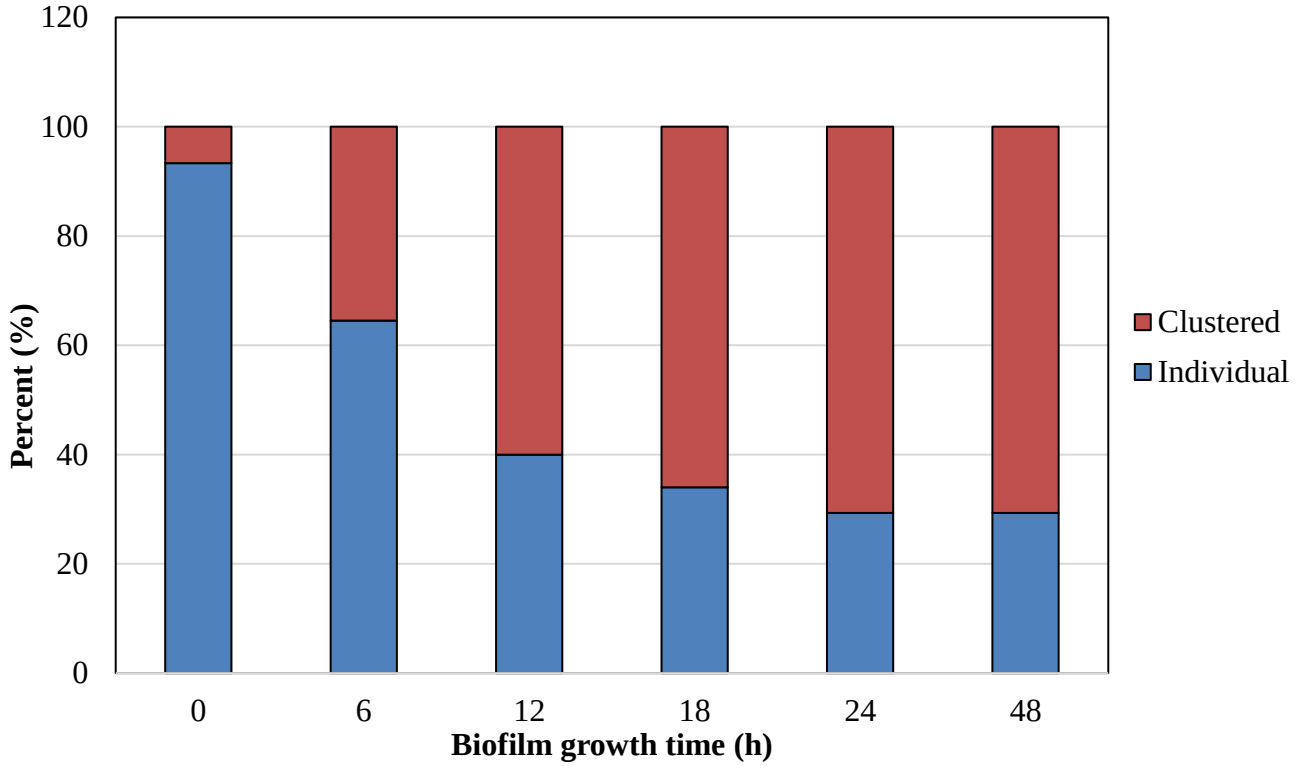

Figure 3.5. Clustered and individual *S.* Enteritidis H4267 cells from sonicated stainless steel discs. Distribution of individual versus clustered cells of *S.* Enteritidis H4267 recovered from sonicated stainless steel discs in 0.1% (w/v) peptone water viewed via wet mount. Stainless steel discs were statically incubated at 37°C in tryptic soy broth inoculated with *S.* Enteritidis H4267 until time point collection.

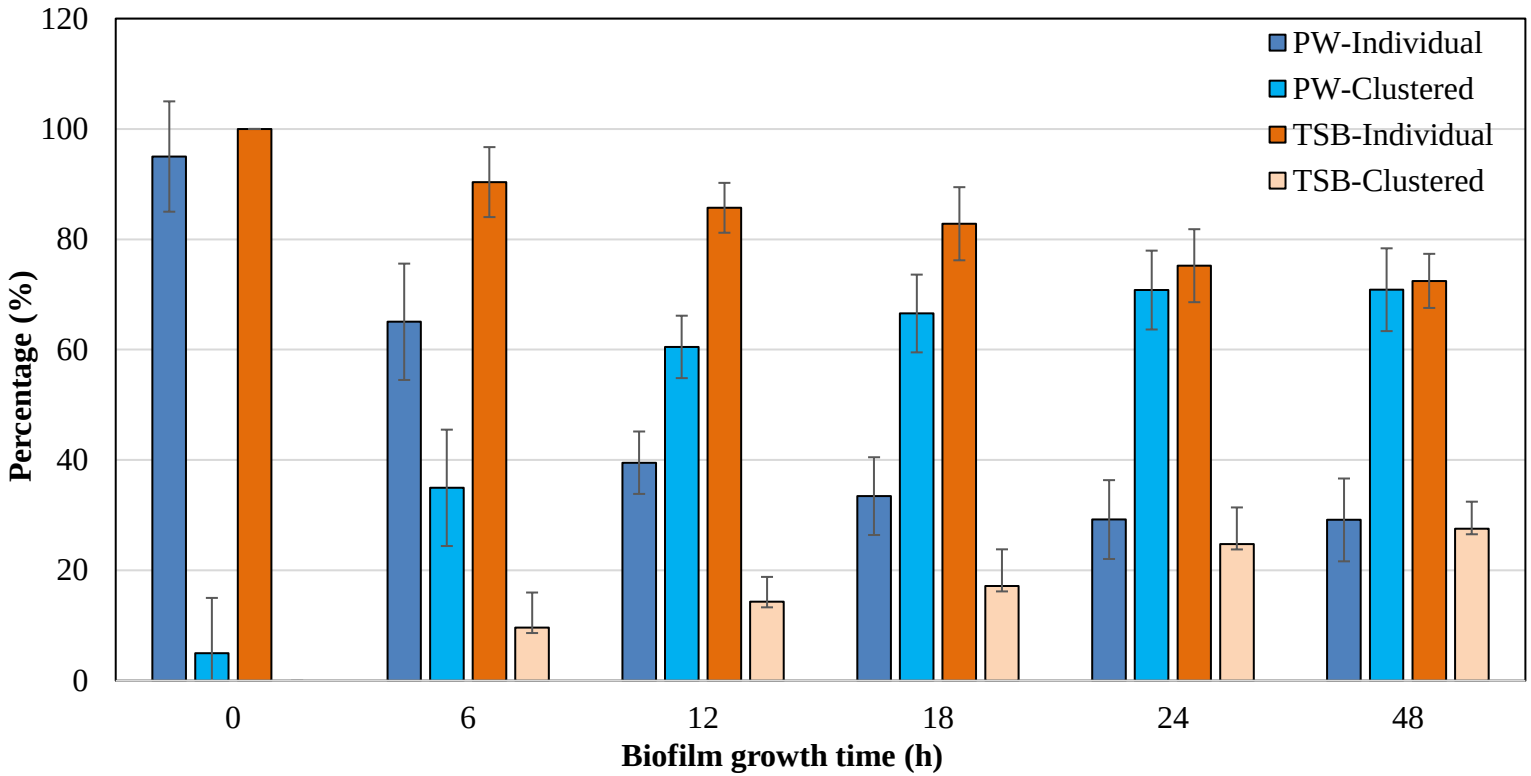

Figure 3.6. Clustered and individual *S.* Enteritidis H4267 cells from collected tryptic soy broth (TSB) and 0.1% peptone water (PW). Clustered and individual cells of *S.* Enteritidis H4267 recovered from TSB and sonicated stainless steel discs in 0.1% (w/v) PW viewed via wet mount. Stainless steel discs were statically incubated at 37°C in TSB inoculated with *S.* Enteritidis H4267 until time point collection. TSB was collected and stainless steel discs were sonicated for 30 seconds in 0.1% PW.

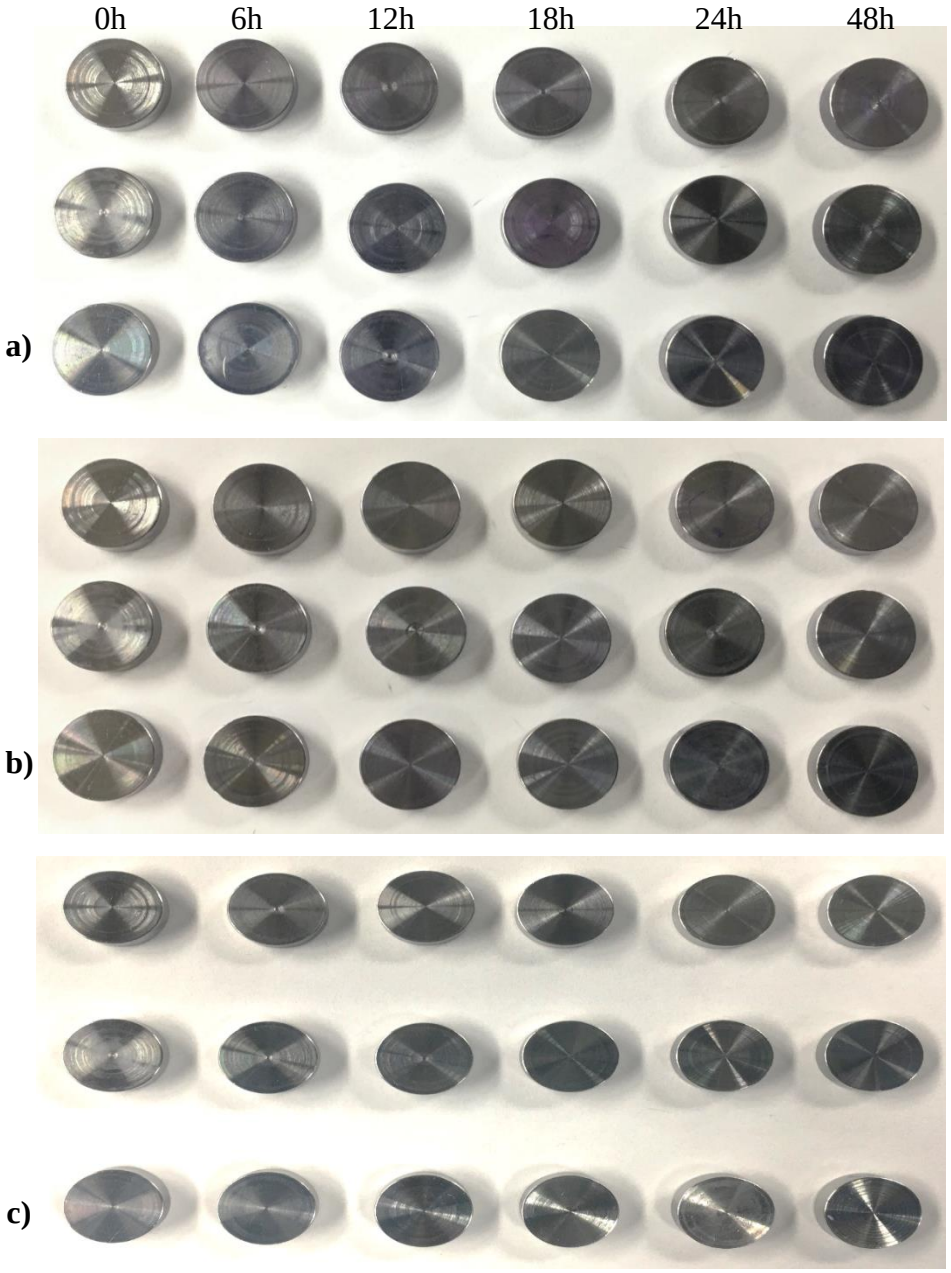

Figure 3.7. Discs stained with crystal violet after *S*. Enteritidis H4267 biofilm growth and various rinsing and sonication methods. Crystal violet stained discs at 0, 6, 12, 18, 24, and 48 hours when a) not rinsed and not sonicated, b) rinsed three times in 0.1% (w/v) peptone water (PW) and not sonicated, and c) rinsed three times in 0.1% PW and sonicated for 30 seconds in 9 ml of 0.1% PW.

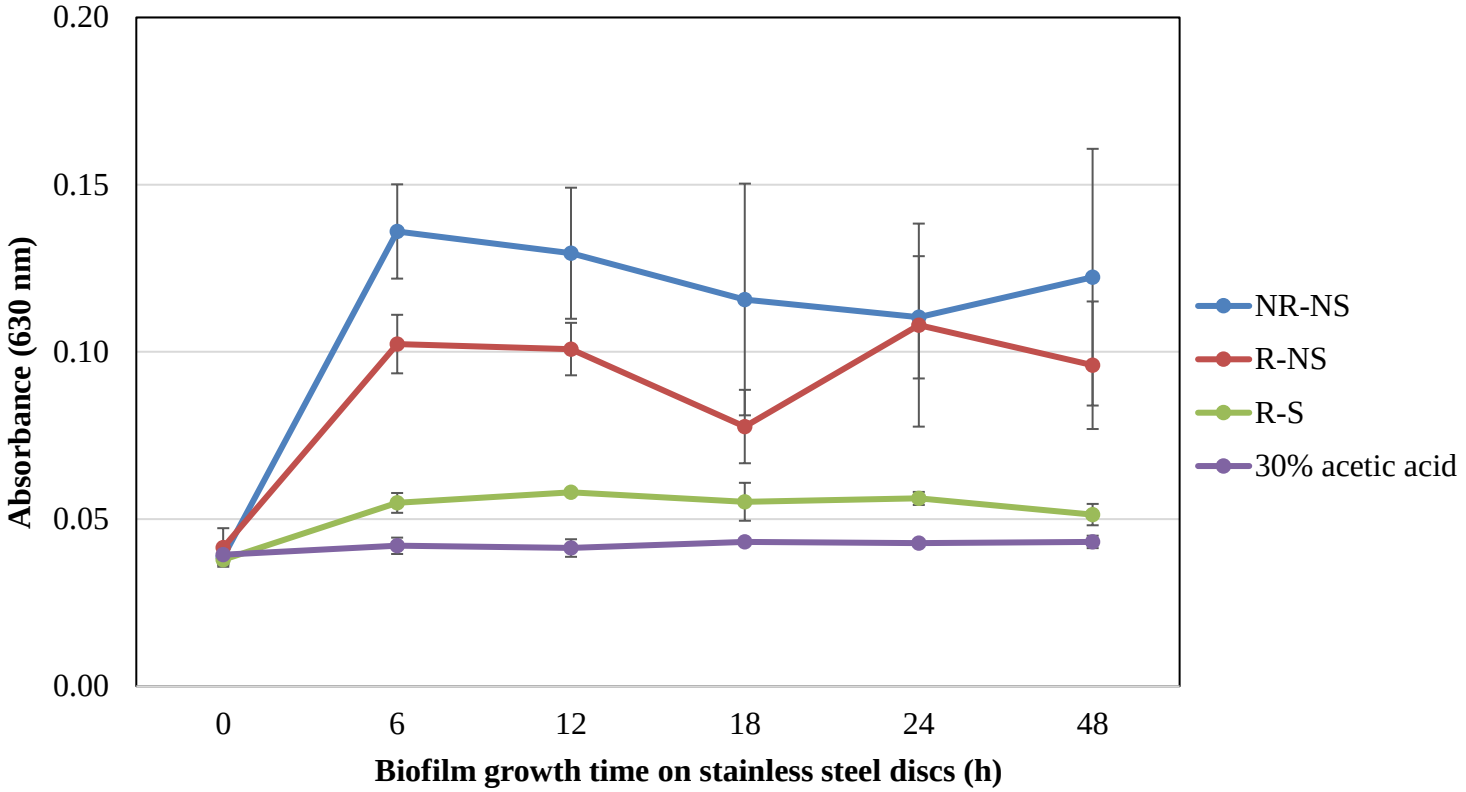

Figure 3.8. Solubilized crystal violet from *S.* Enteritidis H4267 biofilms on stainless steel discs after various rinsing and sonication methods. Absorbance of 30% (v/v) acetic acid from stainless steel discs that were either rinsed and sonicated (RS), rinsed only (R-NS), or neither rinsed nor sonicated (NR-NS).

Chapter 5. Determination of antimicrobial activity of microemulsions containing cinnamon and orange oil on *Salmonella* Enteritidis H4267 biofilms formed on stainless steel disc surfaces

Abstract

Antimicrobial delivery systems to combat *S.* Enteritidis biofilms in the food industry while also avoiding bacterial resistance are of interest. Essential oils (EOs) are attractive systems due to being plant-derived, but colloidal systems, such as emulsions, are needed for utilization due to EOs hydrophobic characteristics. Micro- and nano-emulsions were formed with, on mass ratio, 0 to 2% sunflower lecithin (SL), 3.3 to 20% Tween® 20 or Tween® 80, cinnamon oil (CO, *Cinnamomum zeylanicum*) and orange oil (OO, *Citrus sinensis*) in a 9:1 volume ratio, and deionized water. After treatment for 5 min at room temperature (21±2°C), microemulsions composed of 0 or 2% SL, 5% CO-OO, and 20% Tween 20 had the highest antimicrobial activity against *S.* Enteritidis H4267 biofilms formed on stainless steel discs over 48 h. After 18 h, 2% SL, 5% CO-OO, 20% Tween 20 microemulsions had the greatest antimicrobial activity against *S.* Enteritidis H4267 biofilms ($p<0.0001$). Due to the limitations of time points tested, determining whether emulsion size or surfactant addition affected antimicrobial ability the most could not be determined. However, this study proves that microemulsions composed of CO-OO can have bactericidal activity against *S.* Enteritidis H4267 biofilms present on stainless steel surfaces. This study shows that co-encapsulated EOs in microemulsions could be developed for antimicrobial delivery systems to treat biofilms.

1. Introduction

As microorganisms proliferate, extracellular polymeric substances (EPS) are excreted, encasing the biofilm in a scaffolding matrix.[1] Complexities within the

extracellular matrix of the biofilm arise with continued proliferation, leading to biofilm maturation. This EPS layer can provide an increased extrinsic defense for the biofilm by forming a "coat" on the exterior portion of the biomass of cells.[2] As more EPS are produced, channels and pores begin to form within the biofilm, creating a highway for transporting water, oxygen, and nutrients throughout the biofilm while excreting waste products from the interior portion.[3-5]

Increased resistance to sanitizers and medical antibiotics is also a characteristic of biofilms.[2, 6-7] Sessile cells contained within a biofilm can become more resistant to sanitizing or antimicrobial methods than their planktonic counterparts due to the decreased metabolism.[2, 8-12] Permeability through the complex biofilm matrix may also be related to the amount of biofilm biomass density; the higher the cell density, the lower the diffusion [2, 13-14] When comparing various strains of *S.* Typhimurium, EPS-producing strains in biofilms had higher resistance to quaternary ammonium compounds and chlorine sanitizing solutions compared to planktonic cells.[8]

Antimicrobial delivery systems to combat biofilms in the food industry while also avoiding bacterial resistance to disinfectants are of interest.[15] Plant-derived essential oils (EOs) are an attractive option for antimicrobial systems as EOs do not contribute to drug resistance due to the current mechanism of action focusing on the disruption of bacterial membranes.[16-19] The hydrophobicity of EOs allows the oils to penetrate through a bacterial cell membrane and causes a disruption of cellular metabolic processes and membrane transport systems[17-18] The cell membrane integrity will ultimately breakdown and cause increased membrane permeability.[17-18] Cinnamon oil (CO) has been researched

extensively as an antimicrobial agent against a variety of bacteria, including *Salmonella* and has demonstrated enhanced antimicrobial activity against *S.* Enteritidis in the presence of other EOs.[20-32] Orange oils (OOs) have exhibited antimicrobial activity enhancement of other EOs when used in combination against *Salmonella* spp.[33-35] A limiting factor for the usage of EOs is their hydrophobic characteristic, and colloidal systems, such as emulsions, are needed for utilization.

Colloidal systems with nanoscale oil droplets have unique features that are not found in conventional emulsions.[36-37] Nanoemulsions have droplet diameters between 20 and 200 nm while microemulsions have oil droplet dimensions between 4 and 100 nm.[36] Although nanoemulsions and microemulsions have overlapping dimensions, microemulsions are thermodynamically stable oil-water-surfactant mixtures that are transparent, and nanoemulsions are metastable systems that can be clear or turbid in appearance[36, 38] Due to having a small droplet size and large surface area, microemulsions can penetrate through bacterial cell walls more efficiently, which can increase the antimicrobial activity.[39] While there are several benefits of microemulsions, microemulsions are formed only at a particular set of conditions and require a relatively high level of surfactants.[36-37] Despite these drawbacks, the oil body of microemulsions can be used to dissolve lipophilic compounds, which could be an approach to enhance antimicrobial activity. [40-41] Microemulsions with multiple-component oil phase have also been created and could be utilized for biofilm treatment.[40-41]

Due to the complexity of biofilms, microemulsions that have activity against planktonic cultures may not have activity against a formed biofilm. Additionally, droplet

size is of importance for biofilm treatment to determine if a nanoemulsion would also be able to have an antimicrobial effect on biofilms or if a microemulsion is more appropriate. The application of a microemulsion composed of a mixture of CO and OO (CO-OO) on *S.* Enteritidis H4267 biofilms formed on stainless steel disc surfaces is of interest as it may assist with providing more information towards the realm of food safety.

2. Hypothesis

The hypothesis was that microemulsions containing CO and OO will have a greater antimicrobial effect on *S.* Enteritidis H4267 biofilms compared to control emulsions of a larger droplet size. The addition of surfactants can cause a reduction in emulsion antimicrobial activity and higher oil levels could be needed to overcome this barrier.[22, 24, 42-44] Because of the small droplet size, microemulsions should have greater bioavailability and could have higher antimicrobial ability compared to other emulsions with larger droplets.[32, 36, 45-47]

This hypothesis was tested in two phases. Phase 1 created two types of controls; one set of controls contained surfactants without CO and OO and another set of controls contained CO and OO but were not microemulsions. Once Phase 1 was completed, Phase 2 investigated the antimicrobial effects emulsion treatments on biofilms formed on stainless steel discs that were developed during Objective II.

3. Methods

3.1. Materials

Bacteriological peptone used for 0.1% (w/v) peptone water (PW) was from Becton Dickinson & Company (Franklin Lakes, NJ). Phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific (Waltham, MA) in a 10X-strength powder and was diluted to be 1X strength in deionized water (diH_2O). The pH of PBS after dilution was determined using an Accumet AE150 pH meter (Thermo Fisher Scientific). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased as pre-made powders from Remel Inc. (San Diego, CA) and rehydrated in 1 l of diH_2O prior to autoclave sterilization. Ethanol (200 proof) was from Decan Laboratories Inc. (King of Prussia, PA). Petri plates were from VWR International (Radnor, PA). Conical tubes and 96-well microtiter plates were from Corning Inc. (Corning, NY). Cinnamon oil (CO, *Cinnamomum zeylanicum*) was a product of Sigma-Aldrich Corp. (St. Louis, MO). Orange oil (OO, *Citrus sinensis*) was purchased from Now Essential Oil, Co. (Bloomingdale, IL). Tween 20 and Tween 80 were both products of Acros Organics (Morris Plains, NJ), and sunflower lecithin (SL) was purchased from Perimondo LLC (New York, NY). Stainless steel 316 discs were from BioSurface Technologies Corp. (Bozeman, MT) and were 0.5” diameter, 0.14” height/thickness, and 1.0 g weight.

3.2. Bacterial culture

S. Enteritidis H4267 was obtained from the Department of Food Science culture collection at the University of Tennessee (Knoxville, TN) and was maintained at -20ºC in

25% glycerol. The strain was transferred two times in TSB with 22±2 h incubation at 37ºC prior to use.

3.3. Creation of microemulsions containing CO and OO

Emulsion preparation followed previous work, with modification for compositions.[41] Microemulsions had CO and/or OO added to scintillation vials on mass basis at 1% to 5% levels along with 20% Tween 20 in CO-OO volume ratio of 9:1. A 12% stock solution of SL was prepared with diH_2O by stirring for 8 h at room temperature (21±2ºC) and added to solution vials, on mass basis, at 0% to 2%. Vials were added with diH_2O to bring the total microemulsion mass to 25.0 g and were hand-agitated until visually homogenous, followed by heating in an 80ºC water bath for 5 min. After heating, vials were hand-agitated in an ice water bath (5±0.5ºC).

3.4. Creation of controls

Emulsions to be used as controls against microemulsions were created by combining, on mass basis, 0 to 2% SL, 1 to 5% CO-OO in a 9:1 volume ratio, and 3.3 to 13.3% Tween 80 in a scintillation vial. Controls that did not contain CO-OO were also created using, on mass basis, 0 to 2% SL and 20% Tween 20. Vials had diH_2O added to bring the total mass to 25.0 g and were hand agitated until homogenous.

3.5. Droplet size and zeta (ζ)-potential of control emulsions containing CO and OO

Droplet size. Droplet size distribution of control emulsions were determined using dynamic light scattering (Zeta Sizer S, Malvern Panalytical Ltd., Worcestershire, UK) at a 173º scattering angle. All emulsions were diluted 100-fold in diH_2O to fit instrument sensitivity. Measurements were performed three times for each sample, and emulsions were created in triplicate (n = 3, N = 9).

ζ-potential. The ζ-potential of control emulsions was measured using the same dynamic light scattering instrument utilized for droplet size distribution determination. Samples were diluted 100-fold in diH_2O and were created in triplicate (n = 3, N = 9).

3.6. Preparation of S. *Enteritidis H4267 inoculum*

After *S.* Enteritidis H4267 was incubated for 22±2 h at 37°C, 10 ml of the culture was aliquoted into a 15 ml conical tube and centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 15 min at 1372 x *g*. After centrifugation, the supernatant was discarded, and 10 ml of sterile PBS was added to the conical tube. The pellet was resuspended and dispersed into the PBS by vortexing the tube, and the resuspended pellet underwent centrifugation again. The process was repeated two more times with sterile PBS to have a total of three washes. After the third wash, the supernatant was discarded, and the pellet was re-suspended in 10 ml of sterile 0.1% (w/v) PW.

3.7. Creation of S. *Enteritidis H4267 biofilms on stainless steel disc surfaces*

Ten microliters of the prepared-*S.* Enteritidis H4267 inoculum was diluted to ~10^7 CFU/ml and was inoculated into 10 ml TSB tubes. Tubes were vortexed, and 1 sterile stainless steel disc was added to tubes. Tubes were statically incubated at 37°C and collected at 0, 6, 12, 18, 24, and 48 h time points (*n*=1, N=3). The *S*. Enteritidis H4267 inoculum was also spread plated on TSA at 0 h to verify ingoing concentration to TSB tubes.

3.8. Emulsion treatment of S. *Enteritidis H4267 biofilms on stainless steel disc surfaces*

At each time point, the TSB broth in the tube was transferred to a sterile, labeled tube. To remove loosely attached cells from the disc surface, the disc was aseptically transferred using sterile forceps to a sterile Petri dish containing 20 ml of 0.1% (w/v) PW. The disc underwent two successive rinses in sterile 0.1% PW for a total of three washes. After the final wash, discs were placed in a sterile Petri dish containing 25 ml of either a microemulsion or emulsion formulation. Discs were held in the emulsion treatment for 5 min. After emulsion treatment, discs were aseptically removed and placed in a Petri dish containing 20 ml of 0.1% PW. The treated disc underwent an additional rinse to remove any residual emulsion present on the surface before being placed into a conical tube containing 9.0 ml of 0.1% PW. A control treatment without emulsions was also done where discs were collected at each time point, washed with 0.1% PW three times, and sonicated in 0.1% PW for 30 sec at 25% power (5 kHz).

3.9. Sonication of treated stainless steel discs containing S. *Enteritidis H4267 biofilms*

Conical tubes containing discs were placed in a tube rack in an ice water bath for sonication. The sonicator probe (Vibra Cell VC750 with CV33 probe, Sonics & Materials, Inc., Newtown, CT) was submerged 2 mm below the medium surface in the conical tube, and the tubes underwent sonication (25% power, 5 kHz) for 30 sec to dislodge the attached organisms on the disc surface. The sonicator probe was cleaned with 70% ethanol and rinsed with sterile diH_2O prior to use in another sample.

3.10. Enumeration of S. Enteritidis H4267 post-sonication

The collected TSB and the PW containing the sonicated disc were serially diluted in 0.1% PW and spread plated onto TSA for enumeration. Plates were inverted and incubated at 37°C for 22±2 h. Microbial count data were logarithmically (base 10) transformed.

3.11. Statistical analyses

Logarithmically transformed enumeration data were analyzed using one-way ANOVA to identify statically significant differences ($\alpha = 0.05$). Significant differences were determined using Tukey's test ($p<0.05$). Analyses were conducted using SAS v9.4 (SAS Inc., Cary, NC, USA).

4. Results

4.1. Droplet size distribution and ζ-potential of control emulsions

The surfactant control containing 0 or 2% SL and 20% Tween 20 without CO and OO had small droplet diameters (**Figure 4.1**) (All tables and figures are located in the appendix of this chapter). Nanoemulsions with 5% CO-OO in a 9:1 volume ratio were able to be created using 13.3% (w/w) Tween 80 with 0% or 2% SL. For 1% CO-OO (9:1) formulations, Tween 80 had to be reduced to 3.3% (w/w) to obtain an emulsion that was not within microemulsion droplet diameter range. The 0% SL, 1% CO-OO (9:1), 6.7% (w/w) Tween 80 formulation could be considered a nanoemulsion due to having two intensity peaks, but the 3.3% (w/w) formulation was chosen due to having a greater droplet distribution intensity between 10 and 200 nm. The ζ-potential for all emulsions was slightly negative, with emulsions containing EOs being more negatively charged (**Figure 4.2**).

4.2. Enumeration of **S.** *Enteritidis H4267 after emulsion treatment*

The 0 and 2% SL, 5% CO-OO microemulsions were the only treatments that resulted in bactericidal activity at 0 and 6 h (**Table 4.1**). The 0% SL, 5% CO-OO nanoemulsion also had high antimicrobial activity at 6 h but was not bactericidal (**Figure 4.3**). After 18 h, the 2% SL, 5% CO-OO microemulsion had the greatest antimicrobial effect on biofilm discs ($p<0.0001$). Control discs that did not undergo an emulsion treatment were not different from 5% CO-OO microemulsion treatments until after 18 h ($p<0.0001$).

5. Discussion

Surfactant presence can hinder the antimicrobial ability of EOs when the latter is encapsulated.[22, 48] Bacteria have been shown to utilize the oleic acid moiety in Tween 80 as a carbon and energy source when Tween 80 is above 0.1%.[49-51] Levels of Tween 20 greater than 4% (w/w) of the emulsion solution have shown to act as a nutrient source and contribute to planktonic bacterial growth.[52-56] Despite these drawbacks, there have been studies showing that the presence of Tweens at levels as low as 0.05% were able to decrease *S. enterica* levels from biofilms.[57-58] Tween 20 and 80 can promote the dispersal of *S. enterica* cells from biofilms and cause cell aggregates to break into smaller aggregates or individual cells, making the cells more susceptible to antimicrobials.[57, 59] Bacterial biofilm formation and cell growth have also been shown to be hindered by CO and cinnamaldehyde, a primary component in CO that has antimicrobial and anti-quorum sensing properties.[22, 60-63] While the addition of lecithin above 1.0% (w/v) contributes to microbial growth, lecithin can still play a role in lowering interfacial tension.[64-69] When phosphatidylcholine, a primary component in lecithin, was combined with other surfactants, the mixed system was able to lower the surface tension compared to a single surfactant system.[68, 70-71]

The stage of biofilm development could explain the slight increase in antimicrobial activity seen in a variety of emulsion treatments after 18 h. *Salmonella* sp. biofilms tend to adhere to stainless steel and attach at a fast rate when incubated at 37°C.[72] This has been attributed to the production of curli fibers and fimbriae being

affected by low temperatures, which leads to a decreased rate of biofilm development.[73] By 8 h at 37°C, *S.* Typhimurium can reach the maturation stage of biofilm development, and the detachment phase of biofilm development is already in progress by 24 h.[72] Pieces of a biofilm can detach from the biofilm structure, and this causes the biofilm to be exposed to extrinsic factors.[74] Antimicrobials can take advantage of this exposure and have a greater antimicrobial effectiveness since the EPS layer is no longer intact.[74]

The size of the emulsion also played a role in level of antimicrobial activity as 5% CO-OO microemulsions were the two formulations that had the highest antimicrobial activity over time. The addition of SL and Tween had a role in inhibiting antimicrobial ability, as various emulsions and the 1% CO-OO microemulsion had lower antimicrobial effect than the no-rinse treatment at several time points. The specific cause of the decreased antimicrobial ability is uncertain, whether it be the addition of SL and Tween 20 or the size of the emulsions. All three factors played a role at a variety of time points and convolutes the precise determining factor. Further studies are recommended to include more emulsion-exposure time points, imaging of biofilms pre- and post-emulsion treatment, and a LIVE/DEAD *Bac*Light assay to determine the level of viable and non-viable cells will provide more insight on the action taking place.[75]

6. Conclusion

Microemulsions composed of 0 or 2% SL, 5% CO-OO, and 20% Tween 20 had the highest antimicrobial activity against *S.* Enteritidis H4267 biofilms formed on stainless steel discs over 48 h. The only bactericidal activity seen at both 0 and 6 h were

from 5% CO-OO microemulsion formulations. The absence of emulsion treatments that lowered microbial levels compared to 1% CO-OO formulations after 0 h indicates that the addition of SL and Tween 20 and Tween 80 were unfavorable for the bioactivity of 1% CO-OO. After 12 h, 0% CO-OO control emulsions were among the highest microbial levels. Both 0 and 1% CO-OO results indicate that CO-OO presence at levels above 1% were needed for antimicrobial activity. After 18 h, 2% SL, 5% CO-OO, 20% Tween 20 microemulsions had the greatest antimicrobial activity against *S.* Enteritidis H4267 biofilms ($p<0.0001$). Due to the limitations of time points tested, determining whether emulsion size or surfactant addition affected antimicrobial ability the most cannot be determined. However, this study proves that microemulsions composed of CO-OO can have bactericidal activity against *S.* Enteritidis H4267 biofilms present on stainless steel surfaces and co-encapsulated EOs in microemulsions could be developed for antimicrobial delivery systems.

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Appendix Four

Tables

Table 4.1. Viable cells recovered from stainless steel discs after being immersed in tryptic soy broth inoculated with *S.* Enteritidis H4267 for various incubation times to grow biofilms, treatment by microemulsions and controls for 5 min, and sonication in 0.1% peptone water for 30 s.

Formulation [a]		**Viable cells in biofilms grown on stainless steel discs for different times (log_{10} CFU/ml)** [b, c]					
		0 h	6 h	12 h	18 h	24 h	48 h
0% SL, 1% CO-OO	µE	0.0±0.0 B	1.9±0.5 C	5.7±0.3 BC	5.2±0.3 D	5.3±0.4 C	5.3±0.1 BC
	E	0.0±0.0 B	2.6±0.4 C	5.0±0.1 CD	5.7±0.2 C	5.9±0.3 BC	5.4±0.1 BC
0% SL, 5% CO-OO	µE	0.0±0.0 B	0.0±0.0 D	2.0±0.3 F	3.6±0.1 F	4.0±0.1 D	4.1±0.3 D
	E	0.0±0.0 B	0.5±0.4 D	4.2±0.1 E	4.6±0.1 E	4.1±0.1 D	4.8±0.4 CD
2% SL, 5% CO-OO	µE	0.0±0.0 B	0.0±0.0 D	1.9±0.5 F	3.1±0.2 F	3.1±0.1 E	2.7±0.7 E
	E	0.3±0.5 B	1.8±0.1 B	4.6±0.1 DE	4.2±0.1 E	4.1±0.1 D	4.7±0.2 CD
0% SL (µE control)		0.0±0.0 B	3.5±0.1 B	6.2±0.4 B	6.5±0.1 B	6.3±0.1 B	6.2±0.2 B

Table 4.1 Continued

2% SL (µE control)	0.6±0.7 B	2.6±0.1 C	4.4±0.0 DE	6.6±0.1 B	6.3±0.1 B	6.1±0.1 B
No treatment	0.3±0.5 B	1.5±0.1 D	4.8±0.0 F	5.3±0.1 E	5.9±0.0 D	6.0±0.0 CD
TSB	2.0±0.1 A	5.2±0.3 A	8.7±0.0 A	8.6±0.1 A	8.9±0.0 A	9.0±0.1 A

[a] SL = sunflower lecithin, CO-OO = cinnamon oil and orange oil in a 9:1 volume ratio, No treatment = stainless steel disc without a treatment, TSB = tryptic soy broth. Microemulsions (µE) were composed of 20% (w/w) Tween 20 and varying volumes of deionized water, SL, and oils. Emulsion (E) were composed of 3.3-13.3% (w/w) Tween 80, and varying volumes of deionized water, SL, and oils. µE controls (without oil) contained 0 or 2% SL, 20% Tween 20, and varying volumes of deionized water.

[b] Plate counts are recorded as $\log_{10}$ CFU/ml (N=3).

[c] Different letters indicate statistically significant (α=0.05) means of plate counts on tryptic soy agar within each hour time point.

Figures

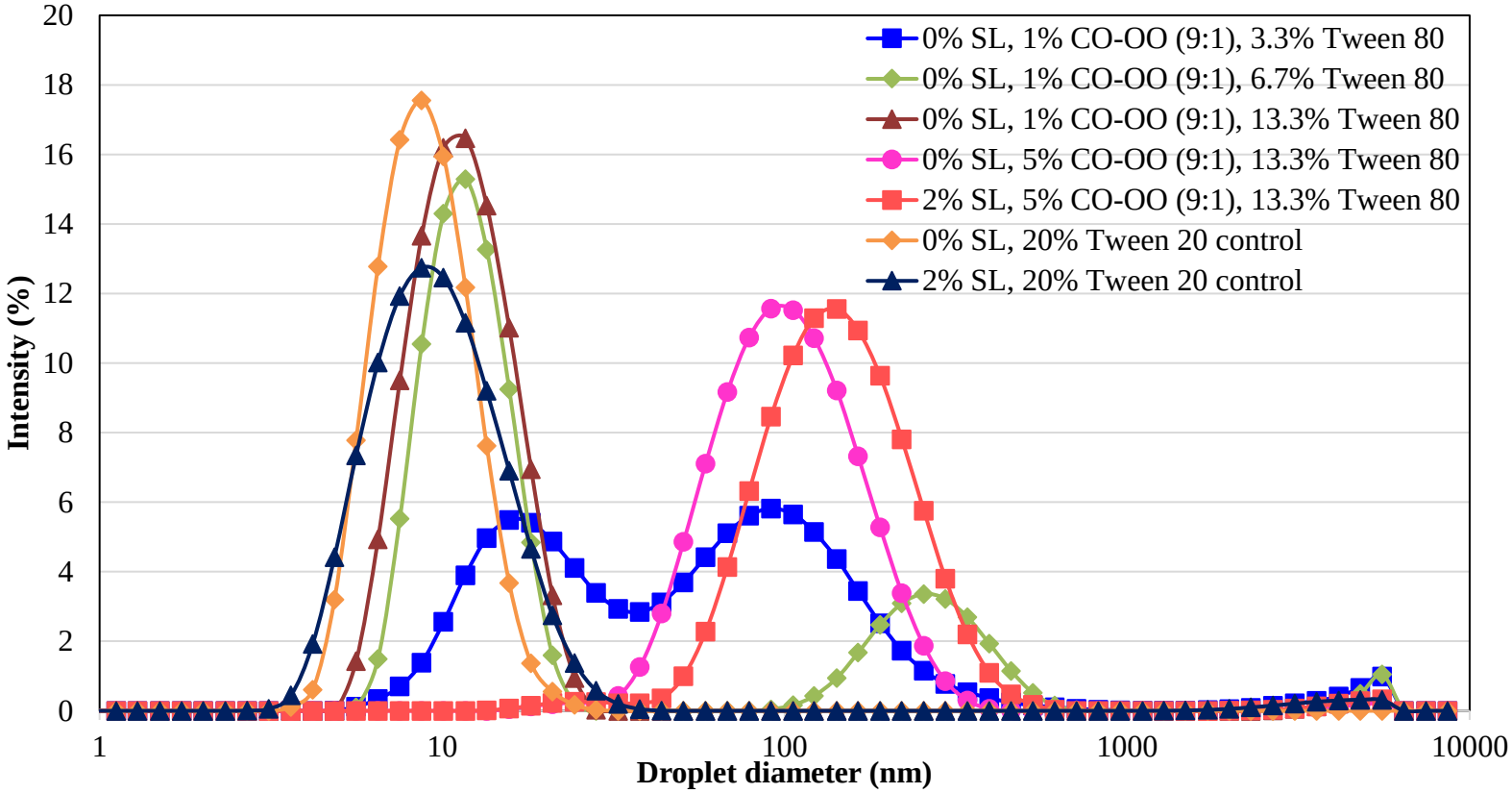

Figure 4.1. Droplet size diameter of controls containing, on mass basis, 0% or 2% sunflower lecithin (SL), 3.3 to 13.3% 80, 5% cinnamon oil (CO) and orange oil (OO) in 9:1 volume ratio, and deionized water. The microemulsion controls with 0 or 2% SL and 20% Tween 20 but without oil are also compared.

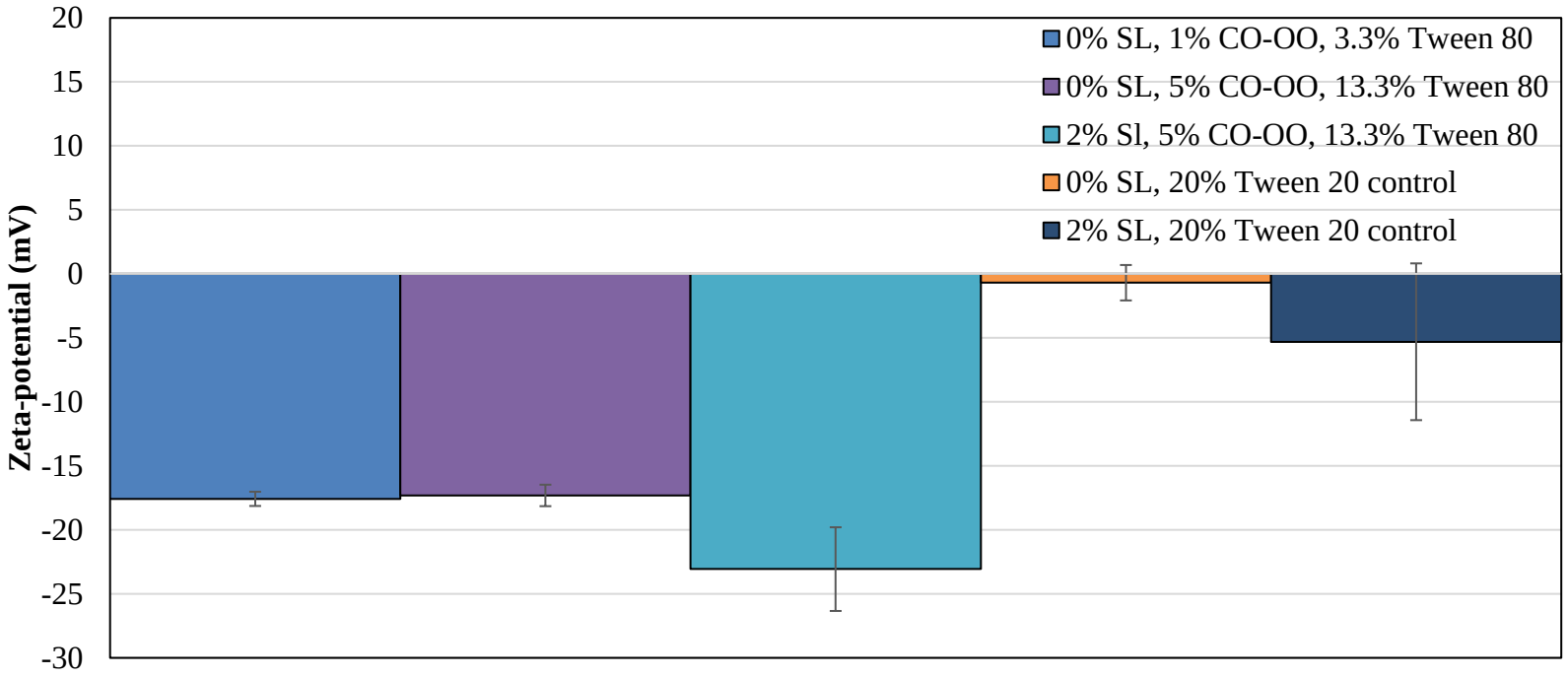

Figure 4.2. Zeta-potential of controls containing, on mass basis, 0% or 2% sunflower lecithin (SL), 3.3 to 13.3% Tween 80, 1 or 5% (w/w) cinnamon oil (CO) and orange oil (OO) in 9:1 volume ratio, and deionized water. The microemulsion controls with 0 or 2% SL and 20% Tween 20 but without oil are also compared.

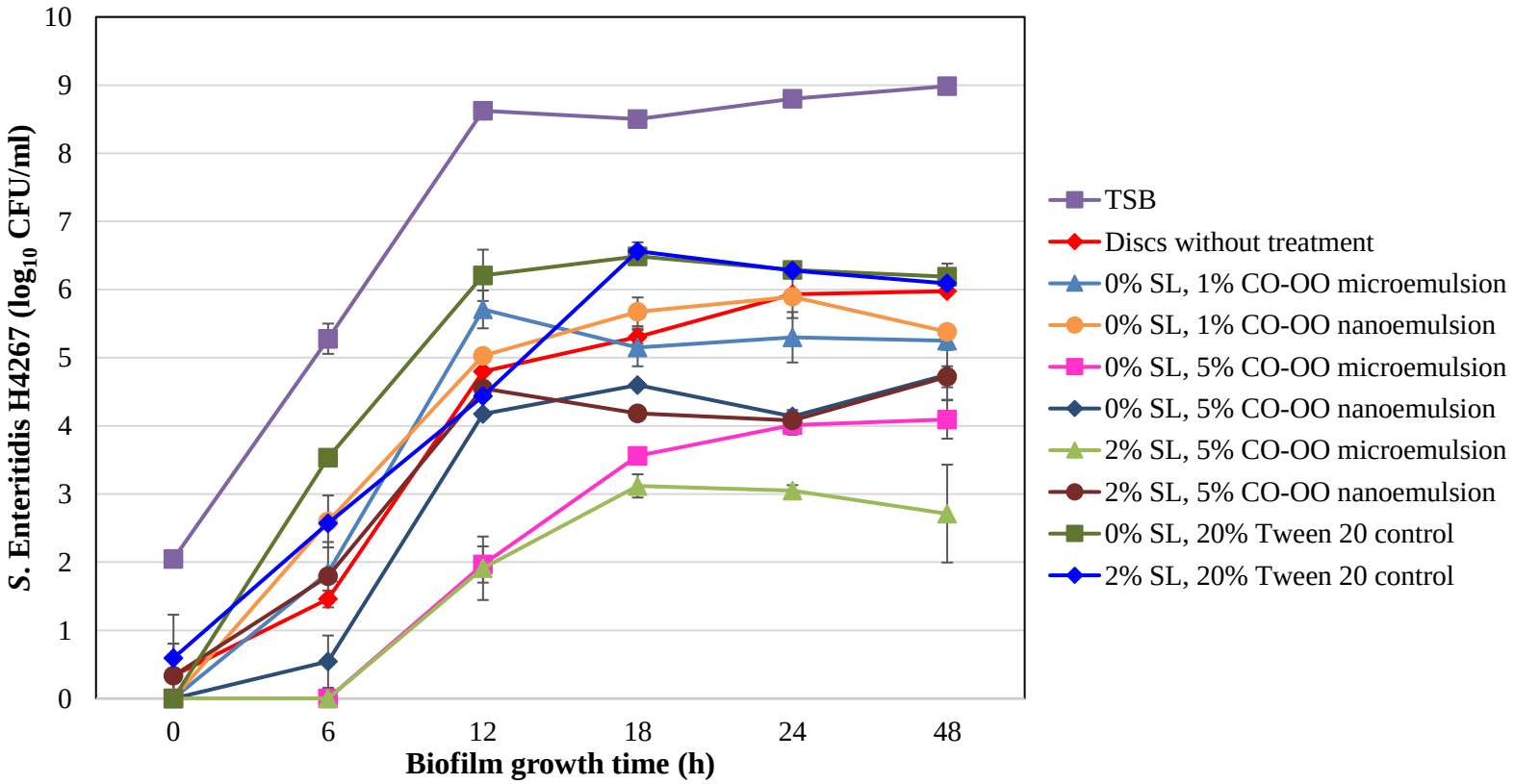

Figure 4.3. *S.* Enteritidis recovery from emulsion treated biofilms formed on stainless steel discs. Stainless steel discs were statically incubated at 37°C in TSB inoculated with S. Enteritidis H4267 until time point collection and discs were exposed to an emulsion treatment for 5 min prior to sonication. Discs without treatment were sonicated for 30 sec without an emulsion treatment.

Chapter 6. Conclusion

Essential oils (EOs) are natural antimicrobials that can be used to develop intervention strategies to inhibit pathogens, but EOs are lipophilic. Colloidal systems, such as microemulsions, are needed for food industry applications. This dissertation focused on the development and characterization of a microemulsion composed of cinnamon oil (CO, *Cinnamomum zeylanicum*) and orange oil (OO, *Citrus sinensis*) to be used against *Salmonella* Enteritidis H4267 biofilms formed on stainless steel disc surfaces.

The CO was found to have a greater antimicrobial effect on *S.* Enteritidis H4267 when used in combination with OO than when used individually ($p<0.001$). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) indicated that CO and OO were optimum in a 9:1 volume ratio (MIC and MBC: 750: 750 ppm) compared to a 1:1 volume ration (MIC and MBC: 1250: 750 ppm). Physical analyses determined that, on mass basis, 0% and 2% sunflower lecithin (SL), 20% Tween 20, 1% and 5% CO-OO (9:1) formulations were transparent, thermodynamically stable, and Newtonian fluids. These formulations were determined to be microemulsions and demonstrated bactericidal activity on *S.* Enteritidis H4267.

The second objective investigated the biofilm forming ability of *S.* Enteritidis H4267 and determined a biofilm removal method for biofilms on stainless steel disc surfaces. Microtiter assays demonstrated biofilm formation on microtiter cell walls as early as 6 h of incubation at 37°C with CV solubilization demonstrating that biofilm

formation increased over 48 h. Rinsing three times prior to sonication in 0.1% (w/v) peptone water removed planktonic cells from disc surfaces while also effectively dislodging biofilms. Sonication at 25% power (5 kHz) for 30 sec and 1 min was shown to dislodge *S.* Enteritidis H4267 cells and/or clusters from disc surfaces without causing extensive cell death. The 30 sec sonication time was selected as it was a shorter sonication time and did not differ from 1 min sonication in terms of *S.* Enteritidis H4267 recovery.

When microemulsions were used to treat *S.* Enteritidis H4267 biofilms formed on stainless steel discs for 5 min, only the formulations with CO-OO at levels above 1% showed antimicrobial activity. The microemulsions composed of 2% SL, 5% CO-OO, and 20% Tween 20 had the highest antimicrobial activity against *S.* Enteritidis H4267 biofilms formed on stainless steel discs, showing consistent activity over 48 h and higher activity than a nanoemulsion control with bigger droplets. Due to the limitations of time points tested, determining whether emulsion size or surfactant addition affected antimicrobial ability the most was not determined.

Despite limitations, this study proves that microemulsions composed of CO-OO can serve as an antimicrobial delivery system against *S.* Enteritidis H4267, either as planktonic cells or as biofilms present on stainless steel surfaces. Co-encapsulating EOs in microemulsions could be further developed for loading capacity and activity of EO antimicrobial delivery systems. Further research into developing microemulsions with positively charged droplets and exposing biofilms to microemulsions for longer times should be investigated. Additional methods, such as scanning electron microscopy, viable

versus non-viable cell identification, and fluorescence microscopy, should also be included to provide a more cohesive view of antimicrobial action.

Vita

Jennifer Vuia-Riser was born in Portland, OR, USA. Her family moved to Axtell, TX, USA when she was young, and she attended Axtell ISD. She obtained her B.S. in Nutritional Sciences at Texas A&M University in College Station, TX, USA. While at Texas A&M University, Jennifer did undergraduate research in Dr. Michael Manson's *Escherichia coli* chemotaxis laboratory and served as a student representative on the Phi Eta Sigma National Honor Society Executive Committee. Jennifer proceeded to obtain her M.S. in Food Science and Technology at Texas A&M University with a focus in food microbiology and safety of fresh and further processed meat and poultry. Her M.S. thesis focus was determining the antimicrobial efficacy of neutralizing Buffered Peptone Water as a rinse fluid for poultry carcass and parts testing for the United States Department of Agriculture (USDA) Food Safety and Inspection Service. Jennifer's M.S. degree was advised by Dr. Thomas Matthew Taylor. Upon completion of her M.S. degree, Jennifer pursued her Ph.D. at the University of Tennessee in Knoxville, TN, USA as a USDA National Needs Doctoral Fellow. While at the University of Tennessee, Jennifer's dissertation work focused on developing an antimicrobial delivery system to combat *Salmonella* Enteritidis H4267 biofilm presence on stainless steel surfaces. Her Ph.D. work was advised by Dr. Qixin Zhong. During her Ph.D. education, Jennifer served as President of the Food Science Club and as a Member at Large for the Institute of Food Technologists Student Association's Board of Directors.